

LABORATORY TECHNIQUES IN CELLULAR AND MOLECULAR MEDICINE



EDITORS

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Cultivation and cryopreservation of cell lines

Anna Janošťáková

I. Introduction

Cell lines are used in many areas of the basic and applied research. They can be developed in-house, acquired from another laboratory or purchased from the cell bank (1). Cell lines are derived from the clinical tumors or from the normal diploid cells using transformation by viral oncogenes, genetic modification or by chemical treatment (1,2).

Cells are most frequently propagated as an adherent monolayer or in a suspension of the freely-floating cells in the culture medium. Cells growing as monolayer attach to the substrate, e. g. to the bottom of culture vessels. The most of normal cell lines are dependent on the anchorage and therefore, they are able to grow only in a monolayer. The exceptions are the hematopoietic cells. On the other hand, the transformed cell lines, cells from malignant tumors and hematopoietic cells can grow in suspension (2). Cell lines are cultivated in a specific growth medium under the precise culture conditions.

The growth media include the relatively simple media such as Eagle's minimum essential

medium (E-MEM), Dulbecco's modified Eagle's medium (D-MEM) or more complex media such as RPMI-1640 medium or medium F12 (2). The growth media usually contain a source of energy (glucose – the primary source of energy and L-glutamine – a secondary source of energy for metabolism), all essential amino acids, cysteine and tyrosine. The other non-essential amino acids may also be added to the growth medium (2, 3). Further, the balanced salt solution (inorganic salts, trace elements) are used to maintain the osmolality and pH of the medium. In this respect, a sodium bicarbonate and carbon dioxide is usually used as buffering system. The concentration of the sodium bicarbonate in the medium must be matched with the level of CO₂ in the atmosphere above the medium. For media containing 1.5 to 2.2 g/l sodium bicarbonate, 5 % CO₂ is used. For media containing 3.7 g/l sodium bicarbonate, 10 % CO₂ is recommended. Sodium bicarbonate also has got significant nutritional role (2).

The media can also contain a variety of other compounds to support the proteins building, cell survival and cell growth (vitamins). Phenol Red can also be added to the growth medium.

This dye serves as a pH indicator. At the low pH levels the color of Phenol Red is yellow while at the high pH levels the color of the compound changes from yellow to red or pink (3). Antibiotics like penicillin, streptomycin or gentamicin can be added to the growth medium to reduce the contamination. However, antibiotics should not be used routinely for the long-term sub-culturing as bacterial strains can develop the resistance. If it is not possible, cell line in an antibiotic-free growth medium should be sub-cultured in parallel as a control (1, 2). Five to twenty percent of the heat inactivated serum (a source of a protein complex, hormones and growth factors) is usually added to the growth medium (3). Serum promotes cell proliferation, contains adhesion factors and anti-trypsin activity.

Other important parameters for the successful sub-culturing of cell lines are physicochemical properties such as temperature, pH and level of CO₂. The temperature of 37 °C, pH of 7.4 and a humidified atmosphere with 5 % CO₂ are usually used for human and warm-blooded animal cells.

A variety of culture vessels can be used for sub-culturing the cells such as bottles, Petri-dishes or multi-well plates. Culture vessels for adherent cells are usually made from the specially treated polystyrene enabling the adhesion of cells to the surface of the vessel. Cells should be regularly checked using the inverted microscope for the density, absence of microbial contamination or for signs of deterioration (e.g. granularity around cell nucleus, cytoplasmic vacuolation or rounding up of the cells) (1, 2). When cells are sub-cultured, they usually follow a standard pattern comprising of the lag period, log phase and plateau phase. The log phase is a period of exponential growth, where the cells consume the nutrients and are sub-cultured (2). Cell counting, manual or electronic, is necessary to determine cell growth and to set up the correct cell density for experiments. For routine counting, hemocytometer and light microscope is sufficient (1).

The very important aspect of work with cell lines of aseptic rules to successfully establish and maintain the cells and tissue cultures. Cell lines are sub-cultured in the flow biological safety cabinet (BSC), preferably Class II biohazard, equipped with the UV lamp. The BSC provides a clean working environment for

the cell lines and protects cell lines, as well as the staff (1). The cell lines can be contaminated by various microorganisms such as bacteria, fungi, yeasts. The effect of microorganisms on the cell culture include pH shifts, turbidity, change of the cellular morphology, change of cellular metabolism and cell death. The most often contamination of cells is caused by *Mycoplasmas* (2). These small bacteria negatively affect cellular morphology and metabolism and result in the unreliable data (1). As *Mycoplasma* contamination is not visible using the standard microscope, cell lines have to be regularly tested for *Mycoplasma* using at least two independent approaches (2).

Cell lines have to be protected from the cross-contamination with a different cell line. Otherwise, contaminating cell line may be better adapted to the culture conditions and can displace the original cell line (4). In this respect, during sub-culturing each cell line should be manipulated separately and the working place should be cleaned between sub-culturing of particular cell lines (1).

In this chapter, three basic procedures connected with the cell lines are described. They involve thawing of the frozen cells, sub-culturing of cell lines and freezing of cells.

II. Materials and instrumentation

Instruments

- Biological safety cabinet Class II, BSC (e.g. Hera safe, Thermo Fisher Scientific)
- Centrifuge (e. g. Rotina, Hettich)
- CO₂ incubator (e. g. Thermo Fisher Scientific)
- Inverted microscope (e. g. Olympus)
- Light microscope (e. g. Zeiss)
- LN₂ cryostorage system (Taylor-Wharton)
- Mr Frosty™ freezing container (Thermo Fisher Scientific)
- Pipettor (Eppendorf)
- Viability Cell Analyzer Vi-Cell™XR (Beckman Coulter)
- Water bath (Mettmert, WNB)
- ThawSTAR (Biocision)
- Ultra-low freezer (New Brunswick)

Materials

Dimethyl sulfoxide (DMSO; A3672.0250, AppliChem), D-MEM (BE12-604F, Lonza), E-MEM (BE12-611F, Lonza), denatured ethanol (85824.360, VWR), fetal bovine serum (FBS; 10270, Gibco), Iscove's modified Dulbecco's medium (0000651383, Lonza), potassium chloride (KCl; 30076-APO, Lach:ner), potassium phosphate monobasic (KH_2PO_4 , 30016-APO, Lach:ner), Penicillin-Streptomycin solution 100x (D910, Diagnostics), sodium chloride (NaCl; 7647-14-5, MikroChem), sodium phosphate dibasic dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$; 30061-APO, Lach:ner), sodium hydroxide (NaOH; 06203, Sigma Aldrich), McCoy's 5A medium (BE12-688F, Lonza), RPMI-1640 medium (BE12-702F, Lonza), TryPLE™ (12563, Gibco™).

Centrifuge tubes - 15 ml (91015, TPP), centrifuge tubes - 50 ml (91050, TPP), Filter Top 500 rapid Filtermax (99505, TPP), needle 18G (REF4665120, B-Braun), serological pipette-10 ml (94010, TPP), serological pipette - 25 ml (94024, TPP), syringe - 20 ml (2023-10-01, B-Braun), sterile cryovial (T309-2A, Simport), tissue culture flasks - T25 cm^2 (90026, TPP), tissue culture flasks - T75 cm^2 (90076, TPP), tissue culture flasks - T150 cm^2 (90026, TPP).

Personal protective equipment (gloves, laboratory coat, laboratory boots), sterile glass bottles, sterile cylinder, sterile beaker, sterile tips 10 μl , 200 μl , 1 ml, racks, marker, disinfectants e. g. , long tweezers, cell counter or hemocytometer, polystyrene box with dry ice or with liquid nitrogen.

III. Procedures

Solutions

Complete growth medium

- Liquid culture medium, e. g. RPMI-1640 medium
- FBS
- Antibiotics

Prepare the complete growth medium under the sterile conditions in the flow BSC. Use the sterile labware for the medium preparation. To prepare the complete growth medium, add FBS to the liquid culture medium to make the final

concentration of FBS of 10 % (e.g. add 50 ml of FBS to 445 ml to the RPMI-1640 medium). ¹

Then, add 5 ml of antibiotic solution (the final concentration of antibiotic solution is 1x) to the prepared growth medium with FBS. Filter the prepared complete growth medium using filter TOP rapid Filtermax 0.22 μm .

Close the bottle with the sterile cap. Store the prepared medium at 4 °C in the dark. Complete growth medium is stable for about 3÷4 weeks at 4 °C.

70 % ethanol in deionized water (working solution)

Mix seven parts of 100 % ethanol and three parts of deionized water. Store at the room temperature (RT).

10× PBS buffer (phosphate buffered saline; stock solution)

- 80 g NaCl
- 2 g KCl
- 32.1 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
- 2 g KH_2PO_4

Dissolve the above mentioned chemicals in 800 ml sterile water (tissue culture quality), adjust pH to 6.8-7.2 with 1M NaOH or HCl and add water to the final volume 1 l. Sterilize in an autoclave (121 °C). Store at 4 °C.

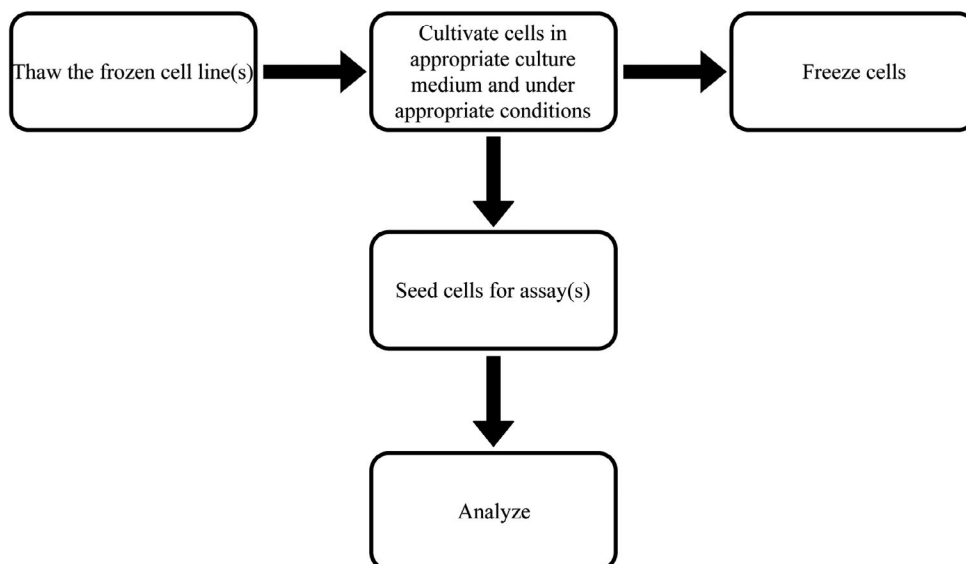
1× PBS (working solution)

Dilute the stock solution of the sterile 10× PBS to 1× PBS in the sterile water (tissue culture quality). Use the sterile glass cylinder, bottle and the sterile bottle cap. Store at 4 °C.

Freezing medium

- Complete growth medium without antibiotics
- DMSO

Add appropriate volume of DMSO into the growth medium. Mix well. The concentration of DMSO depends on the type of the cell line. Use the recommendation of the manufacturer. For example, if the cells require 10 % DMSO in the freezing medium, add 1 ml of the sterile DMSO to the 9 ml of the complete growth medium without antibiotics. Use the sterile labware for the medium preparation. Prepare the freezing medium before the use.



Scheme 1.1. The routine procedures of cell cultivation.

A. Thawing the frozen cell line

1. Pre-warm the complete growth medium in the water bath (37 °C, at least 30 minutes before the work starts).
2. Take the vial with the frozen cell line from the LN₂ container and put it into the polystyrene box filled with the dry ice.
3. Prepare the biological safety cabinet (BSC): wipe the surface of the biological safety cabinet, first with the disinfectant solution and then with 70% ethanol. Switch the UV lamp inside the biological safety cabinet, on for 30 minutes.
4. Put all the necessary materials and instruments such as the culture flask(s) T-75 or T-25, centrifuge tube(s), serological pipette(s), pre-warmed growth medium in the flow BSC. Before placing the materials and instruments inside the cabinet, wipe all of them with 70% ethanol. Switch the UV lamp inside the flow BSC on for 30 minutes.
5. Take out the vial from the transport polystyrene box. Thaw the frozen cell line in the ThawStar device or alternatively, use the water bath set to 37 °C. ²
6. Remove the vial from device/water bath, wipe it with 70 % ethanol and put it into the cabinet. Observe the aseptic rules for all further manipulations in the BSC.
7. Transfer the cell suspension from the vial into the sterile centrifuge conical tube (15 ml) using the pipette. Slowly add the complete growth medium to the final volume of 14-15 ml and close the tube.
8. Centrifuge the cells for 5 minutes at 1,200 rpm at room temperature.
9. Transfer the centrifuge tube back to the cabinet.
10. Carefully remove the supernatant. Add about 2 ml of the complete growth medium into the centrifuge tube.
11. Carefully resuspend the pellet of cells by repeated pipetting.
12. Transfer the cell suspension into the culture flask and add the complete growth medium to the final volume (for flask T25-5 ml, for flask T75-15 ml).
13. Check the cells under the inverted microscope.
14. Place the culture flask into the CO₂ incubator.
15. Take out the complete growth medium out

of the BSC and store it at 4 °C in the dark.

16. Discard solid and liquid waste according to the laboratory rules. Clean with the disinfection. Switch the UV lamp inside the BSC on for 30 minutes.
17. On the next day, check the cells. If necessary, replace the complete growth medium for the fresh one in case of adherent cell line.

B. Sub-culturing the cells

Adherent cells

1. Pre-warm the 1× PBS, TryPLE™ and the complete growth medium in the water bath set to 37 °C (at least 30 minutes before beginning the work).
2. Prepare the biological safety cabinet, (BSC): wipe the surface of the BSC first with the disinfectant solution and then with 70% ethanol. Switch the UV lamp inside the flow BSC on for 30 minutes.
3. Put all necessary materials and instruments such as the culture flask(s) T-75, centrifuge tube(s), serological pipette(s), syringe(s), needle(s), pre-warmed 1× PBS, TryPLE™ and growth medium in the BSC. Before placing the materials and instruments inside the flow BSC, wipe all of them with 70% ethanol. Switch the UV lamp inside the flow BSC on for 30 minutes.
4. Take out the cells grown in the culture flask T-75 from CO₂ incubator. Visually inspect the color of the growth medium, viscosity or turbidity. Check the cells for signs of contamination, debris or unhealthy cells using inverted microscope.³ Return the culture flask back to the incubator.
5. Calculate % of confluence.⁴
6. Take out the culture flask from the incubator and place it in the BSC. Remove and discard the culture medium into the waste container containing the disinfection solution.⁵
7. Wash the flask of cells with 10 ml of the sterile 1× PBS to remove all traces of growth medium and FBS. Remove and discard the PBS solution. Repeat this step once again.
8. Add carefully about 1.5 ml of TryPLE™ to cover a surface of a T-75 culture flask forming a thin layer.⁶ Swirl the flask gently. Incubate the flask 3-5 minutes at 37 °C in CO₂ incubator.⁷
9. Observe the cells under the inverted microscope. Tap on or rock the culture flask

gently to detach all of the cells. The cells should be circular and properly dispersed.⁸

10. Put the flask in the flow BSC. Add 5 ml of the fresh complete growth medium and mix well. Transfer the cells suspension to a 50-ml centrifuge tube.⁹
11. Centrifuge the cells at 1,400 rpm for 5 minutes.
12. Transfer the centrifuge tube aseptically back to the BSC.
13. Carefully remove the supernatant. Add 5-10 ml of the fresh complete growth medium to the pellet and resuspend the pellet by pipetting.
14. Count the cells using a Cell counter or hemocytometer.
15. Based on the cell count, transfer the appropriate volume of the cell suspension into a new culture flask T-75. Add the complete growth medium to the final volume for culture flask T-75 (15 ml). Re-used flask can be used for further cell cultivation or as a back-up if the vented cap is not wet and damaged.
16. Check the cells using an inverted microscope and place it in the CO₂ incubator. Check the cells on the next day.
17. Take out the complete growth medium out of the biological safety cabinet and store it at 4 °C in the dark. Discard solid and liquid waste according to the laboratory rules. Clean the flow BSC properly with the disinfection. Switch the UV lamp inside the biological safety cabinet on for 30 minutes.

Suspension cells

1. Pre-warm the complete growth medium in the water bath set to 37 °C (at least 30 minutes before the work starts).
2. Prepare the BSC: wipe the surface of the BSC first with the disinfectant solution and then with 70% ethanol. Switch the UV lamp inside the BSC on for 30 minutes.
3. Put all the necessary materials and instruments such as the culture flask(s) T-75, centrifuge tube(s), serological pipette(s), syringe(s), needle(s), pre-warmed growth medium inside the cabinet. Before placing the materials and instruments inside the cabinet, wipe all of them with 70% ethanol. Switch the UV lamp inside the cabinet on for 30 minutes.

4. Take out the cells grown in the culture flask T-75 from CO₂ incubator. Visually inspect the color of the growth medium, viscosity or turbidity. Check the cells for signs of contamination, debris or unhealthy cells using inverted microscope.¹⁰ Return the culture flask back to the incubator.
 5. Calculate the cell density and split ratio.⁴
 6. Take out the culture flask from the incubator and put it in the BSC. Transfer the cell suspension to the 15 ml or 50 ml centrifuge tube.
 7. Centrifuge the cells at 1,400 rpmi for 5 minutes.
 8. Transfer the centrifuge tube aseptically back to the cabinet.
 9. Carefully remove the supernatant. Add 5-10 ml of the fresh complete growth medium to the pellet and resuspend the pellet by pipetting.
 10. Count the cells using a cell counter or hemocytometer.
 11. Based on the cell count, transfer the appropriate volume of the cell suspension into a new culture flask T75. Add the complete growth medium to the final volume for culture flask T-75 (15 ml).
 12. Check the cells using an inverted microscope and place it in the CO₂ incubator. Check the cells on the next day.
 13. Take out the complete growth medium out of the BSC and store it at 4 °C in the dark. Discard solid and liquid waste according to the laboratory rules. Clean the BSC properly with the disinfection. Switch the UV lamp inside the biological safety cabinet on for 30 minutes.
- flow BSC. Before placing materials and instruments inside the flow BSC, wipe all of them with 70% ethanol. Switch the UV lamp inside the cabinet on for 30 minutes.
4. Take out the cells grown in the culture flask T-75 from CO₂ incubator. Visually inspect the color of the growth medium, viscosity or turbidity. Check the cells for signs of contamination, debris or unhealthy cells using inverted microscope.¹¹
 5. Before harvesting, take a sample of cell suspension for the mycoplasma assay (0.25 – 0.5 x 10⁶ cells).
 6. Harvest the cells. Before a centrifugation, count the cell number and viability and calculate the final volume of cell for freezing procedure. The final concentration of cells should be 5 x 10⁶ cells/ml.
 7. Centrifuge the cells at 1,200 rpmi for 5 minutes.
 8. Prepare calculated volume of the freezing medium. Prepare an appropriate amount of labelled vials (1 ml of cell suspension per vial, name, passage, date of freezing).
 9. Transfer the centrifuge tube back to the cabinet.
 10. Carefully remove the supernatant and resuspend cells in the freezing medium.
 11. Add 1 ml of cell suspension per one cryovial.
 12. Place the cryovials inside Mr. Frosty or in the polystyrene box. Put it into the ultra-low freezer at -80 °C.¹²
 13. Make your working place clean and tidy. Discard solids and liquid waste following the laboratory rules. Switch UV lamp on for 30 minutes.
 14. Put the vials to LN₂ cryostorage system / container after negative result of *Mycoplasma* testing within a few days.

C. Cryopreservation

1. Pre-warm the complete growth medium without antibiotics in the water bath set to (37 °C at least 30 minutes before beginning the work).
2. Prepare the BSC: wipe the surface of the flow BSC first with the disinfectant solution and then with 70% ethanol. Switch the UV lamp inside the cabinet on for 30 minutes.
3. Put all the necessary the materials and instruments such as the centrifuge tube(s), serological pipette(s), syringe(s), needle(s), pre-warmed growth medium in the

IV. Comments

- ¹ The concentration of FBS depends on the type of the particular cell line. Usually, the final concentration of FBS varies from 5 % to 20 %. In this respect, add appropriate volume of FBS to the liquid culture medium to prepare the growth medium with suitable concentration of FBS.
- ² Thawing should be fast, 2 minutes is usually sufficient.

- ³ Adherent cells should be attached to the bottom of the flask, with membrane refracting light around. Dead cells and damaged cells are smaller, dark and free floating.
- ⁴ Confluence is equal to value of the portion of the vessel covered by cells in percent. Most of the cell lines are ready to sub-culture when the confluence reaches 80 %. Cells should be passaged in a log phase.
- ⁵ During the manipulation, avoid generation of any aerosol.
- ⁶ Instead of TrypLE™, sterile 0.25 % Trypsin-EDTA solution (e. g. T4049, Sigma Aldrich) can be used in the same way as TrypLE™.
- ⁷ The time depends on the type of the cell line. However, it should not exceed 10 minutes. Otherwise, it can result in the increased cell death or slow growth of the cells.
- ⁸ If only part of cells is detached from the flask's surface, incubate the cells for next 3 minutes in the incubator (do not exceed the total of 10-minute incubation). Check the cells using the inverted microscope.
- ⁹ If the cells are not seeded for an experiment, a short version of the protocol without centrifugation can be used. In this case, continue with the step ¹⁴. Alternatively, after addition of 5 ml of growth medium, count the number of cells using a cell counter or hemocytometer. Based on the cell count, leave the appropriate volume of the cell

- suspension in the same culture flask T75 and discard the rest of the cell suspension. Add the complete growth medium to the final volume for culture flask T-75 (15 ml).
- ¹⁰ Suspension cells should be freely floating, round, refracting the light. The dead cells are smaller and darker.
- ¹¹ Only healthy cells without any contamination should be used. The optimal confluence of cells for freezing is 70 – 80 %.

Once the cells have been thawed and sub-cultured, they can be used in many different experiments. It is advisable not to cultivate the particular cell lines longer than 2 months. High number of passages increases a risk of the genetic and other variations of the cells. The staff have to keep records of all cell culture work including the sub-culturing, general cells' appearance and morphology, manipulations such as feeding, counting and seeding. This will ensure that there is no anonymous change in the cell culture. Mycoplasma testing should be done at least twice per month. It is important to reduce the risk of contamination of cell cultures in the tissue culture laboratory to minimum by observing the rules for the work in tissue culture laboratory.

V. Pitfalls

Problem	Cause and cure
Change of morphology or behavior of the cells	• Cross-contamination or <i>Mycoplasma</i> contamination of the cell culture. Discard the cell culture and all solutions. Clean the biological safety cabinet, clean and sterilize CO₂ incubator. Prepare the new solutions and thaw the new vial of frozen cells.
Cells growth is slow	• The complete growth medium is old. Prepare the new complete growth medium. • Cells were not properly thawed. Let the cells to grow without sub-culturing for few days. Change the growth medium every three days. If it does not help, thaw the new vial.

- Seeding concentration was too low. Let the cells to grow without sub-culturing for few days. Change the growth medium every three days. If it does not help, thaw the new vial exactly according to the protocol and change the seeding concentration.

Distinctive bread-like odor

- It indicates yeast contamination. Discard the cell culture, all solutions. Clean the BSC, clean, and sterilize CO₂ incubator. Prepare the new solutions and thaw the new vial of frozen cells.

Bright and yellow-colored medium

- It indicates bacterial contamination. Discard the cell culture, all solutions. Clean the flow BSC, clean and sterilize CO₂ incubator. Prepare the new solutions and thaw the new vial of frozen cells.

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Cell lines and cell culture conditions - 3D cultures

Viswanath Das

I. Introduction

Three-dimensional (3D) spheroid cultures of cancer cell lines play a key role in cancer research and drug discovery (1). Spheroids reproduce the complexities of solid tumors, such as cell-cell interactions, zones of proliferating and quiescent cells, diffusion barriers, multicellular resistance, and regions of hypoxia more effectively than two-dimensional (2D) cultures (1). Number of protocols for 3D cultures of cells for high throughput/high content adaptability presently exist (1, 2). Among these methods, liquid overlay on agarose-coated plates that increases cell-cell interaction by preventing cells to adhere to cell culture plates (3), is a frequently used technique of spheroid formation. However, well-to-well variability in spheroid size, shape, and growth are the frequently encountered challenges that prevent the reliable use of this method for high throughput drug screening (4, 5). Agarose-based cultures are relatively inexpensive compared to the commercially available 3D cell culture plates from different vendors, and much easier to adapt from the standard multi-well plates to microtiter plates than techniques based on scaffolds (6–8).

Liquid-overlay method has been successfully adopted in 384-well plate format for drug screening (7). Additionally, the liquid-overlay method has been miniaturized to 1536-well plates for the generation of uniform spheroids (8). Although miniaturization of assays is highly desirable for high throughput screening, edge effect is one of the common challenges often encountered in microtiter plates (9,10). Longer incubation of plates over 24 h results in faster evaporation of the medium from peripheral wells, resulting in inconsistent readouts (11, 12). Furthermore, small culture volumes in microtiter plates are more prone to evaporation-induced osmolality shifts in the medium that critically affect the growth and metabolic activities of cells (10, 11, 13, 14). To overcome some of these challenges, we have recently developed a few modifications in the liquid-overlay method for spheroid culture (15). These modifications are mainly based on possible ways to prevent uneven medium loss from multi-well plates, using instruments that are commonly found in a high throughput screening laboratory. In this chapter, we present a detailed procedure of our improved method for the generation of uniform-sized reproducible spheroids across 384-well plates.

II. Materials and instrumentation

Instruments

- Filtermax 0.22 μm "rapid" 500 mL filter (99505, TPP)
- Multidrop™ Combi Reagent Dispenser (5840300, Thermo Fisher Scientific)
- Small Tube Dispensing cassette with metal tips (24073295, Thermo Fisher Scientific)
- Standard Tube Dispensing Cassette with plastic tips (24072670, Thermo Fisher Scientific)
- CellCarrier 384-well plates (6057308, PerkinElmer)
- MicroClimb Environmental Lid (LLS-0310, Labcyte)
- SteriStore Rotary Incubator (23641, HighRes Biosolutions)
- Microplate Washer Dispenser (EL406, BioTek)
- CellVoyager High-Content Imaging System (CV7000, Yokogawa Electric Corporation)

Materials

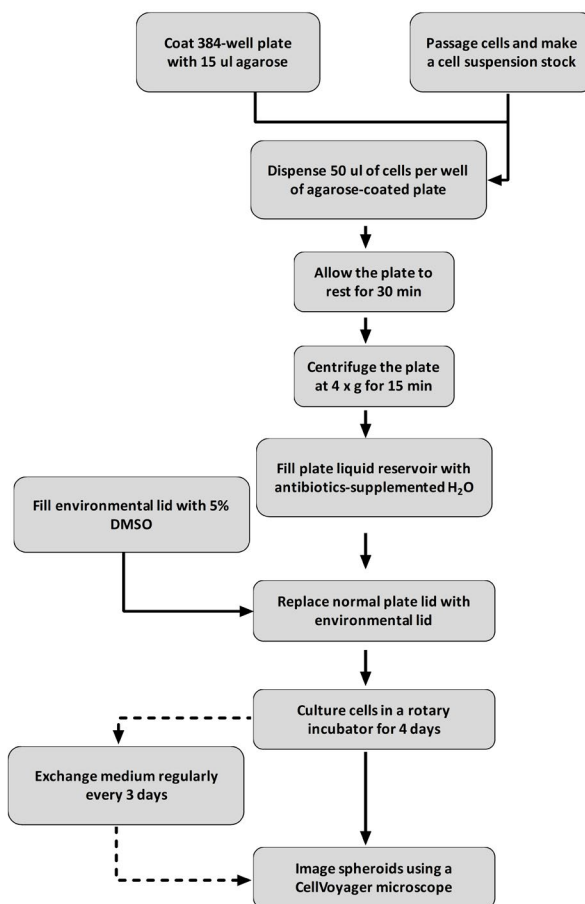
Low melting agarose (Cat. No.: A9414, Sigma-Aldrich), McCoy's 5A medium with or without L-glutamine (16600082, Thermo Fisher Scientific), Fetal bovine serum (FCS; FBS-HI-12A, Capricorn Scientific), L-Glutamine (25030081, Thermo Fisher Scientific), Penicillin-Streptomycin (15140163, Thermo Fisher Scientific), TrypLE™ Express recombinant cell dissociation reagent (12604021, Thermo Fisher Scientific), Dimethyl sulfoxide (DMSO; D4540, Sigma), Phosphate-Buffered Saline (PBS; 10X, pH 7.4, AM9625, Thermo Fisher Scientific), Ethanol (100983, Merck).

III. Procedures

Solutions

Agarose solution

- Add 0.75 g of low-melting-point agarose to 100 ml of McCoy's 5A medium without serum (with or without phenol red). Heat the



Scheme 2.1

solution in a microwave by swirling every 1-2 minutes to completely dissolve the agarose. Sterilize the solution by autoclave.

- Cool the autoclaved agarose to about 70 $^{\circ}\text{C}$ and filter it through a Filtermax 0.22 μm "rapid" 500 ml filter top by vacuum filtration in a flow box.

- Aliquot the filtered 0.75 % agarose into smaller volumes if not using the entire solution at once. Store this ready-to-use agarose solution aseptically for up to 4 weeks in a cold room or 4 °C fridge.

1× PBS (working solution)

- Dilute the stock solution of 10× PBS to 1× in deionized water. Store at 4 °C.

70 % ethanol in deionized water

- Mix 70 ml of 100 % ethanol and 30 ml of deionized water. Store at -20 °C.

5% DMSO in deionized water

- Mix 5 ml of DMSO and 95 ml of deionized water. Store at -20 °C.

A. Preparation of agarose-coated plates

1. Heat a stored aliquot of filtered agarose in the microwave to depolymerize.
2. Attach a small tube dispensing cassette with metal tips to Combi Reagent Dispenser and prime the cassette with 70 % ethanol. Perform this step in a flow box. Wash the cassette with sterile PBS to remove any residual ethanol.
3. Prime the dispensing cassette with agarose solution. Dispense 15 µl of agarose solution per well of a CellCarrier 384-well plate at medium-high dispensing speed.
4. Allow the agarose in the plate to gel for 15÷20 minutes before seeding cells or store the prepared agarose-coated plates aseptically, wrapped in a polyethylene bag in a cold room or at 4 °C, away from direct light.
5. Clean the dispensing cassette by priming with 70°C-80°C sterile water to remove any remaining agarose in the cassette tips and tubes.

B. Spheroid culture

1. Culture the cell line in an appropriate culture medium as described previously (15, 16).
2. Remove the required number of agarose-coated 384-well plates from the cold room/fridge and equilibrate it to room temperature (RT) for 15 minutes.
3. Prepare the Combi Reagent Dispenser for cell seeding by priming a standard tube dispensing cassette with 70 % ethanol followed by washing with sterile 1× PBS.

4. Adjust the seeding volume to 50 µl and dispensing speed to medium using the manual setting buttons on the dispenser. Note that the entire cell seeding is performed under sterile conditions in a flow box.
5. Dissociate adherent cells from the tissue culture flask using a recombinant cell-dissociation enzyme.
6. Make a cell suspension stock in a sterile beaker and seed cells at the desired cell density per 50 µl of the complete growth medium per well. When seeding more than one 384-well plates, stir cells using a magnetic stirrer to prevent cells from settling on the bottom of the beaker.
7. Allow the plates to rest for 30 minutes at RT and then centrifuge them for 15 minutes at 4 x g.
8. Meanwhile, take an evaporation-reducing environmental lid and fill it with 8 ml of 5 % DMSO in sterile H₂O from the short sides (left and right) using a 5 ml pipette (Figure 2A). First, dispense 4 ml of the filling liquid into the left-side trough by sweeping the pipette tip slowly up and down. Repeat the step with the right-side trough.
9. Fill the whole liquid reservoir of the 384-well plate with sterile H₂O supplemented with 1x Penicillin-Streptomycin (Figure 2B) and replace the regular plate lid with the liquid-filled environmental lid.
10. Place the plates in a 37 °C rotary incubator with 95 % humidity, 5 % CO₂, and 20 % O₂, and allow cells to aggregate into spheroids for 4 days. Avoid keeping the incubator door open too long in the subsequent days to prevent the humidity level from dropping abruptly.

Exchange of medium using the automatic dispenser

1. After the formation of spheroids on day 4, add 30 µl of pre-warmed medium per well using an automated microplate washer dispenser.
2. Allow the spheroids to grow until they attain the desired size for experiments. Replace the old medium with the fresh medium regularly every 3 days.

Note that to aspirate a defined volume of medium from each well, empirically adjust the z-height of the washer manifold. Aspirate 30 µl of medium per well and replace it with the fresh, pre-warmed, medium (30 µl).

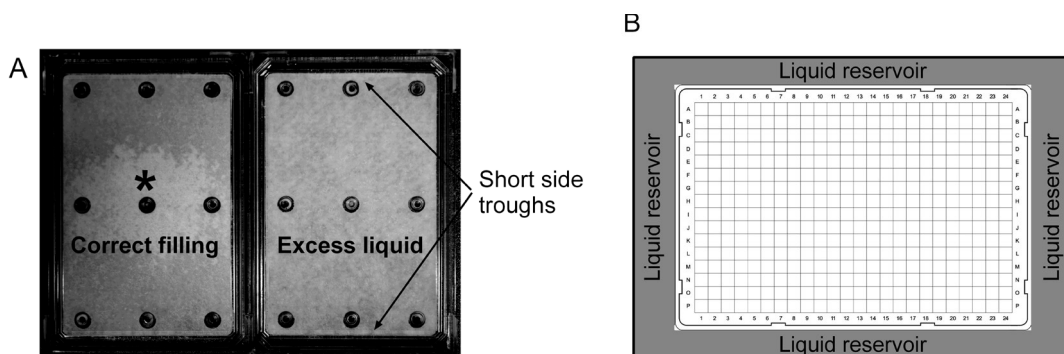


Figure 2. Preparation of environmental lids and a 384-well plate map. (A) Image of an evaporation-reducing environmental lid filled with correct (left) and excess (right) amount of 5% DMSO. The arrow indicates the short-side trough for adding liquid. Asterix shows the gap in the middle of the lid following the addition of a correct volume of DMSO. (B) A plate map showing the liquid reservoir.

Imaging of spheroids and image analysis

1. Image spheroids in CellVoyager High-Content Imaging System using a $4\times$ air objective (numerical aperture 16).
2. Set the exposure time to 11 ms, image type to 16-bit grayscale, and binning to 4×4 . Adjust the number and spacing of z-stacks and the pixel binning as desired for the experiment to capture an entire spheroid per well.
3. Subsequently, process the obtained images as described in Chapter 24.

IV. Comments

- The standard practice in the liquid-overlay method is to use a 1-1.5 % low-melting-point agarose for coating of plates, which requires the agarose and/or dispensing unit to be kept heated to prevent rapid gelling of agarose. However, diluting agarose to 0.75% and then filtering the solution, as described in our method, prevents the rapid gelling of agarose during the plate preparation step. Moreover, the use of filtered 0.75 % agarose prevents the clogging of dispensing cassette tubing and tips. The cassette can be cleaned by simply priming with heated water to remove any residual agarose.
- It is well known that evaporation-induced uneven loss of medium significantly affects the reproducibility of uniform spheroid formation and consequently compromises the outcome of assays (17). The combination of the rotary incubator and evaporation-reducing lids prevents excessive evaporation of medium from the outer wells of 384-well plates, thereby preventing well-to-well variability in drug assays (15). The presented method also results in the formation of uniform-sized spheroids of different cancer and normal cell lines (15). However, since not all cells can inherently aggregate into spheroids on a non-adherent surface, this method may not be suitable for the cell lines that were not tested for the reproducibility of uniform spheroid formation (15).
- Our method mainly focuses on the use of existing laboratory technologies, such as the advanced rotary incubator and evaporation-reducing microtiter plates and plate lids to increase the reproducibility of spheroid formation. These technologies are generally available in the majority of high throughput screening laboratories. Apart from the use of existing technologies, another inexpensive and easy solution to increase the reproducibility of spheroid formation could be the use of embryo-grade mineral oil and breathable sealing tapes or

membranes that prevent evaporation, but allow normal gas exchange (18). It has to be taken into account that agarose adds extra thickness to the plate bottoms, which can be a barrier to high content imaging. However, the depth of the field created by agarose bottom for transmitted light imaging does not hinder the identification of spheroid size by the semi-automated routine described in

Chapter 24. In our opinion, the presented method should be particularly of interest to researchers facing reproducibility issues in liquid-overlay technique of spheroid culture.

V. Pitfalls

Problem	Cause and cure
No spheroids or multiple spheroids per well	• Low cell seeding density. The usual seeding density of HCT116 cells is 2.5×10^4 cells/ml per well; however, optimize the cell density for each cell type • Cells are scattered all around the wells. Allow the plates to rest for at least 30 minutes after seeding cells. • Poor gas exchange. Ensure that the liquid added from the side troughs of environmental lids do not merge at the center of the lid and leave a gap for gas exchange (see Figure 1A). • Old serum stock. Use a new batch of serum.
Unequal medium in outer wells of 384-well plate after 3-4 days following cell seeding	• Leakage of DMSO from environmental lids to outer wells of 384-well plates. Add only 8 ml of 5 % DMSO into environmental lids. Adding an excess amount of 5 % DMSO will result in the seepage of DMSO to the exterior of the lid, and subsequently, into the outer wells of 384-well plates.
Unequal agarose dispensed per well	• Avoid repeated heating of the 0.75 % filtered-agarose solution to prevent a change in the concentration of agarose in the stock solution. Agarose-coated plates can be stored up to 2 weeks when stored under the above-mentioned conditions. The filtered-agarose solution does not require heating during coating of plates.
Loss of spheroids from wells or bubbles in wells after aspiration of the old medium	• Set the speed at which the washer manifold travels down into wells, and the dispensing speed, at the lowest rate, to minimize loss of spheroids and turbulence in the wells.

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Manual cell quantification using Bürker chamber

Natálie Kudlová

I. Introduction

Quantification of cells belongs to the essential procedures in the tissue culture laboratories. It includes various techniques that are used during establishment of the reproducible culture conditions, maintenance of the cell lines, in the transfection experiments, and during the preparation of the cells for subsequent studies requiring the accurate and consistent cell number input (1).

Frequently, the methods that allow to distinguish the living and dead cells are used. Very common variant of such methods is based on Trypan blue staining. This diazo-dye cannot be absorbed by living cells and thus only the dead cells are stained (2). Using this principle, the counting of cells can be performed manually or using automatic analyzers. For manual cell counting, a wide variety of counting chambers can be used. The history of such chambers – hemocytometers, is closely related to the rise of hematology in the mid-1,700s (3). Since that time, the chamber was progressively improved up to today's form (3, 4). Hemocytometers are widely used in both research and clinic laboratories.

They are composed of two chambers; each of them is equipped with a microscopic grid on the glass surface. Before use, it is covered with a coverslip that is attached just 0.1 mm above the chamber bottom and the mixture of cells and dye is sucked into the gap between the chamber and the coverslip. This gap determines the depth of the counting chamber (5,6).

Although the hemocytometers are very common method of cell quantification, other methods can be also used. They include the method based on the wet weights, DNA or protein content determination, evaluation of the rates of synthesis (DNA, protein), and flow cytometry analysis in combination with a fluorescent dye (1). In this chapter, the method based on the manual counting of cells using hemocytometer is described.

II. Materials and instrumentation

Instruments

- Standard handling equipment: pipettes and tips, culture flasks, 1.5 ml and 15 ml tubes

- Bürker chamber (MarienFeld, 0640210)
- Coverslips (Sigma-Aldrich, BR723015)
- Light microscope (e.g. Primovert, ZEISS, 491206-0006-000)
- Tally counter - optional (VWR, 23609-102)

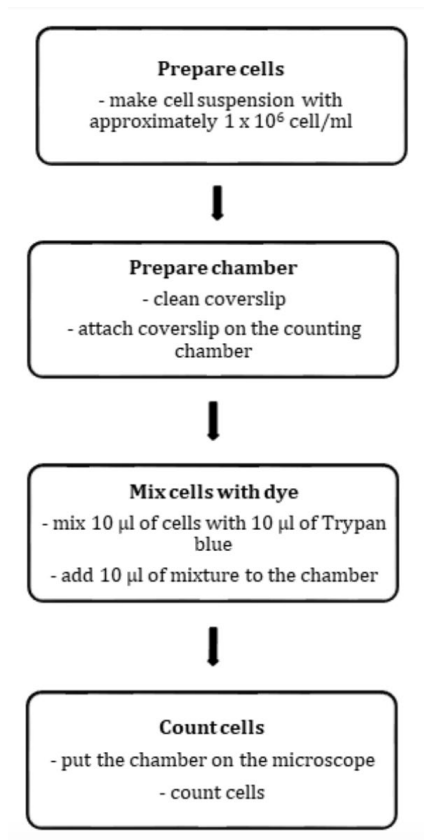
Materials

Sodium chloride (NaCl; 31434, Sigma Aldrich), potassium chloride (KCl; 60130, Sigma Aldrich), sodium phosphate dibasic (Na_2HPO_4 ; S5136, Sigma Aldrich), potassium dihydrogen phosphate (KH_2PO_4 ; P5655, Sigma Aldrich), 1× Gibco™ TrypLE Express Enzyme, no Phenol Red (TrypLE; 12604021, Thermo Fisher Scientific), 0.4 % Trypan Blue Solution (15250061, Thermo

Fisher Scientific), fetal bovine serum (FBS; FBS-22A, Capricorn Scientific), 100× Penicillin-Streptomycin solution (P4333, Sigma Aldrich), sodium hydroxide (NaOH; 06203, Sigma Aldrich).

Other commonly used material such as water (deionized or distilled), cover glasses, pipettes and tips, microtubes, tubes, filters, and immersion oil.

III. Procedures



Scheme 3.1. Scheme shows the simplified steps of the cell counting using hemocytometer.

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Dissolve above mentioned chemicals in deionized water (eight tenths of the final volume), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume. Sterilize in an autoclave. Store at room temperature; after opening, store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

Cell culture growth medium

- Liquid culture medium
- FBS
- Antibiotics, e.g. penicillin and streptomycin

Use medium recommended for the cell line used. Add FBS to the liquid culture medium. The concentration of FBS depends on the type of the particular cell line. Usually, the final concentration of FBS is 10 % or 20 %. In this respect, add 50 ml of FBS to 450 ml of the liquid culture medium to prepare the growth medium with 10 % FBS or add 100 ml of FBS to 400 ml of the liquid culture medium to prepare the growth medium with 20 % FBS. Then, add 5 ml of the antibiotic solution (the final concentration of the antibiotic solution is 1x) to the prepared growth medium with FBS. Filter the prepared complete growth medium using TOP filter Filtermax 0.22 µm. Prepare the complete growth medium under the sterile conditions in the flow box. Use the sterile labware for the medium preparation. Close the bottle with the sterile cap. Store the prepared medium at 4 °C. The complete growth medium is stable for about 3-4 weeks at 4 °C.

70 % ethanol (working solution)

Mix 74 ml of 95 % ethanol and 26 ml of deionized water; store at RT.

Cell counting using hemocytometer

1. Prepare the cell suspension and transfer it into the 15 ml tube. In the case of adherent cell line, detach the cells in a monolayer using e.g. TrypLE solution (for more details see Chapter 1).
2. Mix the cell suspension thoroughly by gentle pipetting.
3. Clean the coverslip with 70 % ethanol, dry it, but do not scratch it.
4. Put the coverslip on the chamber (Fig. 3.1 A).
5. Mix 10 µl of the cell suspension with 10 µl of Trypan Blue in 1.5 ml microtube.
6. Slowly apply 10 µl of the Trypan Blue-treated cell suspension into the chamber underneath the coverslip, allowing the cell suspension to be drawn out by capillary action (Fig. 3.1 B).
7. The second chamber can also be full-filled.
8. Transfer the chamber onto the light microscope, select a 10× objective and focus on the grid lines.
9. Zoom-in and proceed to cell counting under a 40× objective.
10. Count the cells following these rules (Fig. 3.1 C):
 - a. There are 9 large squares - count cells in 4 corner and 1 central square.
 - b. Count both living cells and dead cells (blue), in each target square, omit artefacts.
 - c. Count cells touching the left and upper border, skip the cells touching the right and lower border or vice versa, use this rule in all 5 large squares.
 - d. Calculate all the necessary values (see the step 11).
11. Below is an example of the final calculations: in 5 counting squares, 54 viable cells and 3 dead cells were counted:

a. % of viable cells

$$\frac{\# \text{ of viable cells}}{\text{total \# of cells}} \times 100 = \frac{54}{54+3} \times 100 = 94.7 \%$$

b. average # of cells / square

$$\frac{\# \text{ of viable cells}}{\# \text{ of squares}} = \frac{54}{5} = 10.8$$

c. dilution factor

$$\frac{\text{volume of cells and trypan-blue dye}}{\text{final volume input}} = \frac{200}{100} = 2$$

d. concentration (viable cells / ml)

$$\frac{\text{average \# of viable cells in square} \times \text{dilution factor} \times 10,000}{1} = 10.8 \times 2 \times 10^4 = 2.16 \times 10^5 \text{ viable cells / ml}$$

Factor 10^4 is equal to ratio of 1,000 mm³ (1 ml) and 0.1 mm³ (the volume defined by one square).

IV. Comments

- A wide range of counting chambers which differ in the purpose of use, the grid and accessories is available. In this chapter, the cell counting using Bürker chamber was described. However, it is possible to use variations of Bürker chamber or others like Neubauer, Tuerk, Fuchs-Rosenthal, Nageotte, Malassez, and Thoma. Moreover, other approaches can be used. They include flow cytometry, measurement of DNA content or protein amount. But nonetheless, Trypan blue exclusion method plays still important role in the cell counting.
- If you find out that the chamber is overloaded with cells, dilute the original cell suspension to reach the lower number of the cells for easier counting.
- The work with Trypan Blue dye is dangerous. This solution is hazardous in the case of an ingestion or an inhalation and slightly hazardous in the case of a skin or an eye contact (it is irritant).

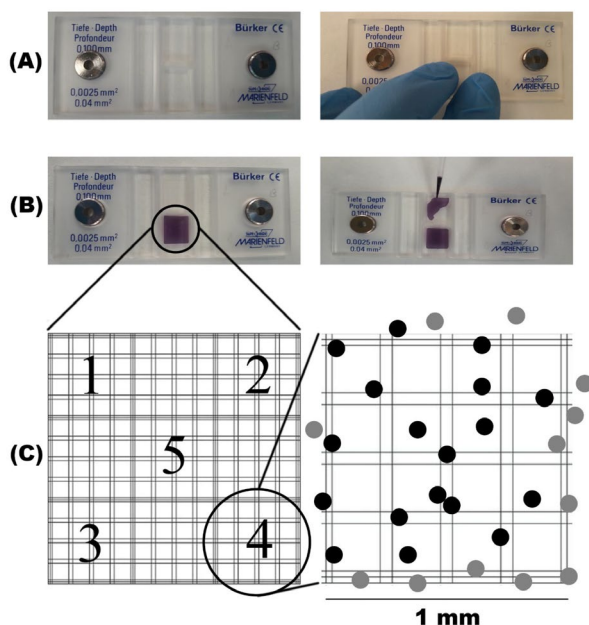


Figure 3.1. Technique for the cell counting using the Bürker chamber.

(A) Bürker chamber from MARIENFELD Germany and the positioning of a coverslip. (B) Adding of a dyed cell suspension. (C) Magnified scheme of a whole grid. The grid (3 x 3 mm) consists of 9 large equal squares which are separated by a triple line from the others. Each (1 x 1 x 0.1 mm) of these 9 squares contains 16 small squares. Numbers 1 - 5 label one central and four corner squares that are used for cell counting. On the right side there is an example of counting in one of total five counting squares. Black dots represent countable cells and grey ones are uncountable. In each counting square you have to follow the rule that describes cell counting only on two from four square sides. For instance, at the beginning decide to count cells on/touching left and upper sides (not right and lower) as it is in the picture.

V. Pitfalls

Problem	Cause and cure
Error in obtaining the sample	Analyzed sample has a different concentration than the original sample. Use a pipette to mix the cell suspension repeatedly and analyze the sample as soon as possible.
Error in loading of cell suspension into the chamber	A small sample was evaluated. Use 4 - 5 grids of the counting chamber, repeat the calculation 3x. More samples mean lower maximum error range.
High statistical error	A small sample was evaluated. Use 4 - 5 grids of the counting chamber, repeat the calculation 3x. More samples mean lower maximum error range.
Error in the distribution of the cells in the chamber	Samples are not distributed equally in the chamber. Shake the samples before loading into the chamber, make sure that the chamber lies horizontally.
Error in dilution	Wrong volumes of solutions were used. Calibrate pipettes and other used instruments.
Error in counting	Aggregates of cells were formed. Count the cells immediately after loading them into the chamber.
Human errors	- Improper visualization method, omission of some cells. Optimize microscope setting and use an appropriate magnification. Loss of counts. Start the counting again. - Use of wrong formula. Check the size of the chamber and correct the formula accordingly. - Counting on all borders. Repeat the calculation using the rules for counting on borders (see the step 10).

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STR profiling for cell line authentication

Jana Stránská

I. Introduction

Authentication of a cell line is the process by which a line's origin is verified by showing to be a genuine one, free of contamination from other cell line(s). Using Short Tandem Repeats (STR) profiling, authentication enables cooperation among all users about the cell line resource and helps to reach valid experimental results. Without scheduled testing, over-subcultured, misidentified or cross-contaminated cell lines are released into the research community resulting in biased, erroneous data (1,2). Skipping cell-line authentication can lead to loss of time, money, and misleading publications owing to cross-contamination and misidentification. For example in 2013, a team had to retract work published in *Nature Methods* because the cells they studied were contaminated with other cells (3,4).

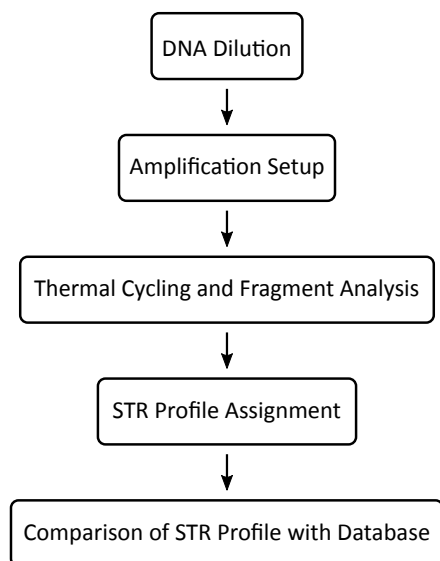
The largest general service culture collection in the world, an American Type Culture Collection (ATCC), not only recommends regular testing of cultured cell lines by STR, but also morphology check by microscope, growth curve analysis, and species verification by isoenzymology (2).

The main test for authenticating cell lines is STR profiling. The same method is used in criminalistics for identification of persons. In principle it is the analysis of microsatellite regions of DNA that have variable numbers of tandem repeats (4 to 5 bp) and are located throughout the genome. PCR amplicons are generated using primers for regions that flank these polymorphic sites. After amplification, PCR products are resolved using capillary electrophoresis, and a profile for these repetitive regions is created. Profile consists of two numbers (corresponding to number of repeats) for each locus. In contrast to STR testing in criminalistics, where DNA profile of individual is unique and does not change in their lifetime, interpretation of STR testing of cell lines is less strict because of genetic changes in cell line during cultivation and passaging. With more than 80% match with a 15-loci profile in cell line database, you can be reasonably confident that you are working with the cell line that you think you are working with (3). Cell lines that match 60%–80% of STR loci need further investigation.

II. Materials and instrumentation

- Cell line DNA
- Laboratory gloves
- Single/Multichannel pipettes
- Aerosol-resistant pipette tips
- 1.7 ml reaction tubes, DNase/RNase-free
- 96-well reaction plates or 0.2 ml tubes compatible with PCR cyclers
- PCR tubes holders
- PCR cycler with silver or gold heating block
- Centrifuge compatible with 96-well plates or reaction tubes
- GeneMarker HID - STR Human Identity Software, SoftGenetics
- Flow-box for pre-PCR work
- Flow-box for post-PCR work
- PowerPlex® 18D System, DC1802 (200 reactions) or DC1808 (800 reactions), Promega Corporation, East Port

III. Procedures



Scheme 4.1

A. DNA Dilution

1. Prepare PCR tubes/strips/plates according to number of your samples.
2. Thaw cell line DNAs, 2800M Control DNA, Amplification-Grade Water. Vortex and

shortly centrifuge DNA tubes.

3. Dilute your positive control DNA (2800M Control DNA, 10 ng/μl) and cell line DNAs to 5 ng/μl into prepared PCR tubes/strips/plates with Amplification-Grade Water. For example, use 1 μl of DNA and add Amplification-Grade Water according to the formula.

$$V_{\text{Amplification-Grade Water}} (\mu\text{l}) = \frac{c_{\text{DNA}}(\text{ng}/\mu\text{l})}{5 \text{ ng}/\mu\text{l}} - 1 \mu\text{l}$$

B. Amplification Setup

1. Thaw all pre-amplification components of PowerPlex® 18D System (PowerPlex® 18D System for Use on the Applied Biosystems® Genetic Analyzers, Technical Manual (<https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/powerplex-18d-system-protocol.pdf?la=en>) PowerPlex® D 5X Master Mix, PowerPlex® 18D 5X Primer Pair Mix, allelic ladder – commercial or home-made by 10000x diluting mixture of STR amplicons from previous run).
2. Prepare PCR tubes/strips/plates according to number of your samples.
3. Vortex the components thoroughly for 15 seconds. Centrifuge tubes briefly, then vortex for 15 seconds before each use. Do not centrifuge after vortexing to avoid the reagents to be concentrated at the bottom of the tube.
4. Make a universal PCR cocktail that ensures that each reaction contains the same PCR amplification mix: determine the number of reactions including positive (2800M Control DNA), allelic ladder, and negative controls (Amplification-Grade Water). Add 1.17x multiple to this number of reactions to compensate for pipetting error.
5. Prepare the PCR amplification mix in 1.7 ml reaction tube by combining the components as in Tab. 4.1:

TABLE 4.1. Composition of PCR amplification mix

Component	Volume per reaction (µl)	Volume for 12 reactions (µl)	Volume for 24 reactions (µl)
Amplification-Grade Water	6.5	91.3	182.5
PowerPlex® D 5X Master Mix	2.5	35.1	70.2
PowerPlex® 18D 5X Primer Pair Mix	2.5	35.1	70.2

- Mix gently PCR amplification mix by pipetting up and down 10 times.
- Pipet 11.5 µl of PCR amplification mix into each reaction well.
- Add 1 µl of controls and DNA to the respective well/tube. In the case of higher amount of the samples, use multichannel pipette.
- Seal the plate or close the tubes. Shortly vortex and briefly centrifuge the plate to bring contents to the bottom of the wells and remove any air bubbles.

C. Thermal Cycling and Fragment Analysis

- Place the tubes/strips/plate in the thermal cycler.
- Run the recommended protocol with the following conditions (Tab. 4.2, in-house validation and cycle number optimization can be required for a different thermocycler):
- After completion of the thermal cycling protocol, proceed with fragment analysis or store amplified samples at -20 °C in a light-protected box.
- Fragment analysis can be performed internally on validated capillary sequencer with compatible lasers. However, it is advantageous to outsource this step to an external

company, e.g. SeqMe Ltd. that has sample collection boxes at the important research centers of the Czech Republic. Prepare your samples into an envelope, add 0.2 ml tube with 0.5 µl/per sample of WEN Internal Lane Standard 500 (ILS500; content of the kit), 0.2 ml tube with 2 µl of PowerPlex® 18D Allelic Ladder Mix (content of the kit), and list of the samples with your identification (following requirements published on SeqMe webpages).

- Mail to lab@seqme.eu one day before that you intend to use collection box or send samples by courier service.

D. Data Analysis

- Data can be downloaded from SeqMe web in the form of .fsa files. Download them to your computer.
- Launch GeneMarker HID (GeneMarker® HID, Quick Start Guide (https://softgenetics.com/PDF/GM_HID_QuickStart.pdf), GeneMarker® HID, User Manual (https://softgenetics.com/PDF/GeneMarkerHID_UserManual.pdf).
- Click *File* → *Open Data* → *Open Data Files*. Navigate to directory containing .fsa files. Select all files by CTRL+A or use CTRL and/or SHIFT keys to select individual samples.
- Click *Open* button in the *Open dialog*. The files selected will appear in the Data File List field.
- Click *OK* button in the *Open Data Files* box and the samples will be uploaded to GeneMarker HID.
- Click *Run Wizard* , set *Template Name* PowerPlex_18D, Panel PowerPlex_18D v1.2, Size Standard ILS500, Standard Color LIZ. Click *Next*>>.

TABLE 4.2. Thermal cycling profile

Temperature	Time	Cycle number
96 °C	2 min	1x
94 °C	10 s	
60 °C	1 min	30x
60 °C	20 min	1x
4 °C	hold or remove	1x

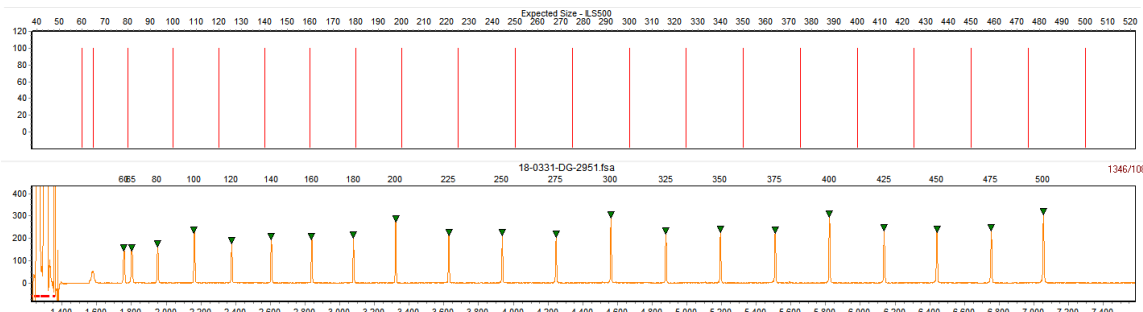


Figure 4.1. Calibration window

7. In *Data Process – HID Analysis*, mark: for *Raw Data Analysis - Auto Range* (frame), Smooth, Peak Saturation, Baseline Subtraction, Pull-up Correction, Spike Removal; for *Size Call – Local Southern*; for *Allele Call – Auto Range*, Peak Detection Threshold Min Intensity 50, Max Intensity 32760, Percentage >20 Global Max. Click *Next>>*.
8. In *Additional Settings – HID Analysis*, uncheck Auto Select Best Ladder, choose your *Allelic Ladder*, choose *Positive Control Template - 2800M*, for *Allele Evaluation* set Peak Score: Reject < 0.00 **Check** 0.20 < **Pass**. Do not tick *Mixture Evaluation*. Click Ok. Data Analysis starts.
9. After analysis is finished, samples (on the left, *Allele Call*) are colored according to their success in size call. Green  means high lane quality, yellow  requires verification, red  sizing did not occur.
10. From main toolbar icons, click *Size Calibration*  and go through all the samples. Add, delete or adjust peaks (right click), if ILS500 peaks in the samples (orange trace, Fig. 4.1) do not correspond to Expected Size Standard (red trace). Green triangle atop the ILS500 peak shows a concord with the expected size. Close Calibration window.
11. On the left, choose Positive Control 2800M only. From *Show Color*  on the main toolbar, choose one color, for example the blue one. From *Marker*  Marker, choose the first locus D3S1358. Expected results for 2800M are D3S1358 17, 18 alleles. Continue with following loci, true alleles for 2800M are shown in Tab. 4.3.
12. The panel can be adjusted if needed. On the main toolbar, go to *Tools → Panel Editor* . Let's start again with the D3S1358 locus.

Zoom in to the D3S1358 locus by left-click, hold and draw a rectangle at the same time.

13. Hold down the ↑Shift key and at the same time place the mouse over the gray marker name bar at the top of the electropherogram. The marker rectangle will be outlined in red and the panel name will be in red font when the adjust feature is active. Drag the marker to align the bins (vertical grey bars) with the peaks (Fig. 4.2).
14. Drag to the next marker with the right-click and horizontal movement and continue with other markers. Save the results.

TABLE 4.3. Alleles of positive control 2800M

Marker	Alleles
D3S1358	17, 18
TH01	6, 9.3
D21S11	29, 31.2
D18S51	16, 18
Penta E	7, 14
D5S818	12, 12
D13S317	9, 11
D7S820	8, 11
D16S539	9, 13
CSF1P0	12, 12
Penta D	12, 13
Amelogenin	X, Y
vWA	16, 19
D8S1179	14, 15
TPOX	11, 11
FGA	20, 23
D19S433	13, 14
D2S1338	22, 25

15. Activate all the samples on the left list by double-click. Open D3S1358 locus again and go through individual alleles and their bins. Moved bins can be adjusted by holding down the ↑Shift key and left-clicking on the gray, vertical bar for the bin at the same time. The bar will turn blue. Use the mouse to drag the blue vertical bar to the center of the peak (Fig. 4.3).
16. Go through all the alleles. Save the aligned panel.
17. Close the *Panel Editor*. All the samples are re-analyzed.
18. Go through individual sample. Control red alleles. Confirm or delete them. Delete alleles in position of artefacts from capillary electrophoresis and/or polymerase chain reaction (Fig. 4.4, *e. g.* stutters, pull-up peaks, blobs, spikes, incomplete adenylation, allele drop-in, drop-out, sister allele imbalance).
19. If any of the allele is out of bin, do not edit whole *Panel* (it unbalances all previous analyses), but click on the peak and use *Edit Allele*. Save the results (*File* → *Save Project*).
20. For comparison with cell line database, click on the main toolbar on *Applications* → *Cell Line Authentication*.
21. On top on the right, set *Show matches* greater than 60%, *Sex marker* Amel.
22. In *Sample* column, set the sample one by one. In *Reference* column, the best match appears. In the table on the right, other matches above 60% appear.
23. Write the results down to your result table. Save the results (possible only for individual sample).

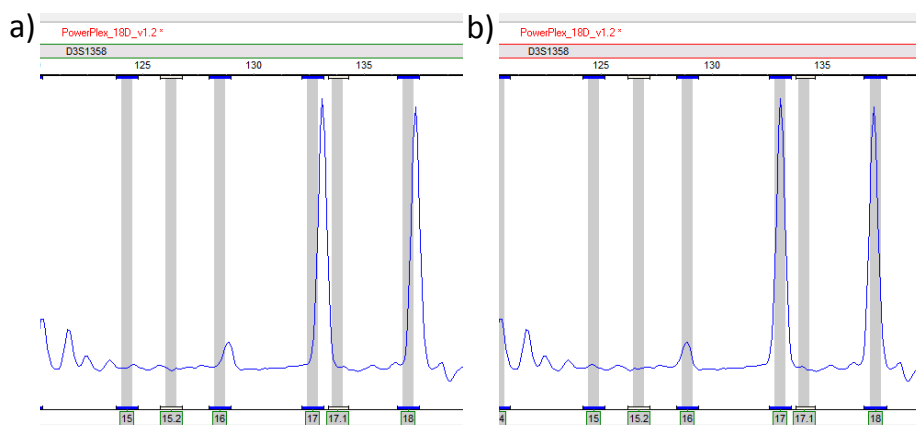


Figure 4.2. Alignment of the whole locus

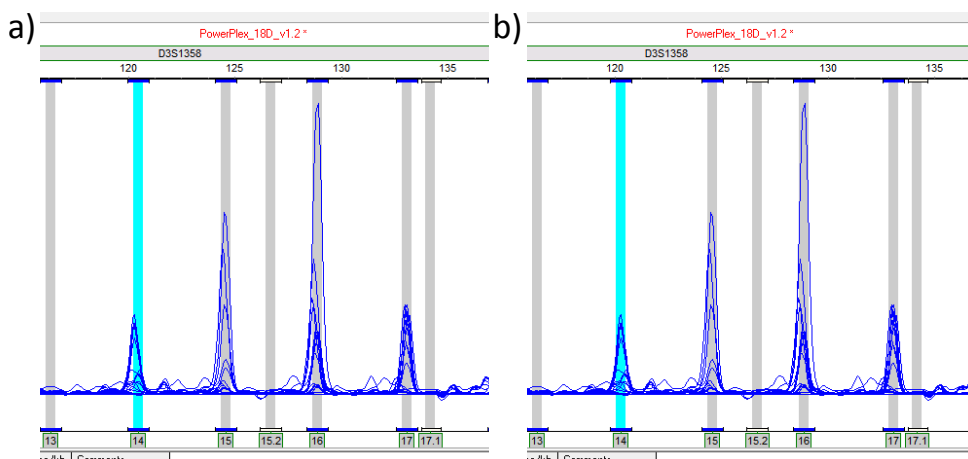


Figure 4.3. Alignment of the individual allele

IV. Comments

Aluminum heating blocks have different heat-transfer properties and may not reach the required temperature uniformly across the block in the programmed time and, therefore, they are not recommended. When forced to use aluminum block, modify PCR program according to <https://worldwide.promega.com/resources/profiles-in-dna/2016/technical-tips-for-str-analyses/>.

When you start with PowerPlex® 18D System, successful amplification could be verified by agarose or capillary electrophoresis of at least few PCR products (see chapter 50).

Use of PowerPlex® 18D Allelic Ladder Mix can

be avoided by preparation of your own allelic ladder by mixing and dilution of the amplicons from the previous run. Diluted mixture is then used as another DNA sample. When allelic ladder is not used (what is not recommended), set Allelic Ladder as “NONE” during data analysis (step 8).

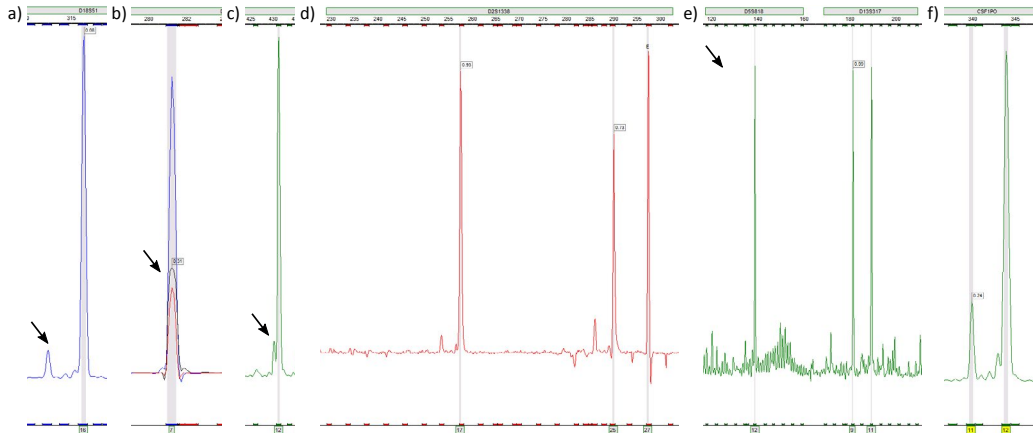


Figure 4.4. Artefacts complicating STR analysis: a) stutter, b) pull-up peaks, c) incomplete adenylation, d) allele drop-in, e) allele drop-out, and f) allele imbalance

V. Pitfalls

Problem	Cause and cure
STR Analysis	• With cancer cell lines it can be difficult to distinguish low-level STR peaks from noise. Using the recommended amount of DNA can help minimize the noise. • Using positive and negative controls helps troubleshoot STR profiles. • Artifacts may require a repeat of the STR protocol. • ‘Stutter peaks’ are usually lower than true allele peaks, but using too much DNA can lead to extremely high stutter peaks, making them harder to distinguish from true allele peaks. • Dye blobs can appear as wide peaks, which the software might assign as regular peaks. The blobs are formed when fluorescent dye tags detach from their primers. Sometimes this can happen if kits are not stored as vendors recommend. • Pull-up or bleedthrough. Pull-up can occur when peak heights are too high or if a poor or incorrect matrix is applied to the sample. • STR profiling software can identify atypically sized allele microvariants. Vendor kits might not cover all microvariants. • Extra peaks visible in one or all color channels can be caused by contamination with another template DNA or previously amplified DNA.
STR Analysis	• Cross-contamination can be a problem. Use aerosol-resistant pipette tips, and change gloves regularly.
PCR Problems	• Storage of the kit, melting, vortexing, PCR cycler, and plastics. Optimization of cycling profile may be needed.

For detailed troubleshooting guide, see Technical manual for PowerPlex® 18D System, Chapter 7 Troubleshooting.

VI. Acknowledgement

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PowerPlex® 18D System for Use on the Applied Biosystems® Genetic Analyzers, [Technical Manual](#)

GeneMarker® HID, [Quick Start Guide](#)

GeneMarker® HID, [User Manual](#)

Detection of mycoplasma contamination in cell lines by PCR

Renata Buriánová and Jana Kotulová

I. Introduction

Mycoplasma is one of the most common contaminants encountered in cell culture laboratories (1, 2). Mycoplasmas are the smallest living self-replicating organisms unrecognizable under phase contrast microscope (3). Frequently, its presence in culture media goes unnoticed up to concentration of 10^8 mycoplasma/ml causing no visible changes in the turbidity or pH changes in the growth media. Mycoplasmas are further characterized by slow generation times and relatively long lag phase. Altogether, it may take weeks to notice mycoplasma contamination. Moreover, these organisms are resistant to most antibiotics often used in laboratories (4).

Mycoplasmas were first thought to be cytoadherent organisms; however, some mycoplasma species can be also found in the intracellular space (5). The most frequent mycoplasma species promoting contamination are listed in Tab. 5.1. Mycoplasma contamination can result in inhibition of cell proliferation due to nutrient withdrawal and secretion of metabolites, alterations in RNA and DNA synthesis, potential changes in cellular metabolism and gene

expression profile, chromosomal aberrations, and increase or decrease of virus propagation. It could even lead to complete degeneration of a cell line. Furthermore, this infection affects cell behaviour practically in every aspect, which could significantly change the outcome of conducted experiments and results. It interferes with a variety of biochemical assays, decreases cell transfection rate, promotes cellular transformation, etc. (reviewed in (6)).

In the past, the predominate source of mycoplasma contamination were fetal calf serum lots provided by commercial suppliers (7). Another source of possible contamination is of human origin, i.e. ascribed to laboratory personnel (6). Nonetheless, the major cause of mycoplasma spreading is nowadays attributed to already mycoplasma-infected cell lines.

Currently, there is a number of means developed for detection of mycoplasma presence at hand, most of them complex and time-consuming. The most recommendable methods from this group are Plasmotest (Invivogen), DAPI staining, and PCR. Plasmotest utilizes a reporter cell line expressing Toll-like Receptor 2 (TLR2). Recognition of

pathogens results in this setup in coloration of the medium that is spectrophotometrically detectable. DAPI staining, is based on microscopic analysis of DNA staining with fluorescent dye. However, regarding to above mentioned mycoplasma characterization, polymerase chain reaction (PCR) is the most sensitive, reliable, reproducible, and convenient method for routine examination of samples (8). The chapter portrayed herein represents routine check for detection of mycoplasma contamination in cultured cell lines by PCR.

TABLE 5.1 Most frequent mycoplasma species promoting contamination (adapted from (6)).

Species	Frequency	Natural host
<i>M. orale</i>	20–40%	Human
<i>M. hyorhinis</i>	10–40%	Swine
<i>M. arginine</i>	20–30%	Bovine
<i>M. fermentans</i>	10–20%	Human
<i>M. hominis</i>	10–20%	Human
<i>A. laidlawi</i>	5–20%	Bovine

II. Materials and instrumentation

Reagents

Proteinase K (Cat. No. 82452, Sigma-Aldrich), Tris-HCl (Cat. No. T3253, Sigma-Aldrich), KCl (Cat. No. 60128, FLUKA), Triton X-100 (Cat. No. 93443, Sigma-Aldrich), EDTA (Cat. No. 11280, SERVA), SDS (Cat. No. 82452, Sigma-Aldrich), 10^x Taq Reaction buffer (Cat. No. AB 0301B, Thermo Fisher Scientific), 50 mM MgCl₂ solution (Cat. No. AB 0301B, Thermo Fisher Scientific), Taq DNA Polymerase (Cat. No. AB 0301B, Thermo Fisher Scientific), DEPC-treated water (Cat. No. AM 9906, Ambion, Thermo Fisher Scientific), 100 mM dATP (Cat. No. U120C, Promega), 100 mM dGTP (Cat. No. U121C, Promega), 100 mM dTTP (Cat. No. U123C, Promega), 100 mM dCTP (Cat. No. U122C, Promega), forward primer

Myco 1 (custom-made, Generi Biotech), reverse primer Myco 2 (custom-made, Generi Biotech), fluorescent probe Myco P (custom-made, Generi Biotech). For oligonucleotide sequence see below.

Consumables

Centrifuge conical tubes 15 ml (Cat. No. 91015, TPP), microcentrifuge tubes 1.5 ml (Cat. No. 3810X, Eppendorf), 8-tube strip for PCR 0.1 ml (Cat. No. B72811, BIOplastics), cell scraper (Cat. No. 99002, TPP).

Instrumentation

- Box Mars Safety classe 2 flowbox (Trigon-plus)
- LightCycler® 480 Instrument (Roche)
- Cetrifuge 5430 (Eppendorf)
- Vortex (Labnet international)
- Dry Block Thermostat (Biosan)

III. Procedures

1. Sample preparation for PCR detection

Solutions

Lysis buffer

- 1 ml 10 mM TRIS-HCl (pH 8.3)
- 1 ml 50 mM KCl
- 1 ml 1% TRITON-X 100

Bring to a final volume of 100 ml with deionized water.

Proteinase K solution

- 40 mg proteinase K lyophilized powder
- 2 ml 10% SDS
- 40 µl 1 M EDTA (pH 8,0)

Bring to a final volume of 20 ml with deionized water. Prepare aliquots, avoid freeze-thaw cycles. Store at -20 °C

Lysis buffer with proteinase K

Mix 1.045 ml of lysis buffer and 55 µl of proteinase K solution, prepare aliquots of 100 µl, avoid freeze-thaw cycles. Store at -20 °C

Steps

1. Ideally, cultivate cells for at least two weeks or three subcultures (whichever is greater) without any antibiotics in culture medium if possible (for further information on cell culture maintenance see chapter 1).
2. Harvest cell culture sample by cell scraper to avoid repeated washing during enzymatic harvesting.
3. Collect cell suspension containing approximately $2.5 \times 10^5 - 5 \times 10^5$ cells.
4. Spin down the sample of corresponding volume at 2,000 RPM for 5 minutes at room temperature.
5. Discard the supernatant and add 100 μ l of lysis buffer with proteinase K, mix thoroughly.
6. Transfer the sample to the 1.5 ml microcentrifuge tube.
7. Incubate at 37 °C overnight, alternatively at 56 °C for 1 hour.
8. To inactivate proteinase K prior the PCR reaction, incubate samples for another 10 minutes at 95 °C.
9. Store the DNA samples at -20 °C or directly use for PCR.

2. Mycoplasma detection by PCR method

For mycoplasma detection by RT-PCR, we use 16 S rRNA gene sequence (996 bp) specific primers and specific Myco P probe can be used.

Solutions

- Myco P probe stock solution (s.s.)**
Dilute lyophilised Myco P probe in DEPC-treated water to a final concentration of 0.1 mM.

- Myco P probe working solution (w.s.)**
Dilute s.s. of Myco P probe in DEPC-treated water to a final concentration of 1.25 μ M.

- Primers Myco 1 and Myco 2 s.s.**
Dilute lyophilised Myco 1 and Myco 2 in DEPC-treated water to a final concentration of 0.1 mM.

- Primers Myco 1 and Myco 2 w.s.**
Dilute s.s. of Myco 1 and Myco 2 in DEPC-treated water to a final concentration of 10 μ M.

- 10 mM dNTPs:**
Dilute 100 μ l 100 mM dATP, 100 μ l 100 mM dTTP, 100 μ l 100 mM dGTP, 100 μ l 100 mM dCTP in 600 μ l of DEPC-treated water.

- Master reaction mix (MRM) for final volume of 25 μ l**

Reagent	Volume /well (μ l)
DEPC treated water	10.7
10 $\times$ Taq Reaction buffer	2.5
50 mM MgCl ₂ solution	2.0
10 μ M Myco 1 Forward Primer	2.0
10 μ M Myco 2 Reverse Primer	2.0
1.25 μ M Myco P	4.0
10 mM dNTPs	0.5
Taq DNA Polymerase (5 U/ μ l)	0.3
(sample DNA – added separately to wells)	1.0

Primers and probes

Myco 1 forward primer	5' GCTGTGTGCCTAATACATGCAT 3'	22 bp
Myco 2 reverse primer:	5' CACCATCTGTCATTCTGTTAACCT 3'	24 bp
Myco P probe:	5' ATCCGCACAAGCGGTGGAGC 3'	20 bp

Steps

1. Upon receiving the lyophilisates of probes, dilute them in DEPC-treated water to 0.1 mM concentration (s.s.), store at -20 °C.
2. Prepare w.s. of Myco 1, Myco 2 and Myco P, store at -20 °C.
3. Prepare MRM for total number of samples plus approximately 10 % more than is the minimum required volume for the experiment. Keep the reagents and MRM at 4 °C during the preparation.
4. Dispense 24 µl of MRM per well in 0.1 ml 8-tube strip, keep it at 4 °C.
5. Transfer 1 µl of sample DNA from 1.5 mL microcentrifuge tube to intended wells, include positive (isolated DNA from mycoplasma positive cells) and negative controls (DEPC-treated water).
6. Centrifuge 8-tube strips at 2,000 RPM for 2 minutes at room temperature to remove any bubbles.
7. Program the thermocycler (LightCycler® 480) using the following parameters:

Step		Temperature	Time
Initial denaturation		95 °C	15 min
40 Cycles	Denaturation	95 °C	45 sec
	Annealing	55 °C	1:40 min
Hold		4 °C	forever

8. Install the strips in the instrument and start the program.

3. Result analysis

The resulting fluorescence signal is measured at 465 – 510 nm channel. Results are automatically analysed in the LightCycler® 480 software. Every sample with fluorescence signal higher than noise band should be considered as mycoplasma positive (Fig. 5.1).

IV. Comments

It is essential to understand the mycoplasma characteristics. Its size of 0.1 – 0.8 µm in diameter together with its lack of a rigid cell wall are distinct features allowing mycoplasmas to penetrate through commonly used filters with 0.2 – 0.45 µm diameter. Despite many possibilities and products on offer, elimination of mycoplasma contamination requires tremendous endeavour which still may turn out to be fruitless. It is therefore imperative to maintain good laboratory practice for aseptic culture technique.

Another possibility to minimize the risk of mycoplasma contamination is prevention of spreading. Newcomer cell lines should always be cultivated separately from continuous cultures, authenticated, and systematically tested before their introduction to the laboratory where the continuous cell lines are cultivated on regular basis.

Lacking cell wall, mycoplasmas are in general resistant to the most of commonly used antibiotics (e.g. penicillin). There are other effective antibiotics available with detrimental impact on eukaryotic cells as well, though. The cost-effectiveness and efficiency of mycoplasma elimination is therefore questionable and recommended only in cases of rare cell lines.

As with every method intended to be routinely used in the laboratory, RT-PCR detection of mycoplasma contamination should be validated. For validation, include for example six negative and seven positive cell lines and use Agilent chip 2100 expert High Sensitivity DNA Assay.

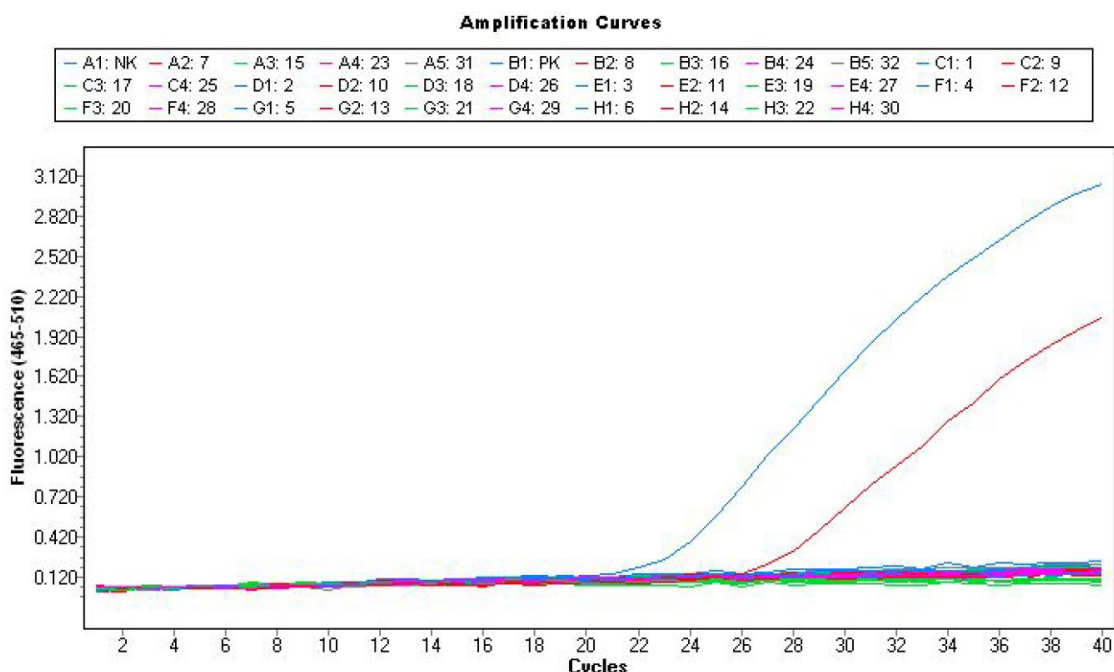


Figure 5.1. Representative example of RT-PCR mycoplasma detection result with 2 positive results above the noise band: positive control with crossing point (CP) = 22.85 and mycoplasma-positive sample with CP = 27.34.

V. Pitfalls

Problem	Cause and cure
False negativity	- Excessive amount of DNA in PCR sample could interfere with the method. It is advisable to strictly follow the cell number per sample in the protocol. - Not even do the commonly used antibiotics fail to successfully treat the mycoplasma infection, they can even serve to mask it and suppress the manifestation of contamination. Correct sample preparation is another crucial step. In order to unmask possible mycoplasma-infected cells, it is eligible to maintain the cells in culture for at least two weeks or 3 – 4 subcultivations in antibiotic-free medium, especially after thawing the cell line.
False positivity	Usual care should be taken to avoid contamination during PCR setup.

VI. Acknowledgement

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Microbial eradication from contaminated cell cultures

Ermin Schadich

I. Introduction

Microbial contamination is a common problem in culturing human and animal cell lines (1, 2). Currently, no cell culture laboratory remains unaffected by this problem because the sources of contamination are ubiquitous and their identification and elimination are difficult. A wide variety of microbial species including bacteria, fungi, protozoa, and viruses may contaminate cell culture. The culture requires a highly nutrient medium, which also provides the minimum requirements for the growth of microorganisms and their spores (3-5). The damages to the cell cultures caused by microorganisms compromise research results and preclude the use of cells for medical therapy such as stem cell therapy (3, 6, 7).

The most frequent microorganisms associated with microbial contaminations are bacteria of genus *Mycoplasma*, other bacterial species and fungi with the contamination incidence of 19, 4, and 8 %, respectively (4, 7-9).

The major source of cell culture contamination is introduction of microorganisms to cultures by unintentional use of non-sterile supplies

such as media and solutions, chemicals and equipment, and specifically by aerosols that fall out during transportation and incubation (6, 10). Specifically, the fetal bovine serum obtained from commercial suppliers has been identified as a major source of mycoplasma contamination (9). The contamination can cause the loss of several plates or flasks and the complete loss of experiments. Further, cross-contamination can occur when working with multiple cell lines at the same time (4, 11, 12).

The microbial contamination can be identified as macroscopic and microscopic changes before the massive cell deaths occur (Tab. 6.1). The bacterial contamination causes the rapid increase in turbidity and drop in pH indicated by a change in the color of the medium of the affected culture (1, 4, 8). Bacterial cells are much smaller than eukaryotic cells and they appear as dark rod-like structures or coccoid or spiral structures. They may exist as single cells, in pairs, chains, or clusters (1, 10). The fungal contamination appears as thin filamentous mycelia and clumps of spores, while yeast cells appear as oval or round particles (1, 2, 8) (Tab. 6.1). More specific identification of bacterial

and fungal microbes can be done by histological staining with Hoechst or Giemsa stains, fungal specific staining and distinctive qPCR tests (4, 9, 10). Viruses and mycoplasma cause mostly invisible changes in the contaminated cultures,

and their final effects can be seen only in the later stages (5, 9, 13). Specifically, viral contamination causes the rapid development of massive cellular necrosis and loss of cell cultures (5).

TABLE 6.1. Characteristics of contaminated cell cultures.

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drops	pH drops in heavy infections	pH drops in some infections
Cloudy medium: Under microscope (100–400 x)	rods, cocci, and spirals	round or ovoid particles that bud off small particles	thin filamentous mycelia; sometimes clumps of spores

All of the microbial contaminations should be eliminated as soon as detected (3, 6, 14). All of the contaminated cultures should be treated by autoclaving or hypochlorite and discarded (3, 4, 8). However, if the contaminated cultures are irreplaceable, they can be considered for treatments for eradication of microbial contamination (3, 10). In this chapter, three different procedures for the eradication of bacterial, mycoplasma, and fungal contamination of tissue culture are described.

II. Materials and instrumentation

Instruments

- Phase contrast inverted microscope (IX51, Olympus)
- Pipettor (9907200, Hirschman)
- Vortex Lab Dancer (0003365000, IKA)
- Laboratory shaker ThermoMixer® C (5382000015, Eppendorf)
- The positive displacement pipette for the pipetting of viscous liquids (for example Microman ME100, FD10004, Gilson)
- Centrifuge (5810R, Eppendorf)

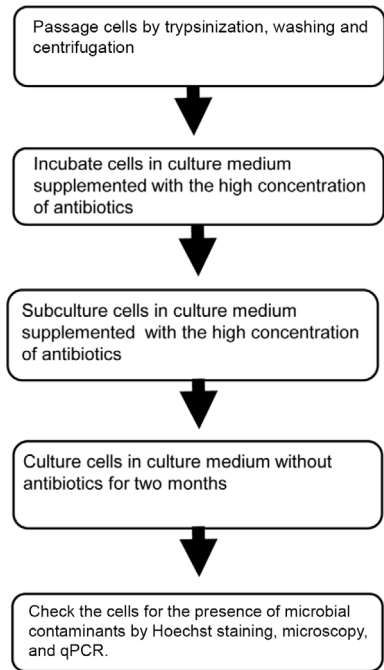
Materials

Dimethyl sulfoxide (DMSO; 34943, MERCK), dimethylformamide (DMF; 227056, MERCK), Hank's Balanced Salt Solution Powder (BSS; 10474672, HyClone™), Luria Bertani powder

(LB, L3022, MERCK), TrypLE Express (12604013, Thermo Fisher Scientific), penicillin-streptomycin (15140122, Thermo Fisher Scientific), ampicillin (A6140, MERCK), erythromycin (114-07-8, MERCK), gentamicin sulfate (E6376, MERCK), kanamycin sulfate (10106801001, MERCK), neomycin sulfate (1405-10-3, MERCK), streptomycin sulfate (S6501, MERCK), ciprofloxacin (Ciprobay 100, Bayer), tetracycline hydrochloride (T3383, MERCK), amphotericin B (A9528, MERCK) tylosin tartrate (T6271, MERCK) and nystatin (N6261, MERCK).

In addition, the commonly used consumables such as tweezers, parafilm, Petri dishes, serological pipettes, tips, 1.5 ml microcentrifuge Eppendorf tubes, 10 and 50 ml Falcon tubes and tissue cultures flasks are required. Deionized sterile water is needed for the preparation of solutions and microbiological media.

III. Procedures



Scheme 6.1 A common scheme of procedures used for eradication of bacterial, mycoplasma, and fungal contamination of tissue culture. This process should be performed by experienced personnel in a laminar flow biological safety cabinet (BSC) that is not used for routine cell culture.

TABLE 6.2. List of antibiotics used for tissue culture.

Antibiotic	Solvent for stock solution	Stability at 37 °C	Effective concentration	Effective against
Penicillin-Streptomycin	Water	3 days	0-100 U/ml penicillin and 50-100 µg/ml streptomycin	Bacteria and fungi
Ampicillin	Water	3 days	100 µg/ml	Bacteria
Erythromycin	Ethanol	3 days	100 µg/ml	Bacteria
Gentamicin sulfate	Water	5 days	50 µg/ml	Bacteria including mycoplasma
Kanamycin sulfate	Water	5 days	100 µg/ml	Bacteria including mycoplasma
Neomycin sulfate	Water	5 days	50 µg/ml	Bacteria
Streptomycin sulfate	Water	3 days	100 µg/ml	Bacteria
Tetracycline hydrochloride	Water	4 days	10 µg/ml	Bacteria

Amphotericin B	DMSO	3 days	2.5 µg/ml	Fungi including yeast
Nystatin	DMF	3 days	50 µg/ml	Fungi including yeast
Tylosin	Water	3 days	8 µg/m	Bacteria including mycoplasma

Solutions

HBSS

Dissolve 1 g of HBSS powder in 1 l of deionized water and adjust pH to 6.5. Sterilize by filtration and store at 4 °C.

Antibiotic Stock Solutions

All stock solutions of antibiotics are prepared by dissolving the powder in appropriate solvents according to the guidelines provided by the manufacturer (Tab. 6.2).

Dissection Balanced Salt Solution 1 (DBSS1)

Add the solution of penicillin (250 U/ml), streptomycin (125 µg/ml) and kanamycin (50 µg/ml) to 500 ml of sterilized HBBS and store at 4 °C.

Dissection Balanced Salt Solution 2 (DBSS2)

Add the sterile solution of ciprofloxacin (10 µg/ml) to 500 ml of sterilized HBBS and store at 4 °C.

Dissection Balanced Salt Solution 3 (DBSS3)

Add the solution of gentamicin (50 µg/ml) and amphotericin B (2.50 µg/ml) to 500 ml of sterilized HBBS and store at 4 °C.

High Antibiotic Medium 1

Add the solution of penicillin (250 U/ml), streptomycin (125 µg/ml) and kanamycin (50 µg/ml) to 500 ml of the complete growth medium and store at 4 °C.

High Antibiotic Medium 2

Add the sterile solution of ciprofloxacin

(10 µg/ml) to 500 ml of the complete growth medium and store at 4 °C.

High Antibiotic Medium 3

Add the sterile solution of gentamicin (50 µg/ml) and amphotericin B (2.50 µg/ml) to 500 ml of the complete growth medium and store at 4°C.

Eradication of bacterial contamination

1. Carefully collect the contaminated culture medium from the affected cell cultures and dispose it into the waste container for autoclaving.
2. Wash the cells with DBSS1 twice according to their adhesion to the culture vessel surface as follows:

(a) Adherent cell lines

- Wash the cellular monolayer three times with 10 ml of DBSS1.
- Remove the DBSS1 and add 1 ml of TrypLE Express to dissociate cells from the culture vessel surface.
- Place flask in incubator for 5 min.
- Check cells under microscope to confirm that cells are detached from the surface.
- Add 10 ml of culture media to inhibit TrypLE Express.
- Transfer the cell suspension into the 10 ml Falcon tube.
- Centrifuge the cell for 5 min at 150 x g and room temperature. Remove the supernatant and add 10 ml of DBSS1.
- Resuspend the cell pellet and repeat the previous step again.

- Resuspend the cell pellet of cells in 10 ml of high antibiotic media 1.

(b) Suspension cell cultures

- Transfer the cell suspension into the 10 ml Falcon tube.
 - Centrifuge the cell for 5 min at 150 x g and room temperature. Remove the supernatant and add 10 ml of DBSS1.
 - Resuspend the cell pellet and repeat the previous step two more times.
 - Resuspend the cell pellet in 10 ml of high antibiotic media 1.
3. For seeding the cells into a new culture flask, adjust the concentration of cells to the lowest seeding density.
 4. Change high antibiotic medium 1 every two days.
 5. When the cells reach confluence, subculture them according to steps 1 - 4. Repeat the subculturing three times.
 6. Check the cell culture for the presence of bacterial contamination by visual inspection and under a microscope (Tab. 6.1), by Hoechst staining and distinctive qPCR test (9).
 7. Cultivate the cells in culture medium without antibiotics for two months and check them for the presence of bacterial contamination.

Eradication of mycoplasma contamination

For eradication of mycoplasma contamination, repeat all of the steps for the protocol concerning eradication of bacterial contaminations with these modifications:

- Use DBSS2 instead of DBSS1 in step 2.
- Use the high antibiotic medium 2 instead of the high antibiotic medium 1 in step 4.
- Accordingly, repeat the subsequent use of the DBSS2 and the high antibiotic medium 2 for the subsequent subculturing of cells three times (step 5).

- For identification of mycoplasma contamination in the cell culture, use Hoechst staining and mycoplasma specific qPCR test (see Chapter 5).

Eradication of fungal contamination

For eradication of fungal contamination, repeat all of the steps for the protocol concerning eradication of bacterial contaminations with the following modifications:

- Use DBSS3 instead of DBSS1 in step 2.
- Use the high antibiotic medium 3 instead of the high antibiotic medium 1 in step 4.
- Accordingly, repeat the subsequent use of the DBSS3 and the high antibiotic medium 2 for the subsequent subculturing of the cells three times (step 5).
- Check the cell culture for the presence of fungal contamination according to the characteristics provided in Tab. 6.1, and by Hoechst staining and fungal qPCR tests.

IV. Comments

The eradication of microbial contamination from the tissue culture by antibiotic and/or antimycotic treatments should be used only to save the unreplaceable tissue cultures. After treatments, further care should be considered in the post-eradication handling of the treated cell cultures that have been confirmed decontaminated as they must be often re-checked for the presence of microorganisms (3). Apart from the treatments of contaminated cultures, the antibiotics and antimycotics in the tissue culture laboratories should be used only when it is necessary as their extensive use can lead to emergency of chronic contaminations and to the generation of bacterial and fungal strains with resistance to antibiotics and antimycotics (3, 6). It is also worth to assure that strict preventive measures and aseptic techniques are in place in order to preclude the re-occurrence of microbial contaminations (12). Specifically, the cell lines received from other sources must be also tested for microbial contamination before they are placed in a common incubator with other cell lines (12, 15).

V. Pitfalls

Problem	Cause and cure
Poor bacterial elimination	The problem is very rare but it can occur due to the different reasons including bacterial resistance to certain antibiotics. If possible, the sample of contaminated media should be cultured in autoclaved LB broth (20 g of LB powder in 1 l water) in early stages of procedures and bacteria should be tested for sensitivity to a range of antibiotics (Tab. 6.2). Accordingly, the high antibiotic medium with effective antibiotics should be used for treatments.
Persistent fungal growth	The resistance of some fungal strains to amphotericin B is rare. If it happens, use nystatin in high antibiotic medium (Tab. 6.2).

VI. Acknowledgement

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Transformation of bacteria

Martin Ondra

I. Introduction

In molecular biology, we often need to prepare many copies of specific pieces of DNA or even whole genes. This task can be accomplished by PCR, gene synthesis (1) or, traditionally, by DNA cloning. DNA cloning is a horizontal gene transfer and consists of DNA isolation, restriction, ligation into plasmid, transformation, and plasmid isolation (2). One of the major steps in DNA cloning is transformation of bacteria with plasmid containing DNA of interest. By this process, plasmid with a foreign DNA is introduced into cells, mainly into bacteria. Transformed bacteria will make many copies of plasmid and can be used not only for replication of plasmids, but also for studies of bacteria, to express large amounts of proteins and enzymes, generation of cDNA libraries, in DNA linkage studies, or as a DNA storage. To be sure that our bacteria were indeed transformed with appropriate plasmid, we use chromogenic substrate or antibiotic selection which is borrowed to bacteria by inserted plasmid bearing antibiotic resistance (3-5).

Not all bacteria are able to take in exogenous DNA from their environment. At present there

exist two major ways how to acquire competent cells for insertion of plasmid. The first method for transformation of bacteria was chemical one published in 1970 by Mandel and Higa (6). To enhance permeability for DNA and create competent cells, bacteria are treated by CaCl_2 and after that, heat-shock is used to create thermal imbalance across the cell membrane that helps DNA enter into cells through cell pores or the damaged cell wall (7). The second method is electroporation, where pulse of high-voltage electricity field is used to enhance the uptake of DNA after application to the cells (6). In a principle, electric field increases the cell membrane potential. As a result, charged molecules like DNA can enter the cell (8).

This chapter describes heat-shock method which is generally the most used procedure how to get plasmid into competent bacteria.

II. Materials and instrumentation

Reagents

S.O.C. Medium (catalog number: 15544034; Invitrogen™), LB Broth (catalog number: L3022; Sigma), Bacteriological agar (catalog number: A5306; Sigma-Aldrich), Kanamycin sulfate (catalog number: 60615, Sigma), Qubit™ dsDNA BR Assay Kit (catalog number: Q32853; Invitrogen™)

Equipment

Julabo® water baths at 42 °C (catalog number: Z615471; Aldrich), shaking incubator at 37 °C (catalog number: 757; Amerex Instruments), stationary incubator at 37 °C (catalog number: E5537314; Memmert), ice, Petri dishes (catalog number: P5606; Sigma), 1.5 ml tubes (catalog number: 0030120086; Eppendorf), sterile glass spreader (catalog number: S4522; Sigma-Aldrich), Qubit™ Assay Tubes (catalog number: Q32856; Invitrogen™), Qubit® 2.0 Fluorometer (catalog number: Q32866; Invitrogen™)

Consumables

NovaBlue Competent Cells (catalog number: 69825; Novagen), plasmid to be transformed.

III. Procedures

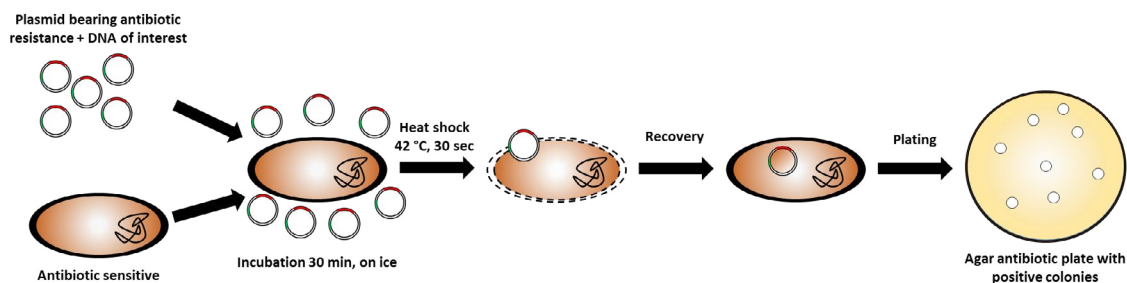
Steps

LB agar plate preparation (5 plates):

1. Weight 1.5 g of bacteriological agar and dissolve it in 100 ml of LB medium (25 g of LB powder in 1l of H₂O).
2. Put the bottle with LB and agar in microwave and heat it till the agar is completely dissolved in LB medium.
3. Let it cool down for 2 minutes.
4. Add appropriate antibiotic. Type and concentration of antibiotic depends on plasmid's antibiotic resistance which you use (for example Kanamycin 50 µg/ml).
5. Pour the liquid into 10 cm Petri dish (20 ml/plate). Let it solidify.

Fluorometric measurement of plasmid's concentration:

1. Pipet 198 µl of Qubit dsDNA BR buffer into Qubit™ Assay Tubes.
2. Add 1 µl of your sample.
3. Add 1 µl of Qubit dsDNA BR reagent.
4. Vortex it for 5 seconds. Let it incubate in dark place for 2 minutes.
5. Measure the plasmid concentration on Qubit 2.0 fluorometer.



Scheme 7.1.

Transformation:

1. Take out three 50 µl stocks with competent bacteria from -80 °C and incubate it on ice for 15 minutes to thaw (1x negative control without plasmid, 1x positive control with control plasmid, 1x bacteria with plasmid of interest).
2. Add plasmid DNA (50 ng) to bacterial stock and gently mix it by finger flick. Let it incubate on ice for 30 min.
3. Put the tube into 42 °C water bath for 30 seconds.
4. Immediately put it in the ice for 2 minutes.
5. Add 150 µl S.O.C. media without antibiotic to the bacteria/plasmid mixture. Put the whole tube into 37 °C shaking incubator for 30 minutes.
6. Gently pipet 50 µl of the mixture on LB agar plate with antibiotic. Spread it with sterile spreader.
7. Incubate plates at 37 °C stationary incubator overnight.
8. Check the grown colonies. Negative control should not have any colonies. Positive control should have single colonies. Store the agar plate with colonies in 4° C.

Transformation efficiency:

Transformation efficiency is a number of colony forming units (cfu) which would be produced by 1 µg of supercoiled plasmid DNA used in the transformation reaction.

1. Count all grown colonies on antibiotic agar plate.
2. Calculate transformation efficiency (TE) using the formula below:

$$TE = \frac{\text{number of grown colonies on plate}}{\text{amount of plated DNA (ng)}} \times 1000 \text{ (cfu/}\mu\text{g)}$$

IV. Comments

- This protocol describes bacteria transformation with supercoiled plasmid DNA which has regular size between 4 kb - 15 kb. For bigger plasmids it is necessary to optimize the protocol.
- All materials and reagents coming into contact with bacteria must be sterile.
- Concentration of plasmid DNA should be measured in different room (post PCR area) to avoid contamination of different samples (for example gDNA).
- All transformation steps should be done in GMO lab.
- It is highly recommended to plate several different dilutions of each transformation culture on antibiotic plate.
- When transformation efficiency is counted do not forget about dilution and actual amount of DNA which was plated on antibiotic agar plate.
- The pUC19 vector (catalog number: SD0061; Thermo Scientific™) is suitable as control. The transformation is efficient when 10 ng of DNA is used.
- Every freeze/thaw cycle of cells significantly reduces transformation efficiency of competent cells. Make aliquots of cells for only one use to avoid loss of transformation efficiency.
- Avoid contaminants such as salts in plasmid DNA by use of column purification methods. Contaminants could interfere with transformation.
- Spread transformation solution properly on selective agar plate to avoid satellite colonies which are not true transformants. Also plating too many cells may give rise to the same problem.

V. Pitfalls

Problem	Cause and cure
No single colony	• Check that you used appropriate antibiotic which resistance is coded by plasmid you used. • Do not forget to use positive control to be sure that there is no problem in any step of transformation. Positive control is different plasmid than yours which is known to work well with transformation protocol and chosen strain of bacteria.
Only few colonies	• Try different transformation method, for instance electroporation. • Decrease used DNA plasmid concentration. Often you will get higher transformation efficiency with less DNA.
Overgrown agar plate (not possible to distinguish single colonies)	• Too much cells were plated on agar plate. • Bacteria cells were not spread properly on plate. • Dilute sample or load less sample. • Incubation time in 37 °C was too long. Decrease the time in stationary incubator after plating.

VI. Acknowledgment

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Transient transfection

Agáta Kubíčková

I. Introduction

Generally, transfection is a process of introduction of the foreign genetic material (DNA or RNA) into eukaryotic cells by non-viral methods. These systems transiently open pores in the cell membrane and permit the entry of the transfected DNA or RNA. Transfection can be mediated chemically using for example calcium phosphate, cationic polymers, liposomes or dendrimers, or by non-chemical methods such as electroporation, microinjection, or receptor mediation (1). Transfection allows for transient expression of a gene of interest in a target cell line and can be useful for short term studies of protein function (2). The result of transient transfection is the presence of foreign DNA (vector) in the cell nucleus. In this case, the foreign DNA is not integrated into the nuclear genome. In the absence of plasmid replication, the foreign DNA is degraded or diluted over time (3). The problem with the degradation or dilution of the foreign DNA can be solved by (i) shortening the duration of the subsequent experiment to 8 - 48 hours post-transfection or (ii) using vectors with the viral-based origins of replication (ORIs), because there are no “natural”

mammalian ORIs. The presence of viral ORIs greatly reduces the rate of plasmid dilution but does not guarantee 100 % transfection efficiency. For example, if you need to express a vector which contains EBV or SV40 ORI, you should use cell line expressing nuclear antigen 1 (EBNA1) or the SV40 large-T antigen (4).

The term transduction is used to describe virus-mediated gene transfer into eukaryotic cells. Both transfection and transduction can lead to the transient or stable expression of foreign DNA, depending on the method used or on the viral tool. Transient transduction is mainly used for efficient RNA (mRNA, short hairpin RNA, guide RNA, etc.) delivery to cell lines which cannot be easily transfected by chemical transfection methods or electroporation (5). This RNA delivery tool is based on a bacteriophage-lentivirus chimera (6).¹

In this chapter, the protocol for a general method for the transient transfection of human U2OS cells using non-liposomal multi-component reagent X-tremeGENE 9 DNA Transfection Reagent is described. The described approach can be adapted for the different cell

lines and for the use with the different transfection reagents as well.

II. Materials and instrumentation

Instruments

- Beckman Coulter VI CELL XR Cell viability analyzer
- Confocal microscope (for example Carl Zeiss LSM 780 with UV laser and ELYRA. PS1 module)
- Biological safety cabinet (BSC), preferably Class II biohazard (for example HeraSafe KS)
- Pipetman Hirschmann
- CO₂ incubator HeraCell 150i
- Water bath Julaba W8

Cell line

- U2OS (HTB-96, ATCC)

Materials

X-TremeGENE 9 DNA Transfection Reagent (6365809001, Sigma-Aldrich), EndoFree Plasmid Maxi Kit (12362, Dynex), McCoy's 5a medium (BE12-688F, Lonza), heat-inactivated fetal bovine serum (FBS; FBS-12A, Capricorn Scientific), Penicillin-Streptomycin (10,000 U/ml; 15140163, Gibco™), Opti-MEM™ I Reduced Serum Medium (11058021, Life Technologies), pipettes (94010, TPP), 10 cm – plates/dishes (93100, TPP), sodium chloride (NaCl; 31434, Sigma Aldrich), potassium chloride (KCl; 60130, Sigma Aldrich), sodium phosphate dibasic (Na₂HPO₄; S5136, Sigma Aldrich), and potassium dihydrogen phosphate (KH₂PO₄, P5655, Sigma Aldrich)

Solutions

McCoy's 5a complete medium

- 445 ml McCoy's 5a medium
- 50 ml heat-inactivated fetal bovine serum
- 5 ml Penicillin-Streptomycin (10,000 U/ml)

Prepare the complete growth medium under the sterile conditions in the flow biological safety cabinet (BSC). Use the sterile labware for the medium preparation. Add FBS to the liquid culture medium to a final concentration of 10 %. Store at 4 °C.

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

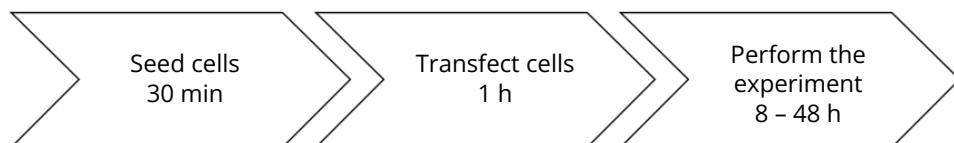
Dissolve appropriate amounts of the above-mentioned chemicals in deionized water (eight-tenths of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Sterilize in an autoclave. Store at room temperature; after opening, store at 4 °C.

1× PBS (working solution)

- Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

Protocol

1. Seed U2OS cells at 2× 10⁶ cells per 10-cm Petri dish in McCoy's 5a complete medium.²
2. Incubate the cells at 37 °C, 5 % CO₂ for 24 h.³
3. Prepare the transfection reagent: vector mixture. Prior to use always equilibrate the vial of X-tremeGENE 9 DNA Transfection Reagent to room temperature and briefly vortex. Dilute 20 µl of X-tremeGENE 9 DNA Transfection Reagent in 1 ml of Opti-MEM™ I Reduced Serum Medium and gentle mix. Then, add 10 µg of vector



Scheme 8.1.

DNA.⁴ Incubate the transfection reagent: vector complex for 30 minutes at room temperature.

4. Gently aspirate culture medium and add 9 ml of fresh McCoy's 5a complete medium.
5. Add the transfection mixture to the cells in a dropwise manner to avoid the release of cells.
6. Gently swirl the Petri dish to ensure the even distribution of the mixture over the whole dish surface. Once the transfection reagent: vector mixture has been added to the cells, there is no need to replace it with fresh medium.
7. Incubate the cells for 18 - 72 hours before measuring the protein expression. The duration of incubation will depend on many factors, including the transfected vector construct, the cell type being transfected, the culture medium, cell density, and the type of protein being expressed. After the incubation period, measure the protein amount using an appropriate assay.

IV. Comments

¹ For a generation of these hybrid viral particles, you can use protocol from chapter 9 with only one major difference, which is the use of the following

plasmids: pRLP transfer plasmid, pRLP-MCP packaging plasmid, pVSVG (Vesicular Stomatitis Virus Glycoprotein) envelope plasmid (6).

² Cells should have a low passage number to achieve the best results. Use cells that are regularly passaged, proliferating well in a log-growth phase and plated at a consistent density. Cells must be healthy and free of any contamination, especially *Mycoplasma* contamination. Accurately quantify cell concentration using a hemocytometer or automated system. For the transfections, fetal bovine serum with low endotoxin levels is recommended.

³ U2OS cells should be 50 % confluent.

⁴ Isolate plasmids with high-quality DNA preparation kits to obtain endotoxin-free DNA. Prepare the plasmid DNA solution using sterile and endotoxin-free TE (Tris/EDTA) buffer or sterile water at a concentration of 0.5 to 2.0 µg/µl. For replicable results, accurately determine the plasmid DNA concentration using 260-nm absorption and DNA purity using a 260 nm/280 nm ratio (the optimal ratio is 1.8).

V. Pitfalls

Problem	Cause and cure
Low DNA transfection efficiency	• Optimize the volume of the transfection reagent and the amount of vector DNA. Increase the volume of transfection reagent first. If it is insufficient, increase the amount of DNA. • Use a plasmid containing a common reporter gene such as luciferase or green fluorescent protein as a positive control. • Preferably, use DNA at a concentration of 0.5 to 2 µg/µl. • For cells known to be difficult to transfect, start the transfection by using 1.5 × of the suggested amount of DNA. If you observe toxicity, decrease the DNA amount.

Low DNA transfection efficiency

- Use the high-quality plasmid, free of proteins and RNA ($OD_{260/280} > 1.8$).

Cellular toxicity

- Analyze transfection at an earlier time point (e.g. at 24 h instead of 48 h).
 - Wash cells with 1× PBS 4 h after transfection.
 - Decrease the amount of vector DNA added per Petri dish.
 - Decrease the volume of transfection reagent.
 - Ensure that the vector DNA is endotoxin-free.
 - Verify the toxicity of the expressed protein. If the expressed protein is toxic for the cells, reduce the amount of vector DNA.
-

VI. Acknowledgement

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Stable transduction by lentiviral particles

Agáta Kubíčková

I. Introduction

Stable transfection (non-viral gene transfer) or transduction (virus-mediated gene transfer) are mainly used for extended pharmacology research, large scale protein production, research on the long-term genetic regulation, or gene therapy (1, 2). At the beginning, an exogenous DNA is inserted into a eukaryotic cell. This exogenous DNA may or may not become a part of the cellular genome. If the inserted DNA segment is integrated into cellular DNA, it becomes an inherent part of the host genome. The introduced DNA is subsequently replicated and expressed, even in progeny. Expression of the foreign genetic material is continual and the transfection procedure is termed a stable transfection or transduction (3). If the host cell rejects the inserted DNA segment, the transfection or transduction is transient because the introduced genetic material is not expressed during the subsequent generations (4). In this respect, stable transfection or transduction increases reproducibility of particular experiments as it eliminates the variation associated with the repeated transient transfection of cells (5).

Successful stable transfection and transduction requires also a selectable marker such as resistance to a specific antibiotic (puromycin, geneticin, zeocin, etc.) and/or production of visible signal (green fluorescent protein, yellow fluorescent protein, mCherry, etc.) as only fraction of cells can stably express the gene of interest (2). In this respect, only approximately one of 10^4 transfected cells will stably integrate exogenous DNA. The efficiency varies depending on the cell type and whether the linear or circular DNA is used. Integration is most efficient when the linear DNA is used (6).

There are two ways how to obtain stably transfected cell lines: (i) transfection of cells with a vector containing gene of interest and a marker gene, which gives the cell a selectable advantage or (ii) transduction of the host cells using a lentiviral vector, which naturally integrates its DNA into the host cell genome in a random manner (7). Lentiviral vectors also contain marker genes.

In this chapter, the protocol for the production of lentiviruses and cell transduction is described. The protocol of preparation of vectors is described in chapter 7.

II. Materials and instrumentation

Instruments

- Beckman Coulter VI CELL XR Cell viability analyzer
- Confocal microscope Carl Zeiss LSM 780 with UV laser and ELYRA.PS1 module
- BD FACSAria II Sorpe flow cytometer/sorter
- Biological safety cabinet (BSC), preferably Class II biohazard (for example HeraSafe KS)
- Centrifuge Hettich 460RS
- Pipetman Hirschmann
- CO₂ Incubator HeraCell 150i
- Water bath Julaba W8

Bacterial strains

- NEB Stable Competent *E. coli* (High Efficiency) (C3040H, New England Biolabs) or One Shot Stbl3 Chemically Competent *E. coli* (C737303, Thermo Fisher Scientific)

Cell lines

- 293T/17 (CRL-11268, ATCC)
- Cell line used for transduction

Vectors

- pMD2G (12259, Addgene)
- psPAX2 (12260, Addgene)
- pMH0001 (85969, Addgene)

Materials

jetPRIME DNA and siRNA transfection reagent (114-15, VWR), EndoFree Plasmid Maxi Kit (12362, Dynex), Dulbecco' modified Eagle medium (DMEM; BE12-614F, Lonza), heat-inactivated fetal bovine serum (FBS; FBS-12A,

Capricorn Scientific), polybrene (TR-1003-G, Merck), pipettes (94010, TPP), 75 cm² tissue culture flask (90076, TPP), syringe filter 0.45 µm, 33 mm in diameter (99745, TPP)

III. Procedures

Solutions

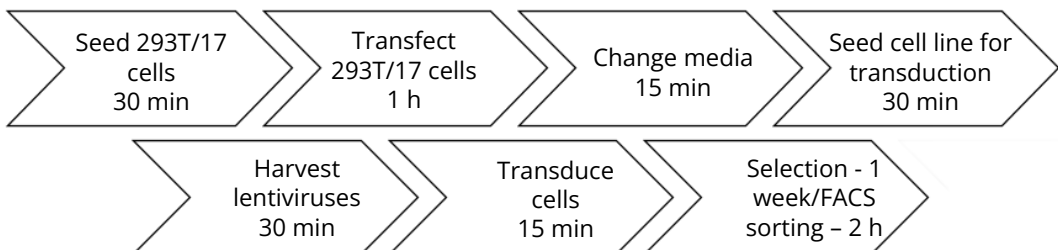
DMEM complete medium

- 445 ml DMEM medium
- 50 ml heat-inactivated FBS
- 5 ml Penicillin-Streptomycin (10,000 U/ml)

Prepare the complete growth medium under the sterile conditions in the flow BSC. Use the sterile labware for the medium preparation. Add FBS to the liquid culture medium to a final concentration of 10 %. Store at 4 °C.

Protocol

1. Seed 15×10^6 293T/17 cells per 75 cm² culture flask in DMEM complete medium.¹
2. Incubate the cells at 37 °C, 5 % CO₂ for 24 hours.²
3. For the transfection of one 75 cm² flask, prepare the transfection reagents and the equimolar ratio of the packaging and expression lentiviral vectors according to Tab. 9.1.³
4. Add all 3 vectors to the jetPRIME buffer (1.5 ml) and gentle vortex the mixture.
5. Add the jetPRIME reagent (90 µl) to the solution from the previous step. Vortex it for 10 seconds and centrifuge it for 2 minutes at $300 \times g$. Incubate 30 minutes at room temperature.
6. Exchange the culture medium for the fresh one. The final volume of the culture medium with the transfection mixture must be 10 ml.



Scheme 9.1.

7. Add the transfection mixture to the culture flask. Swirl the flask gently and incubate for 16 hours.
8. Remove the culture medium and add 20 ml of the fresh culture medium. Allow the viral production to continue for 72 hours post-transfection before harvesting lentiviruses (ca 100 % confluence of cells in 3 days).^{4,5} Ideally, the transfection efficiency should be more than 90 %.⁶
9. Seed the cell line to be transduced 2 days after the transfection of 293T/17 cells.
10. Collect the medium from 293T/17 cells and centrifuge the solution for 5 minutes at 300 × g.
11. Filter supernatant through 0.45 µm filter. Approximately 15 ml of a solution containing lentiviruses will be obtained.
12. Proceed immediately with the transduction. Remove the culture medium from the cells and add the lentiviral mixture containing 8 µg/ml polybrene.⁷ Incubate the cells overnight.
13. On the next day, change the culture medium and incubate cells at 37 °C, 5 % CO₂. Cells can be split or selected by FACS (Fluorescence-activated cell sorting) or by antibiotics 48 - 72 hours after transduction.

IV. Comments

¹ Lentiviral titer highly depends on the health of 293T/17 cells and transfection efficiency. Do not allow the 293T/17 cells to be over-confluent. Trypsinize the cells to obtain single cells. Clumpy 293T/17 cells will produce lower virus titer.

² Cells should be 80 - 90 % confluent at the day of transfection.

³ All lentiviral vectors should be propagated in NEB Stable *E. coli* or Stbl3 bacterial strains. Otherwise, there is a high risk of vector recombination due to the repetitive sequences mainly in LTR (long terminal repeats) regions. The viral titer also depends on the size of the lentiviral construct. Large constructs that approach or exceed the natural maximum of HIV (Human Immunodeficiency Virus)/lenti LTR to LTR packaging limit have low titers.

⁴ Virus-producing cells have a distinct rounded phenotype. The medium is fairly depleted by 72 hours (it is possible to add 50 % more fresh media 48 hours post-transfection).

⁵ If a visible marker is present in the plasmid, transfection efficiency can be assessed visually 48 - 72 h post-transfection.

⁶ From this step, all the next steps are done in BSL 2 laboratory observing the rules for the work with the infectious material. In this respect, decontaminate all surfaces and liquids with 70 % ethanol.

⁷ Polybrene (hexadimethrine bromide) is a cationic polymer used to increase the transduction of cells. It is cytotoxic to some types of cells, for example for those derived from neuronal tissue.

TABLE 9.1. Specification of the amount of lentiviral vectors used for transfection of 293T/17 cells.

Vector name	Vector size (bp)	Moles of DNA	DNA amount (µg)/75 cm ² flask
<i>pMD2G</i>	5,824	1.5 pmol	5.5
<i>psPAX2</i>	10,668	1.5 pmol	10
<i>pMH0001</i>	14,756	1.5 pmol	14

V. Pitfalls

Problem	Cause and cure
Low rate of infected cells	Problems with virus preparation. • Use fresh low-passage of 293T/17 cells. • Preferably use vector DNA at a concentration of 0.5 to 2 µg/µl. • Use high-quality plasmid preparation, free of proteins and RNA ($OD_{260/280} > 1.8$). • Check for the fluorescence marker (if present in vector) in 293T/17 cells. Target cells are difficult to infect. • Check infection of a control cell line (such as HEK 293). • Concentrate virus using spin-filtration or ultracentrifugation.

VI. Acknowledgement

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Analysis of cytotoxicity by tetrazolium salt reduction test

Soňa Gurská

I. Introduction

Cytotoxicity assays are widely used both in the basic research as well as in the drug discovery research to monitor the response of the cells to the treatment with various compounds or to various stimuli. There are a variety of methods that can be used to estimate the number of viable cells, based on various cell functions such as (I) cell membrane permeability (dye exclusion methods, e.g. Trypan blue dye), (II) metabolic activities (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2 H - t e t r a z o l i u m - 5 - c a r b o x a n i l i d e (XTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), Alamar Blue, calcein AM, LDH release assay, G6PD release assay), (III) ATP production (ATP assay), (IV) assays based on DNA synthesis or cell proliferation markers (5-bromo-2-deoxyuridine, 5-ethynyl-2-deoxyuridine, PCNA, phosphohistone H3, Ki-67 detection), and (V) co-enzyme production (protease viability marker assay) (1, 2).

In this chapter, the tetrazolium reduction assay

measuring an enzymatic activity as a marker of viable cells is described. This assay is based on the mitochondrial activity and the supposition that the number of viable cells is linearly proportional to the mitochondrial activity. Viable cells with the active metabolism reduced tetrazolium salts into a purple colored formazan crystals by mitochondrial NAD(P)H-dependent oxidoreductases; the death cells lost this ability. Higher purple color intensity denotes higher number of viable cells (3, 4). These assays are very popular even though they contain many steps, have the variable sensitivity depending on cell type, and cannot differentiate cytotoxic from cytostatic properties of tested compounds.

Most commonly used tetrazolium compounds are: positively charged MTT, and negatively charged XTT, MTS, and WST-1. The MTT assay was the first cell viability assay developed for high-throughput screening (HTS). The disadvantage of MTT assay is the production of insoluble formazan crystals. The formed crystals have to be solubilized before a measurement for example using dimethyl sulfoxide (DMSO) or sodium dodecyl sulfate (SDS). Therefore, the tetrazolium reagents forming an aqueous soluble

formazan were developed. The negative charge of these MTT analogues - MTS, XTT and WST-1, contributes to the solubility but limits the cell permeability of the tetrazolium. For this reason, these tetrazolium reagents are used in combination with the electron acceptor reagents, phenazine methyl sulfate (PMS) or phenazine ethyl sulfate (PES). Since the formazan crystals are directly dissolved in the cell culture medium, the last liquid handling steps during the assay procedure can be excluded saving the time and eliminating the potential errors. This makes MTS assay more appropriate for HTS (5-7).

In general, the MTS is reduced by metabolically active cells into formazan soluble in the tissue culture medium. The quantity of the produced formazan is directly proportional to the number of living cells in culture and is measured using absorbance at 490 nm directly from the assay plate (6, 8-10).

II. Materials and instrumentation

Instruments

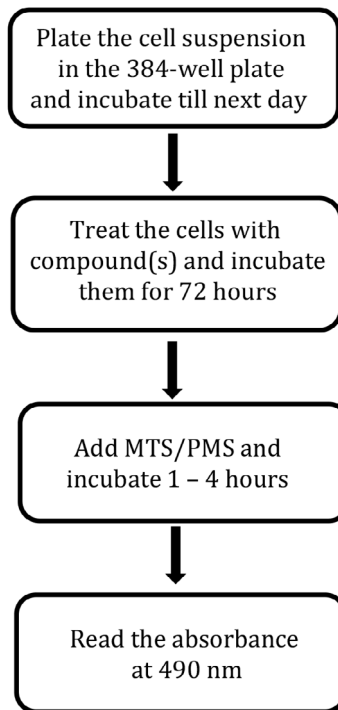
- Magnetic hotplate stirrer VMS-C7 (442-0665, VWR)
- Automated laboratory incubator SteriStore (HighResBiosolution)
- Multidrop Combi Reagent Dispenser (5840300, ThermoFisher Scientific)
- Liquid handler ECHO 555 (Labcyte)
- Multimode plate reader EnVision (2105, PerkinElmer)
- Dotmatics software for data analysis
- Centrifuge (5810R, Eppendorf)

Materials

Sodium chloride (NaCl; 7647-14-5, MikroCHEM), potassium chloride (KCl; P9541, Sigma Aldrich), sodium phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 30388-APO, LACHNER), potassium dihydrogen phosphate (KH_2PO_4 ; 30016-APO, LACHNER), sodium hydroxide (NaOH; 06203, Sigma Aldrich), MTS (G1111; CellTiter96 Aqueous MTS Reagent Powder, Promega), PMS (P9625, Sigma Aldrich), actinomycin D (Act.D, A4448, APExBIO), dimethyl sulfoxide (DMSO; D8418, Sigma Aldrich), mitomycin C (Mit.C;

A4452, APExBIO), 384-well source plate (LP-0200, Labcyte), clear 384-well assay plate with lid (6007658, PerkinElmer), 0.2 μm filter (VACUUM FILTRATION "RAPID"-FILTERMAX, 99955, TPP), RPMI-1640 (D840, Diagnostovum), ethanol 96 %, denatured (85824.360, VWR), Hydrochloric acid (HCl; 7647-01-0, mikroCHEM).

III. Procedures



Scheme 10.1

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na_2HPO_4
- 18 mM KH_2PO_4

Dissolve appropriate amounts of the above mentioned chemicals in deionized water (eight tenth of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Sterilize in an autoclave. Store at room temperature (RT); after opening, store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

70 % ethanol

Mix seven parts of 100 % ethanol and three parts of deionized water. Store at RT.

2 mg/ml MTS (working solution)

In the light-protected container dissolve MTS in the 1× PBS buffer (pre-warmed to RT). Mix at moderate speed on a magnetic stir plate until the MTS is completely dissolved. Adjust the pH with 1 M HCl, the optimal pH is between 6.0 – 6.5. Sterilize by filtration through a 0.2 µM filter into a sterile, light-protected container. Aliquot and store at -20 °C.

15 mM PMS (working solution)

Dissolve PMS in 1× PBS buffer. Aliquot and store at -20 °C.

MTS/PMS (0.15 mM PMS in MTS, the final solution)

Mix 1 part (e.g. 1 ml) of 15 mM PMS with 99 parts (e.g. 99 ml) of 2 mg/ml of MTS. Mix gently on a magnetic stir plate. Prepare the MTS/PMS final solution just before the use.

0.8 mM Actinomycin D (positive control, working solution)

Dissolve Actinomycin D in DMSO. Aliquot and store at -20 °C.

30 mM Mitomycin C (positive control, working solution)

Dissolve Mitomycin C in DMSO. Aliquot and store at -80 °C.

10 mM tested compounds, e.g. LEM 12731 (stock solution)

Dissolve compound in DMSO in barcoded vials and store at -20 °C. Prepare the working solutions of compound (1 mM and 0.1 mM) before the treatment by dilution of 10 mM stock solution in DMSO.

A. Cells plating on 384-well plate

1. Prepare the cell suspension according to the particular cell type and the expected target density (500 – 4,000 cells/well based on the cell growth characteristics, Tab. 10.1).

2. Dispense the cells into columns 2 - 23 of 384-well assay plate (30 µl/well) by Multidrop Combi.
3. Dispense the medium as a blank into the first and twenty forth columns of the assay plate.
4. Incubate the assay plate at 37 °C in atmosphere of 5 % CO₂, 20 % O₂ and 95 % humidity till the next day.

B. Compound treatment

1. Add the tested compound(s) into the compound source plate in three concentrations (10 mM, 1 mM and 0.1 mM), (Fig. 10.1 A).
2. Add controls (working solutions) into the control source plate (Fig. 10.1 B).
3. Centrifuge the source plates using centrifuge to avoid air bubbles (60 s, 1,000 rpm).
4. Transfer the compound(s) from the compound source plate to the assay plate by the ECHO (dose-response software).

TABLE 10.1. Cell density according to the particular cell type plated into the 384-well plate.

Cell line	nr. of cells/ well (30 µl)	nr. of cells/ml
<i>CCRF-CEM</i>	1.2×10^3	4×10^4
<i>CEM-DNR</i>	5×10^2	1.6×10^4
<i>K562</i>	8×10^2	2.7×10^4
<i>K562-TAX</i>	5×10^2	1.6×10^4
<i>A549</i>	8×10^2	2.7×10^4
<i>HCT116</i>	8×10^2	2.7×10^4
<i>HCT116 p53-/-</i>	8×10^2	2.7×10^4
<i>U2OS</i>	8×10^2	2.7×10^4
<i>Bj</i>	2.1×10^3	7×10^4
<i>MRC5</i>	2.1×10^3	7×10^4

Test 7 different concentrations of the tested compound(s) (Tab.10.2, Fig. 10.2).

5. Transfer the controls (100 nl) from the source plate to the assay plate by ECHO

(plate reformat software) (Fig. 10.2).

6. Incubate the plate for 72 hours (37 °C, 5 % CO₂, 20 % O₂, 95 % humidity).

	1	2	3	4
A	CMP1 10 mM	CMP1 1mM		
B	CMP1 0.1 mM			
C	CMP2 10 mM	CMP2 1 mM		
D	CMP2 0.1 mM			

A

	1	2	3	4
A	DMSO	MIT. C		
B	ACT.D			

B

Figure 10.1. Schematic example of the source plates layout. A) the compound source plate layout, B) the control source plate layout.

Source concentration (mM)	Transfer volume (nl)	Final concentration (µM)
10	150.0	50.000
10	37.5	12.500
10	10.0	3.333
1	22.5	0.749
1	5.0	0.167
0,1	15.0	0.049
0,1	5.0	0.017

Table 10.2. Volumes of the tested compound solutions transferred from the source plate to the assay plate using ECHO Liquid Handler.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A			MIT. C																					
B	B	D	CMP	1	50 µM	→	0.017 µM		CMP	8	50 µM	→	0.017 µM		CMP	15	50 µM	→	0.017 µM					B
C	L	M	CMP	1						CMP	8					CMP	15							L
D	A	S	CMP	2						CMP	9					CMP	16							A
E	N	O	CMP	2						CMP	9					CMP	16							N
F	K		CMP	3						CMP	10					CMP	17							K
G			CMP	3						CMP	10					CMP	17							
H			CMP	4						CMP	11					CMP	18							
I		A	CMP	4						CMP	11					CMP	18							
J		C	CMP	5						CMP	12					CMP	19							
K		T	CMP	5						CMP	12					CMP	19							
L		D	CMP	6						CMP	13					CMP	20							
M			CMP	6						CMP	13					CMP	20							
N			CMP	7						CMP	14					CMP	21							
O			CMP	7						CMP	14					CMP	21							
P																								

Figure 10.2. The assay plate layout. This plate layout allows testing 21 compounds (CMP) in two replicates across 7 concentrations per one assay plate.

C. MTS assay

1. Prepare the fresh MTS/PMS final solution.
2. Dispense the MTS/PMS final solution into the assay plate (4 μ l/well) by Multidrop Combi.
3. Incubate the assay plate for 1 – 4 hours in the SteriStore incubator (37 °C, 5 % CO₂, 20 % O₂, 95 % humidity).
4. Measure the absorbance at 490 nm using the plate reader EnVision.
5. Calculate the IC₅₀ value using e.g. Dotmatics software. The IC₅₀ from the example experiment in the Fig. 10.3 is 0.572 μ M.

IV. Comments

- Cells used in the assay must be in a good condition with the stable metabolism and cell cycle.
- Perform the assay in the laminar flow box or in other sterile working area.
- To avoid the non-homogeneous results due to the errors in dispensing steps, prime the dispensing cassette of the Multidrop Combi

apparatus with the sufficient volume of the dispensing liquid. Tubing of dispensing cassette can be washed with 70 % ethanol and 1× PBS. Before dispensing of the cell suspension, prime the dispensing cassette with sufficient volume of RPMI-1640 medium.

- The rest of the fresh MTS/PMS final solution can be stored at – 20 °C. Avoid multiple freezing-thawing cycles and storage the solution at 4 °C as it can results in the slight change of the color of MTS/PMS final solution and consequently in the higher background.

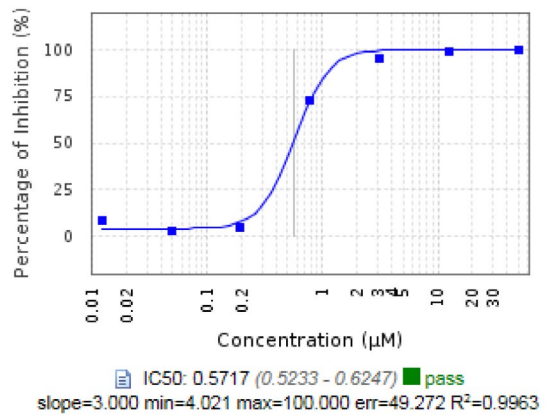


Figure 10.3. Effect of the compound LEM 12731 on the cell viability.

V. Pitfalls

Problem	Cause and cure
Low absorbance readings	• Check the status/condition and seeding density of the cell • Add PMS or PES to the MTS just before the use. • Increase the incubation time with MTS/PMS solution.
Poor replicates	• Increase the priming volume during dispensing cells as well as MTS/PMS solution by Multidrop Combi. • Ensure that no bubbles are present in the wells. • MTS and PMS or PES must be fully dissolved before use.
High background	• Check the proper storage of the MTS or MTS/PMS solutions. • Decrease the incubation time with MTS/PMS solution. • Ensure that the culture medium is free of microbial contamination.

VI. Acknowledgement

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Laser microdissection

Pavla Flodrová and Patrik Flodr

I. Introduction

Laser microdissection (LMD) is a valuable tool with an important role in basic research and translational medicine, first described in 1976 (1). Using a laser, LMD allows to get single cells, subcellular components (chromosomes), microorganisms (2) or areas of interest from complex tissues (e.g. histological tissue section), blood smears, cytologic preparations, or live cell cultures. This procedure dramatically increases specificity and sensitivity of follow-up research methods (DNA, RNA, protein and metabolite analyses) and is therefore exploited in genomics, transcriptomics, proteomics, or metabolomics.

Laser is coupled with a microscope and the procedure is permanently performed under a visual control. The sample is placed on a normal glass slide, a glass membrane slide or a frame slide, according to the individual LMD system. It should be modified for visualization of specific structures, mostly stained with hematoxylin and eosin, the most common histological staining. Other methods can be used as well, e.g. various special histological staining or immunohistochemistry. Fresh reagents and clean coplin jars must be used

during the staining procedure in order to avoid contamination. Visualization of histological and cytological features is more difficult during LMD as coverslips cannot be used on the LMD slides. Apart from bright field microscopy, phase contrast, polarization, or fluorescence microscopy can be applied.

Two basic types of LMD systems exist. Infrared (IR) capture systems and ultraviolet (UV) cutting system. In the first type, the microdissected area is attached to a thermosensitive membrane on the specific cap through the infrared laser. Normal glass slides and membrane slides are applicable. The second system with the UV laser is more widespread and it is equipped with a high-energy laser in the UV spectrum, which cuts the tissue or the complex tissue-membrane (according to the individual type of the LMD system). There are several approaches for transport of the microdissected area of interest. Collection is carried out via gravity (Leica LMD system), laser catapult (PALM MicroBeam LMD system/Zeiss) or at an adhesive cap (MMI CellCut LMD system). Gravity-assisted and laser pressure catapulting are non-contact transport methods (3; 4).

During the “cutting procedure” the laser beam follows the trajectory determined by the user. Every LMD system also offers a software for controlling the laser via a touchscreen, often with function of automatic cell recognition, collecting detailed information about the LMD procedure and microdissected areas or for taking images and videos.

In this chapter we will focus on a procedure using the MMI CellCut LMD system.

II. Materials and instrumentation

MMI CellCut laser microdissection system; Molecular Machines & Industries Germany / Switzerland / Hong Kong / USA Supplier for the Czech Republic is Olympus Czech Group, s.r.o. MMI Membrane Slides (Prod. No. 50103) or MMI Membrane Slides RNase free (Prod. No. 50102), MMI Isolation Transparent Caps 50 pieces, vol. 0.2 ml (Prod. No. 50208), vol. 0.5 ml (Prod. No. 50204) or vol. 1.5 ml (Prod. No. 50212). Supplier for the Czech Republic is Olympus Czech Group, s.r.o.

III. Procedures

1. Tissue section is placed on the MMI MembraneSlide and visualised as required (hematoxylin and eosin for common histological visualisation).
2. Membrane slide is placed onto a normal glass slide to create a protection of the tissue section against contaminations.
3. The pack of membrane slide-sample-glass slide is then placed onto the stage of the MMI CellCut LMD system. Make sure it is firmly fixed.
4. The adhesive MMIIssolation Cap is placed into the cap holder and positioned above the slide.
5. Area of interest is marked using a touchscreen (freehand tool or predefined geometrical shapes) or mouse.
6. By pressing a button, the cutting phase starts.
7. Once the area is carved, it is stuck by lowering and lifting of the adhesive cap.

8. After transporting the sample onto the lid, it can be visualised for control (Fig. 11.1).

IV. Comments

LMD as a precise procedure brings benefits to a wide range of diagnostic and research disciplines thanks to gaining specific material of high purity, ideal for further analyses. DNA, RNA or proteins or metabolites can be subject of interest and it is possible to extract them from almost every type of original sample, including problematic specimens like frozen tissue, live cell culture or archive formalin-fixed paraffin embedded tissue blocks. On the other hand, LMD procedure may be quite time-consuming and requires a morphology-experienced person (5).

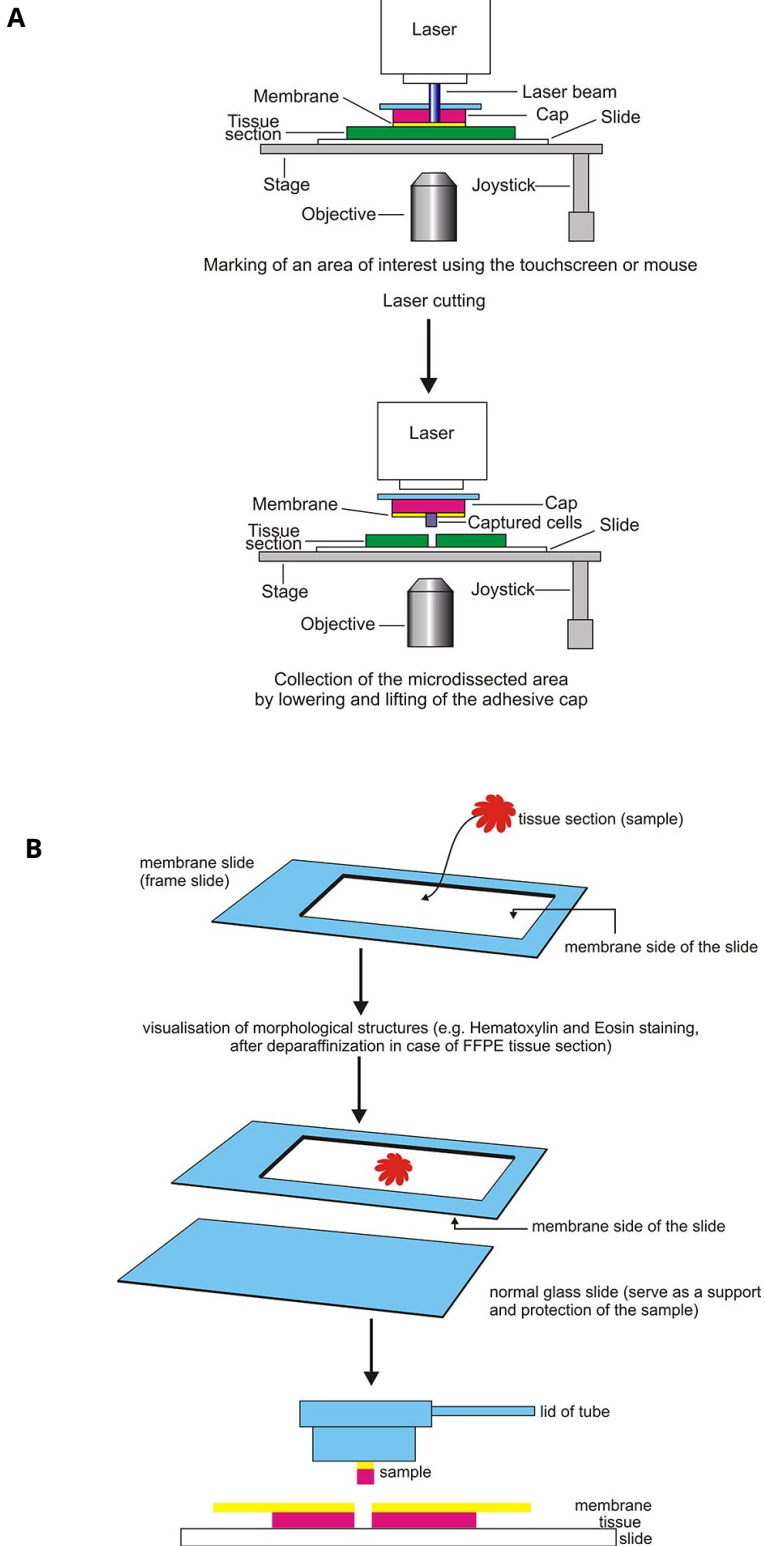


Figure 11.1 Laser microdissection with an adhesive cap. A) Handling with the tissue section and orientation of the adhesive cap, membrane and captured tissue. B) Overview of the laser microdissection system.

V. Pitfalls

Problem	Cause and cure
Selected area does not remove from the slide	• This problem may arise due to insufficient dehydration of the tissue section after staining (let them dry). • When the laser beam cutting was incomplete (repeat the lowering/lifting the cap, repeat the cutting or check the planned trajectory if it is suitable).
Selected area is not cut out	• Check if the laser is on.
Cutting trajectory differs from the planned one	• Make sure that the slide is firmly fixed on the stage. • Check laser setting (setting of the laser position must be done every time you start work and should be checked if a problem occurs).
Area of interest cannot be found on the slide	• This problem with optical resolution can be caused by lacking the coverslip which may lead to altered morphology (use more specific staining for a better visualization of individual structures)

VI. Acknowledgement

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Immunohistochemistry

Daniela Kurfürstová and Gabriela Kořínková

I. Introduction

Immunohistochemistry is widely used method applying immunological principles and techniques to study tissues and cells. The beginning of immunohistochemistry is usually dated back to 1941 when Coons and colleagues used fluorescently labeled antibody to identify an antigen in tissue section. Importantly, Emil von Behring, assistant of Robert Koch, discovered serum antibodies in 1890 and used them to cure diphtheria and tetanus for which he received the first Nobel prize in physiology and medicine in 1901. The first immunohistochemical techniques started to be used in diagnostics in 1950's. Major improvement was achieved by the advent monoclonal antibodies in 1970's. Nowadays, recombinant antibodies are becoming more and more popular because of their high specificity, sensitivity and stability, which may easily be optimised by genetic and chemical modifications (1).

Two main principles are being used, direct and indirect immunohistochemistry. The simple direct one requires antibody directly conjugated with a label; it is used for antigens which are

present in sufficient amounts in tissues. This method is much less sensitive than the indirect one which is based on sequential incubation with primary and secondary antibodies. Primary antibody finds the specific antigen in the tissue, while the secondary antibody (e.g. conjugated to an enzyme) finds the Fc-fragment of primary antibody amplifies the signal. If the amount of antigen in tissue is very low, three step indirect method can be used where primary antibody is found by non-labeled secondary antibody which is detected by another labeled antibody in the final step. The process may also be automated which leads to better reproducibility; however, at a higher cost (e.g. Benchmark Ultra from Ventana/Roche, Bond-Max from Leica or Autostainer Link 48 from Dako/Agilent) (2).

In any case, primary antibodies are crucial for proper and reliable evaluation of the protein/antigen of interest. The quality and consistency of data generated through the use of antibodies vary greatly (3) and may even cause misleading conclusions (4). International recommendations for antibody validation have been formulated and producers should adhere to these guidelines (5). Currently, it is highly advisable to perform

in-house validation (verification, e.g. by testing on positive and negative cell lines embedded in paraffin) for all new antibodies in the laboratory. In this chapter, simple and relatively universal method is presented.

II. Materials and instrumentation

Antibodies

Primary antibodies are used in appropriate working dilutions usually in the range from 1:10 to 1:1000 (or as ready to use antibodies - RTU). Suitable dilution in antibody diluent EnVision™ FLEX antibody diluent (K8006, Dako, HPST) or Dako Real Antibody Diluent (S2022, Dako, HPST) should be tested in a dilution series to achieve the optimal staining and to avoid unspecific reactions.

Secondary antibody (i.e. dextran coupled with peroxidase molecules and goat antibody molecules against rabbit and mouse primary immunoglobulins) EnVision™ FLEX/HRP (SM802, Dako, HPST). Amplification of the primary antibody signal is possible with EnVision™ FLEX+ Mouse LINKER (K8021, Dako, HPST) or EnVision™ FLEX+ Rabbit LINKER (K8009, Dako, HPST).

Reagents

EnVision™ FLEX Target Retrieval Solution 50x, High pH or Low pH (DM828 or K8005, Dako, HPST), EnVision™ FLEX Wash Buffer 20x (DM831, Dako, HPST), EnVision™ FLEX Peroxidase-Blocking Reagent (SM801, Dako, HPST), EnVision™ FLEX DAB+ Chromogen (DM827, Dako, HPST), EnVision™ FLEX Substrate Buffer (SM803, Dako, HPST), hematoxylin solution and general laboratory reagents for mounting process – 96% ethanol, xylene, acetone and Pertex® (HI-00801, HistoLab, Bamed s.r.o.).

Other materials

Microtome with disposable blades, water bath for paraffin sections, laboratory incubator, programmable water bath for histology (Dako PT Link, Dako, HPST), microscope slides TOMO IHC Adhesive (07098928, Matsunami, Roche)

and coverslips, humidified chamber (plastic box with a lid and wet tissue paper at the bottom) and general laboratory equipment.

III. Procedure

Reagents of the Dako Flex system enable two or three step indirect immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections (FFPE). They can be used in the manual staining process, which is described in this procedure or can be used for an automatic staining in the Dako Autostainer Link instrument. Prior to the staining, deparaffinization of the sections is commonly performed by immersing into a dewaxing solution (such as xylene) which is followed by hydration (using alcohols and distilled water) and heat-induced epitope retrieval (HIER) in the buffer pH 6 or pH 9 to unmask the target antigen. In this procedure, deparaffinization, hydration and heat-induced epitope retrieval (HIER) are combined in one step using EnVision™ FLEX Target Retrieval Solution (pH 6 or pH 9). Activity of the endogenous peroxidase is blocked with peroxidase-blocking reagent to reduce unspecific reactions. Next step is incubation with the primary antibody at appropriate dilution. Then the dextran backbone-secondary antibody binds primary mouse or rabbit antibodies (the two step procedure) which can be amplified using a mouse or rabbit linker (the three step procedure). The brown product is generated due to the chromogenic reaction of the diaminonobenzidine (DAB) substrate buffer with the peroxidase molecules conjugated to the secondary antibody. Finally, blue nuclear counterstaining by hematoxylin and mounting with the Pertex® are performed. Examples of immunohistochemical staining are shown in Figure 12.1.

Section Preparation

1. Using the microtome and pre-heated water bath containing distilled water prepare the tissue sections with optimal thickness of 3÷5 µm and place them on positively charged microscope slides.
2. Dry the sections in the laboratory incubator at 58 °C for one hour, then at 37 °C up to the next morning.

Staining of Sections

1. Incubate FFPE tissue sections in the EnVision™ FLEX Target Retrieval Solution (working solution) at 97 °C 20 minutes using the PT Link water bath.
2. Leave the sections to cool down in the PT Link to 65 °C.
3. Wash 3 times the tissue sections in the EnVision™ FLEX Wash Buffer (working solution in a Coplin jar) for 5 minutes at room temperature.
4. Incubate the tissue sections with 100 µl of the EnVision™ FLEX Peroxidase-Blocking Reagent for 5 minutes at room temperature. Wash 3 times (step 3).
5. Amplification step (optional). Incubate the tissue sections with 100 µl of the EnVision™ FLEX+ Mouse or Rabbit LINKER in the humidified chamber for 15 minutes at room temperature. Wash 3 times (step 3).
6. Incubate the tissue sections with 100 µl of the primary antibody in the humidified chamber for 20 minutes at room temperature. Wash 3 times (step 3).
7. Incubate the tissue sections with 100 µl of the EnVision™ FLEX/HRP secondary antibody in the humidified chamber for 20 minutes at room temperature. Wash 3 times (step 3).
8. Incubate the tissue sections with 200 µl of the fresh substrate solution 10 minutes at room temperature. The DAB-containing substrate solution is prepared by mixing 1 drop of the EnVision™ FLEX DAB+ Chromogen with the 1 ml of the EnVision™ FLEX Substrate Buffer. Wash 3 times (step 3).
9. Counterstain with the hematoxylin solution.
10. Wash 5 minutes using tap water.
11. Mount in Pertex® (or any aqueous or organic-solvent-based mounting medium).

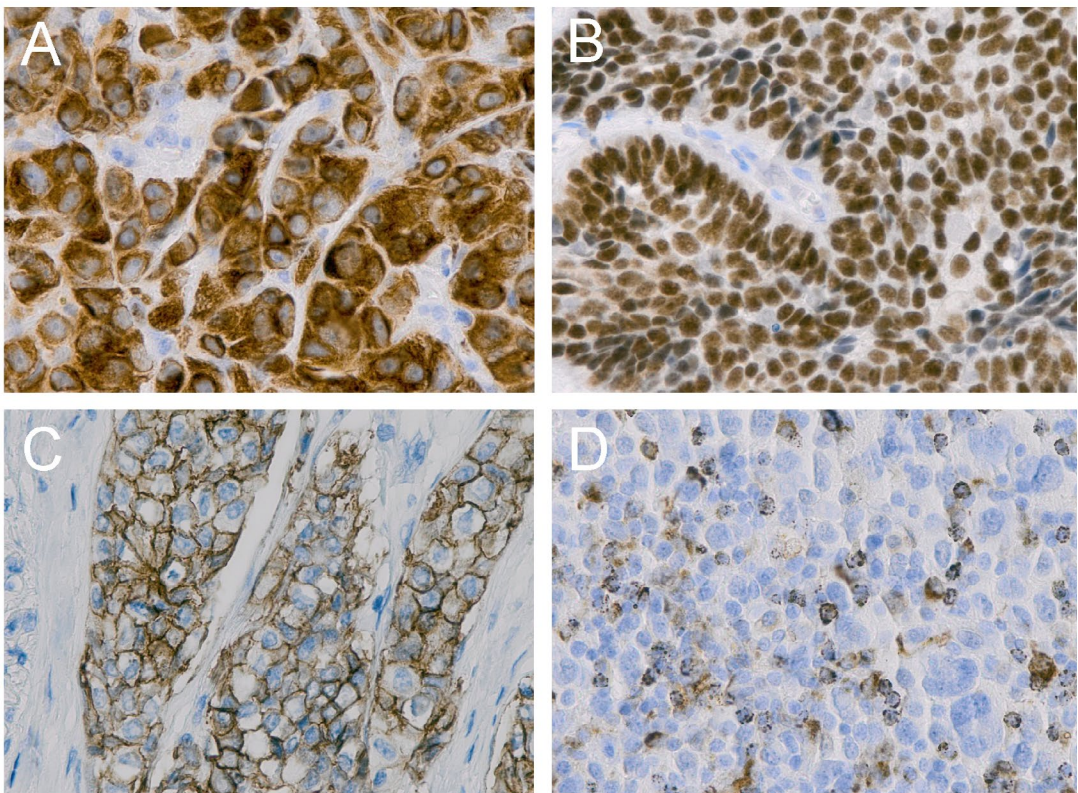


Figure 12.1. Examples of cytoplasmic, nuclear, membrane and perinuclear positivity. A, Cytoplasmic expression of cytokeratin 18 in salivary gland ductal carcinoma. B, Nuclear expression of p40 in squamous cell carcinoma. C, Typical membrane expression of Her2/Neu in invasive ductal breast cancer, NST. D, Perinuclear granular expression of TIA1 in peripheral T-cell lymphoma NOS.

V. Pitfalls

Each step of immunohistochemistry method can lead to false negativity or positivity if not performed properly. Additional problems may cause overfixation which can damage some epitopes. On the other hand, ischemia or cold effect may lead to degeneration of proteins

and enzymes prior to tissue fixation. Storage of tissue sections is also important as epitopes can be degraded by oxidation and water content in the tissue. Evaluation of immunohistochemistry staining requires high qualification and knowledge about the protein of interest in order to recognize potential artefacts and pitfalls.

Problem	Cause and cure
High background staining	• The tissue sections dried out during the staining process. • The noise is too high, decrease the amplification and depending on the software analysis, enhance the contrast. • Make sure that the incubation with the peroxidase-blocking reagent was done properly. • Optimise conditions of the primary antibody incubation (concentration of the antibody and length of the incubation). • Try shorter HIER time (up to 20 minutes). • Try incubating with 5 % human serum to form AB donors.
Poor specific staining	• Check preanalytic conditions. • Use well-fixed tissues (optimal duration 24-72 hours; rapid fixation of small tissue pieces with enough amount of the fixative). • Use freshly cut sections or store them in the dry box at the 4 °C prior to staining. • Try longer HIER time (up to 40 minutes). • Try pretreatment with the proteinase K instead of the HIER. • Amplify the signal using Linker solution.

VI. Acknowledgement

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Multiplex immunohistochemistry

Mariam Gachechiladze

I. Introduction

Multiplex immunohistochemistry (IHC) is an advanced protein detection methodology that allows simultaneous assessment of multiple proteins of interest on one formalin-fixed and paraffin-embedded (FFPE) tissue section, using standard IHC antibodies. Antibodies can be linked with chromogenic dyes, fluorescent dyes or heavy-metals for visualisation, from which fluorescent labelling is the most widespread method. Multiplex IHC has several advantages, including evaluation of multiple biomarkers on limited tumor biopsy samples, evaluation of the composition of tumor microenvironment, mapping out the spatial relationship between different subtypes of immune cells and/or tumor cells, detection and visualisation of co-expression of several functional proteins and their sub-cellular localisation, such as signalling, cell cycle or DNA repair markers.

There are ample different methods developed for multiplexed IHC staining (1). Generally, staining is performed as a one-step procedure or as a successive cycles of a sequential antibody staining on one FFPE slide (2). Earlier

methods of multiplex IHC are mainly based on performing multiple stainings at the same time, using animal-specific secondary antibodies, directed against primary antibodies raised in different species (3,4). However, due to limited variety of antibody sources it is difficult to achieve high level of multiplexing using this method. The highest level of multiplexing is achieved by recently developed method, so called “next-gen immunohistochemistry”, or Imaging Mass Cytometry that uses secondary ion mass spectrometry to image antibodies tagged with isotopically pure elemental metal reporters combined with multiplexed ion beam imaging. It is possible to analyse up to 100 targets using this method (5–7). However, method is labour intensive and advanced bioinformatic approaches are needed for efficient analysis of data obtained from simultaneous multiple target detection.

Current state-of-art method for multiplex biomarker detection on FFPE tissue sections has been developed by PerkinElmer and named as Opal multiplex IHC method (8). The method uses individual tyramide conjugated fluorophores to detect multiple targets. Staining is performed

sequentially and includes antibody labelling and stripping steps, which allows multiplexing by primary antibodies from similar species, without cross-reactivity (Scheme 13.1). Up to 7 targets can be visualised using this method. Besides Opal staining kit, PerkinElmer also provides systems for spectral imaging with linear unmixing. The method is now mostly used for immune-oncology applications (9); however, it can be used for detection of any combination of targets of interest. The method is also frequently used for the validation of the results obtained by imaging mass cytometry (10) and nowadays it is considered as the method with the highest potential for clinical application, amongst other multiplexing approaches. Recently, Ijsselstein *et al.* also developed a tyramide free multiplex staining method (11), however, this procedure still requires further validation. Here we present the methodological guidance for multiplex tissue phenotyping, using PerkinElmer developed Opal staining method.

II. Materials and instrumentation

Samples

Formalin-fixed and paraffin-embedded (FFPE) whole tissue blocks or tissue microarrays (TMAs). Human tissues are usually retrieved from the archives of clinical pathology laboratory and are already embedded in paraffin. Fixation and embedding procedures vary in different laboratories. Widely accepted method of fixation is using of 10% neutral buffered formalin, with variable fixation times from 4 to 48 hours. In our laboratory, paraffin embedding is performed overnight using automated tissue processor (Tissue-Tek VIP®, Sakura). TMAs are constructed for research purposes by research staff.

Materials and Reagents

Microtome; hot water bath; adhesive glass slides (Superfrost Plus or analogue); slide processing jars; solvents for deparaffinization and rehydration of FFPE tissue: xylene and serial dilutions of ethanol (100%, 90%, 70%) respectively; slide incubation/humidity tray; hydrophobic barrier pen; glass coverslips;

distilled water; TBST wash buffer (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20, pH 7.6); antigen retrieval buffers, e.g. citrate buffer, pH 6 (10 mM sodium citrate, 0.05% Tween 20, pH 6.0), EDTA buffer, pH 9 (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0), or others; commercial antibody diluent for both antibodies and fluorochromes and blocking reagent; 1× plus amplification diluent; DMSO; spectral DAPI solution; mounting medium for fluorescence (e.g. ProLong® Diamond Antifade Mountant (Thermofisher) or analogue).

Equipment for Antigen Retrieval

Heat induced antigen retrieval is well accepted method. Following equipments can be used for antigen retrieval: water bath, microwave, pressure cooker, automated rapid microwave processor (e.g.: Histos 5 from Milestone or analogue). Using of rapid microwave processor is highly recommended for best results.

Antibodies

Monoclonal or polyclonal primary antibodies (Abs) for targets of interest must be suitable for antigen detection on FFPE samples. Antibody dilutions and antigen retrieval conditions have to be optimised in each laboratory. Optimisation of single staining for each antibody is recommended before performing multiplex staining. Universal HRP (horseradish peroxidase) conjugated secondary antibodies (rabbit/mouse) are available from some suppliers (e.g. Dako, PerkinElmer). In case of using primary antibodies produced in other species (goat, rat, etc.) relevant secondary antibodies must be selected and dilutions should be optimised.

Fluorophores

Standard fluorophores used in Opal 7-plex kit are: Opal 520 (494-525 nm, Green), Opal 540 (523-536 nm, Yellow), Opal 570 (550-570 nm, Red), Opal 620 (588-616 nm, Amber), Opal 650 (627-650 nm, Orange) and Opal 690 (676-694 nm, Clear), plus spectral DAPI (358-461 nm, Blue) for nuclear counterstaining.

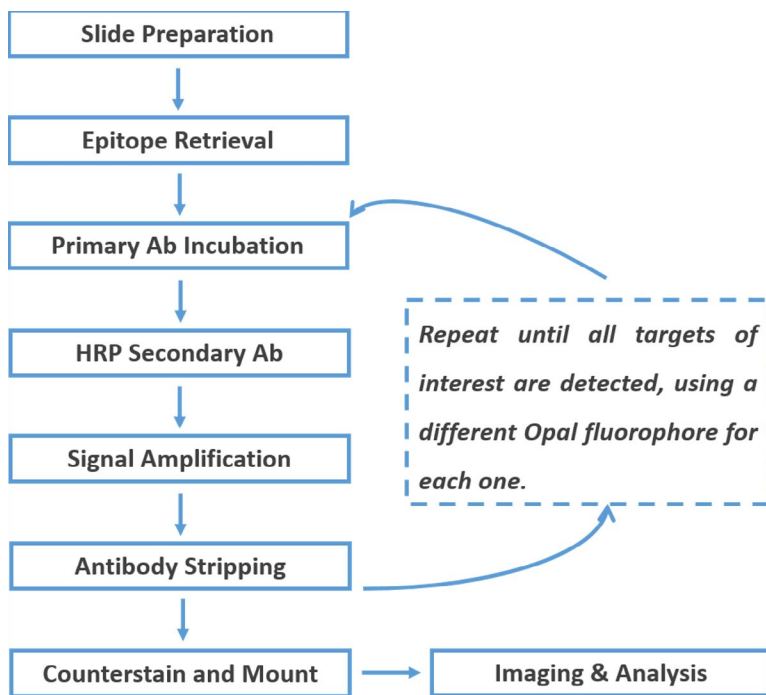
Microscopy and image analysis

Microscope equipped with the appropriate filters to view immunofluorescence specimens is necessary. PerkinElmer provides three imaging systems: Vectra 3.0 Automated Quantitative Pathology Imaging System, Matra Quantitative Pathology Workstation and novel, Vectra Polaris Automated Quantitative Pathology Imaging System. These systems detect multiple fluorophores as well as autofluorescence and increase signal-to-noise ratio. InForm® image analysis software is provided by PerkinElmer, together with imaging systems.

III. Procedures

Sequential Multiplex Staining Steps

1. Cut 2-4 μm sections from FFPE tissue or TMA blocks and mount onto adhesive glass slides.
2. Incubate overnight at 37 °C temperature.
3. Deparaffinize in xylene – 3 $\times$ 10 min.
4. Rehydrate in serial dilutions of ethanol: 100%, 90%, 70% - 5 min., 5 min., 2 min.
5. Wash with distilled water – 2 min.
6. Perform antigen retrieval using relevant buffer.
7. Cool down slides together with buffer – 15 min.
8. Wash with distilled water – 2 min.
9. Wash with TBST – 3 $\times$ 2 min.
10. Draw line around the tissue with PAP pen.
11. Apply blocking buffer – 10 to 30 min. depends on the blocking reagent used.
12. Carefully remove blocking solution without washing.
13. Add diluted primary Ab. and incubate in a humid chamber – 60 min.
14. Wash with TBST – 3 $\times$ 2 min.
15. Add HRP conjugated secondary Ab. – 30min.
16. Wash with TBST – 3 $\times$ 2 min.
17. Add diluted (in amplification diluent) fluorophore – 10 min.
18. Wash with TBST – 3 $\times$ 2 min.
19. Perform antibody stripping – the same procedure as antigen retrieval.



Scheme 13.1 Schematic representation of multiplexed fluorescence staining (Opal Method, PerkinElmer).

20. Cool down slides together with buffer – 15 min.
21. Wash in distilled water – 2 min.
22. Wash in TBST – 3×2 min.
 - In case of multiplexing restart the whole procedure from the step 10, as many times as required, using different primary Ab. and different fluorophore for each repetition.
23. Add DAPI – 5 min.
24. Mount with medium for fluorescence and let dry – 30 min.
25. Store in a cold room and protect from light.

Imaging and Analysis Steps (Mantra system)

1. Turn on the fluorescence illuminator and wait for 30 min until warmed up.
2. Open Mantra Snap Software.
3. Select: "settings" => "load protocol" => "DAPI" (this will activate fluorescence mode automatically).
4. Slide beam block to cover the brightfield illuminator.
5. Select: "Snap Rules" => "exposures" => chose imaging bands (e.g. DAPI, Cy3, Cy5, Fluorescein, etc.) => "autoexpose" => "done"
6. Locate the slide into the stage, choose objective and epifluorescence filter, set the level of illumination.
7. Select: "save" => "destination folder" => enter file name in "Base Filename" and choose "starting number".
8. Focus image and snap as many images as necessary.
9. Open InForm software.
10. Load project.
11. Prepare images.
12. Select: "tissue segmentation" => "trainable tissue segmentation".
13. Mark areas of interest manually, e.g. tumor area, stromal area.
14. Train classifier.
15. Run classifier on other images and revise images to check if system can correctly differentiate the areas of interest.
16. Improve tissue marking manually if necessary, until satisfactory classification is reached.

17. Select: "find features" => "cell segmentation".
18. Mark cells of interest manually: e.g. DAPI, single positive, double positive, triple positive, etc., each with your colour of choice. at least 10 cells should be included in each group.
19. Run automated cell segmentation on other images and revise them if the system can correctly differentiate the cells of interest.
20. Improve cell marking manually if necessary, until satisfactory classification is reached.
21. Select: "score". The number of cells of interest in the selected areas of interest will be automatically counted.
22. Select: "export". Data will be exported as an MS Excel file.

IV. Comments

For this method, we use an automated rapid tissue processor and citrate buffer pH6 for antigen retrieval, which is suitable for unmasking of most of epitopes. The optimal dilutions of each antibody must be first optimised in a single-plex staining. Appropriate controls are very important for successful analysis, including one unstained tissue slide, for the assessment of autofluorescence which should be processed in the same way as other slides and negative control slides, without incubation with primary antibody for each fluorophore used.

V. Pitfalls

Problem	Cause and cure
Low signal	- Wrong Ab and/or fluorophore concentration - titer primary Ab and fluorophore for optimal concentration. - Insufficient incubation time – increase primary antibody and fluorophore incubation time. - Inefficient antigen unmasking – use additional steps of antigen retrieval.
Excess signal	- High concentration of primary Ab – decrease the concentration of primary Ab. - High concentration of fluorophores - decrease the concentration of fluorophores. - Long incubation - decrease primary antibody and fluorophore incubation time.
High background	- Wrong Ab and/or fluorophore concentration - titer primary Ab and fluorophore for optimal concentration. - Old buffers – prepare fresh buffers. - Insufficient blocking – increase blocking time. - Insufficient washing – increase number and/or length of washes.

VI. Acknowledgement

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Quantitative immunofluorescence microscopy

Tereza Buchtová and Martin Mistrík

I. Introduction

Quantitative immunofluorescence (IF) microscopy is a powerful tool used for localization and quantification of molecules (usually proteins) and their complex structures by determining their mutual interactions and relations using fluorescence-tagged antibodies. Quantitative results are potentially expressed as signal intensity, area, shape, and/or count. The method has multiple advantages compared to manual microscopic evaluations. Relatively large amount of data is produced during the quantitative analysis and, once set, the procedure is not time-consuming. Software routines are also devoid of subjective bias. These are important prerequisites for proper statistical testing.

Antibodies are glycoproteins with a pivotal role in humoral immunity (1). In combination with fluorescence methods, antibodies have proved to be very efficient research tools, due to their binding ability against specific antigens.

Immunoglobulins G are commonly used antibodies in the method. They are composed of two light and two heavy chains and include antigen-binding site recognizing antigen

epitopes. Generally, three types of antibodies are utilized for the usage in the IF methods (2-4). A Monoclonal antibodies give an added guarantee in constant quality. They are the products of one clonal plasmatic cell which is immortalized by fusion with mouse myeloma cell and thus can be propagated indefinitely providing unlimited source of the specific antibody. B Polyclonal antibodies are easier to produce and have ability to bind more than one epitope which might be advantage (target protein is less prone for antibody invisibility due conformational changes) and also disadvantage (polyclonal antibodies generally display higher promiscuity and can bind more off targets). Main disadvantage for polyclonal antibodies is in the fact that each batch differs in the quality. C Recombinant antibodies are provided with a different structure, for example only as fragments responsible for recognition of epitopes. Recombinant technology has multiple advantages but generally is used only for antibodies produced in large quantities such as those for medical applications.

For successful labelling of internal cellular structure, the sample needs to be permeabilized with a suitable surfactant or special acetone-based

fixative must be used. Permeabilization disrupts membrane structures to allow antibody entrance inside the cells.

It is possible to incubate the specimen with more than one antibody of different origin (mouse, rabbit, goat, etc.) to get multicolour (multichannel) readout.

Immunofluorescence microscopy usually uses so called indirect - two step - immunofluorescence. As the first step, primary antibodies tag the substrates. Subsequently, secondary fluorescence-tagged antibody is added. This secondary antibody recognizes the constant regions of the primary antibodies. Advantage of this approach includes stronger signal (signal amplification) and omitting the relatively demanding process of primary antibody tagging. An alternative is direct immunofluorescence based on fluorescently tagged primary antibodies; however, this approach is used only rarely, in cases where time is an issue as the staining process is faster (5).

The detection of fluorochrome-tagged antibodies recognized molecules is enabled by the light emission analysis after exposure to appropriate wavelengths. Fluorochromes absorb photons. That leads to a change in electron energy states. The electrons return to their ground state and emit light with less energy (longer wavelengths). This phenomenon is called Stokes shift and is due to the vibratory relaxation of the electrons (6, 7).

As the fluorescent tags are commonly used organic molecules with aromatic cores containing conjugated double bonds (delocalized electrons and non-bonding orbitals) that can be excited from the ground state by using the energy of photons. The energy required for excitation depends on the number of conjugated bonds. With the presence of a higher bond number, lower energy is needed to supply in the system (6).

In the case of researching more than one molecule, it is necessary to use different fluorescent dyes that do not overlap in the emission spectra. Also the used microscope needs to have the ability to visualize more emission spectra and must be equipped with appropriate excitation/emission filter set.

The signal from the sample is visualized by a fluorescence microscope. The modern

microscopes usually use the laser or LED as the light source for the dye excitation and thus excitation filters are no longer necessary. Emitted light from the specimen is reflected back to the objective and hits the filter cube, where the emitted light is allowed to enter to the photomultiplier detector (in case of laser scanning microscopes), camera or oculars while excitation light is blocked due to the presence of a dichroic mirror (7).

The acquired digital images consist of picture elements (pixels). The amount of photons, detected on each pixel, are expressed as intensity values. The pixel intensity value correlates with the amount of fluorophores molecules coming from the particular sample region which correlates with the amount of detected substrate (e.g. protein). From the spatial and intensity information within the acquired image, various variables such as signal area, distance between signals, circularity and other shapes of the signal area, maximal, minimal and mean intensity values, signal integrated density and more can be calculated and statistically tested (8).

There is plethora of software solutions for the quantitative image analysis available on the market including freeware. For the image analysis, every software follows usually the same principles including background and noise subtraction, region or edge detection, signal quantification, and data analysis. The segmentation, resulting in a region and edge detection, is the most challenging step and specialized software solutions already contain pre-programmed detection routines. In the recognized areas the software provides information about the count, size, and intensity differences within the signal. The segmentation of fluorescence images is usually based on intensity threshold methods (region determination) and intensity difference methods (edge determination) (9). A good example of this process is usage of the freeware „Image J” for the quantitative analysis of local protein accumulations within sites of DNA damage in individual cell nuclei (10). In this study, the cell nuclei were recognized based on DAPI signal (one channel) using intensity threshold which generates binary mask. This mask is then used in corresponding channel to score and attribute individual foci of protein of interest to individual nuclei. Final readout is the number of foci per nucleus corresponding to the

amount of DNA damage within the cells.

Very similar approach but using another software solution (ScanR, Olympus) is described in details on the following images.

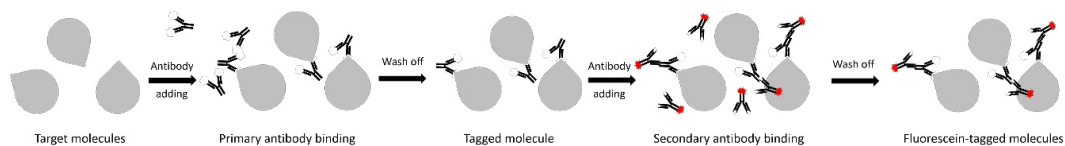


Figure 14.1. The principle of the immunofluorescence method: primary antibodies bind directly to the target molecules afterward secondary antibodies tag the complex.

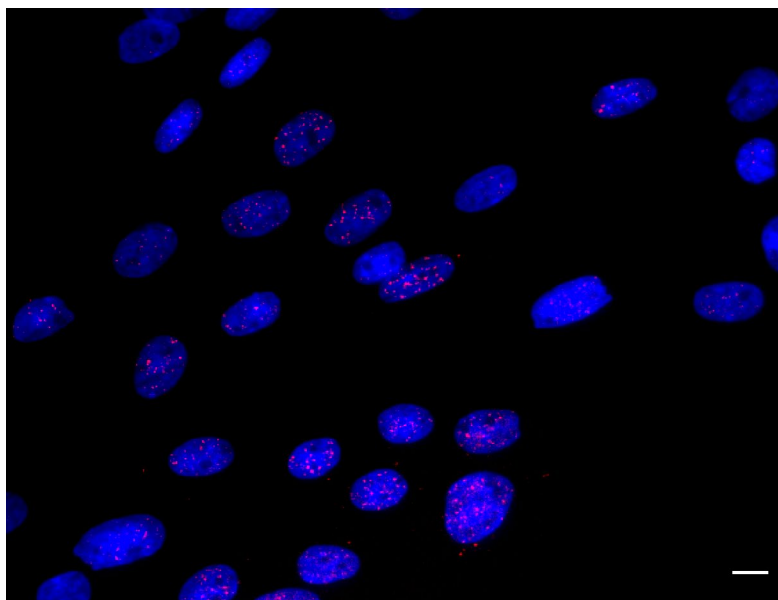


Figure 14.2. Cells stained for 53BP1 protein using specific primary and secondary antibodies: The secondary antibody is conjugated to Alexa Fluor 568 dye (red channel). Cells were irradiated by gamma irradiation (total dose 4 Gy) one hour before fixation to induce 53BP1 foci formation. Cell nuclei were counter stained with DAPI stain (blue channel).

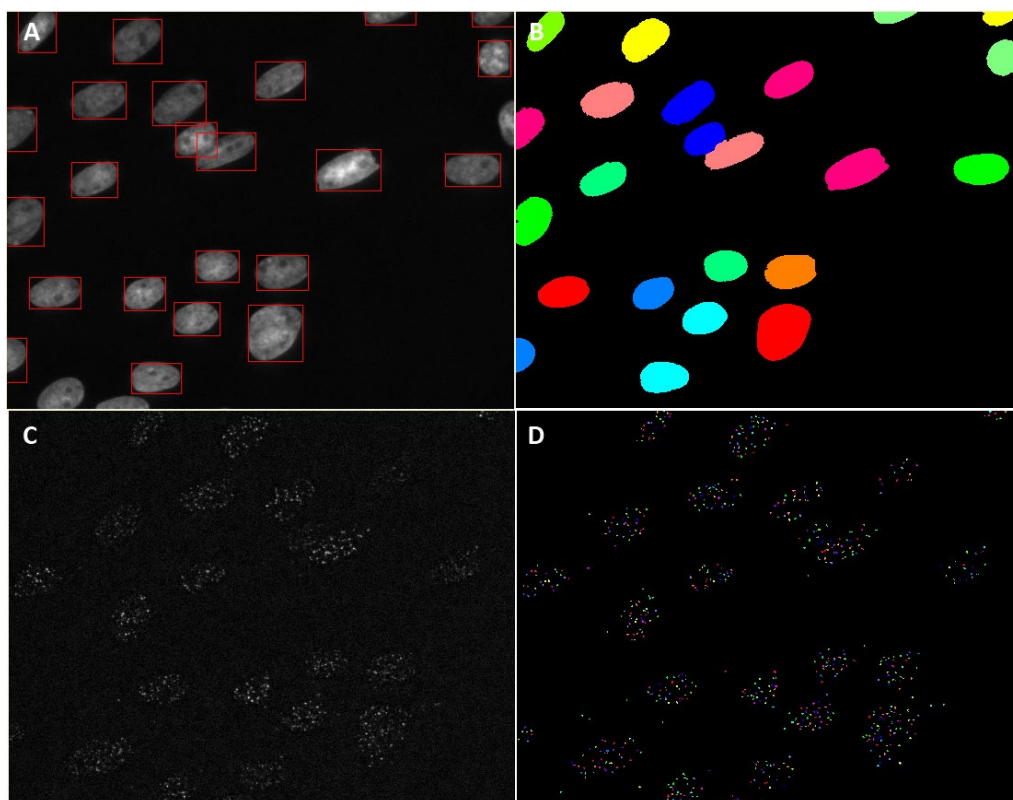


Figure 14.3. Quantitative analysis by ScanR Analysis programme: (A) Separated nuclei are recognized based on intensity and size thresholds. ScanR software uses also watershed algorithm (11) for better segmentation of objects which are in close contact. (B) With the appropriate setup, the algorithm recognizes shape, size and intensity, and creates a binary mask of each nucleus. The mask is applied on corresponding channels. (C) Corresponding channel (Alexa Fluor 568 signal) contain signal in the form of foci formed by the detected 53BP1 protein. (D) The individual foci are also recognized based on intensity and size thresholds. The number of foci and their values (area, intensity, and circularity) are then related to individual nuclei and expressed numerically or in plot diagrams (see below).

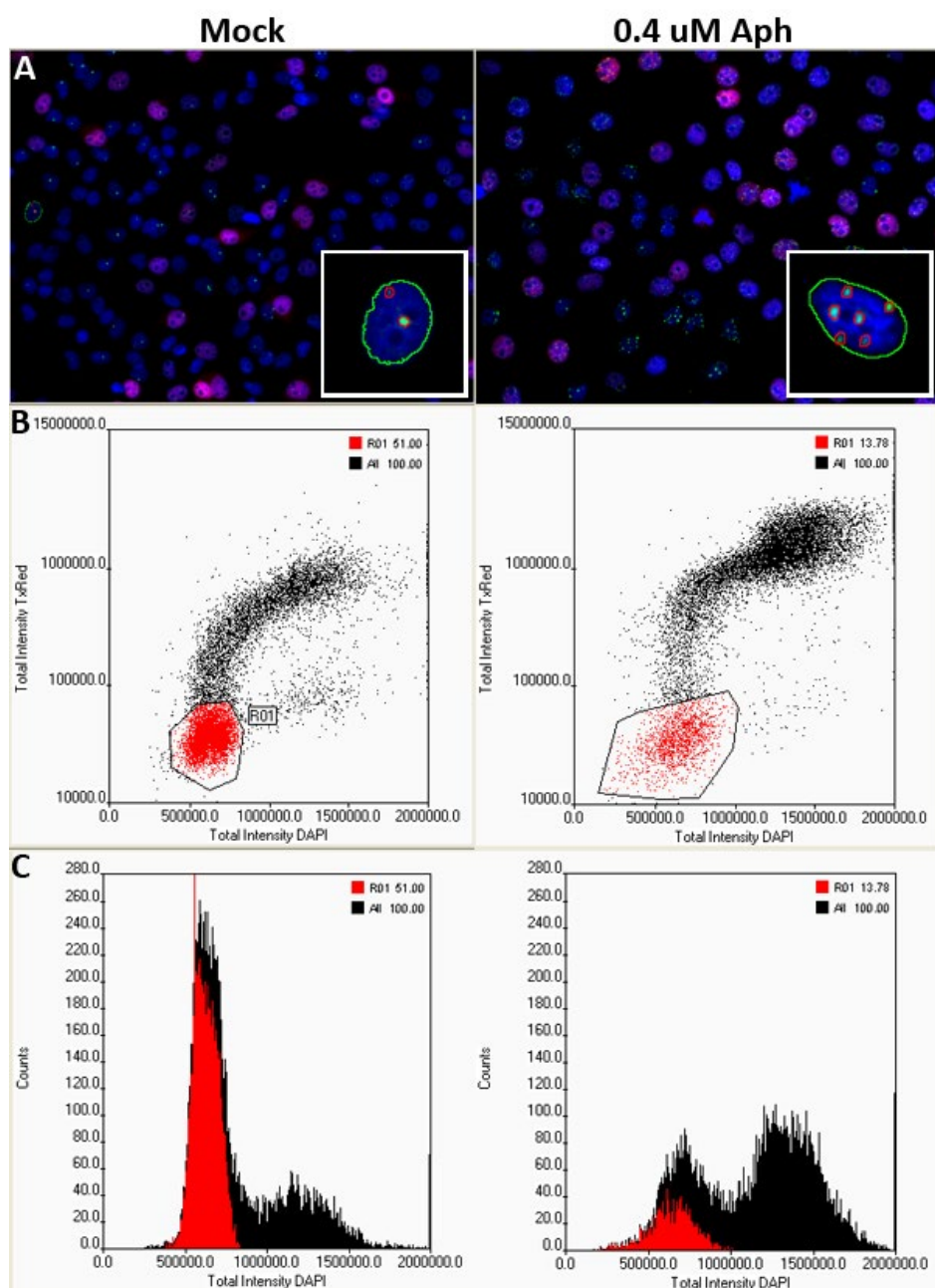


Figure 14.4. Example of three fluorescent channels acquisition and graphical data output (dot plot and histogram diagrams) provided by the ScanR Analysis software: (A) Samples were stained for 53BP1 protein which serves as DNA damage marker (green channel), cyclin A which marks cells specifically in S and G2 phases of the cell cycle (red channel) and for DNA highlighting the individual cell nuclei (DAPI stain – blue channel). Cells were treated either with mock or 0.4 μ M aphidicolin and were analysed for the amount of 53BP1 foci in cyclin A negative cells (G1 cells). (B) Data from S and G2 phases were excluded by gating the cyclin A negative cells. (C) Histograms of the cell cycle profiles (based on DAPI signal). The gate R01 highlights only the cells in G1 (based on Cyclin A negativity) within the histograms. Nuclei outside of the gate were excluded from the final analysis which scored the amount of 53BP1 foci.

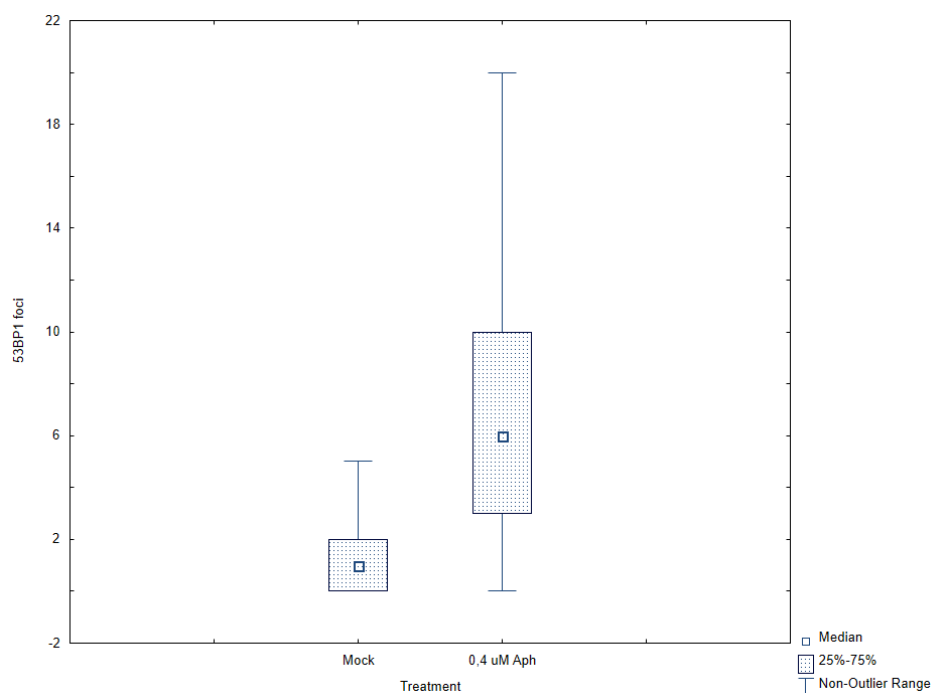


Figure 14.5. Box plot graphical output of numeric data from the analysis introduced in Fig 14.4., processed in Statistica 13 software. Amount of 53BP1 foci in Cyclin A negative cells is shown as box plot chart.

II. Materials and instrumentation

Instrumentation

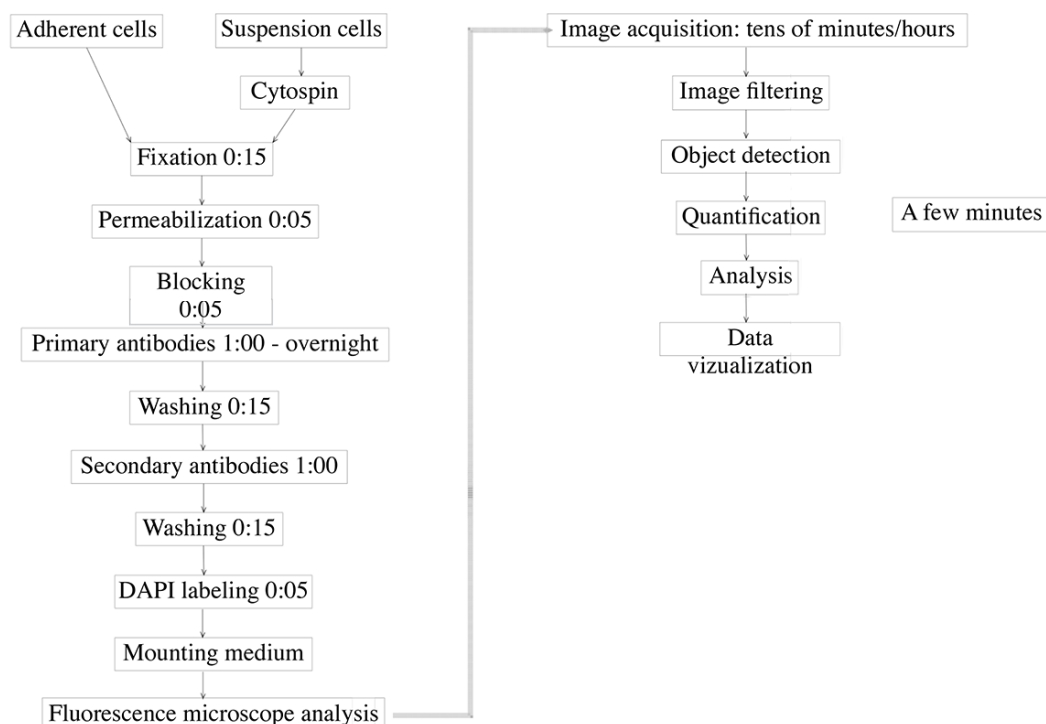
Automatic pipettes, computer with appropriate programmes for quantitative analysis, fluorescent microscope, humidity chamber, orbital shaker, and refrigerator

Reagents:

4% Formaldehyde (HT501128-4L; Sigma Aldrich); Sodium azide (71289; Sigma Aldrich); DAPI (D9542-10MG; Sigma Aldrich); DMEM High Glucose w/ Stable Glutamine, W/ Sodium

Pyruvate (LM-D1110/500; Biosera); FBS Fetal Bovine Serum 10270 (10270-106; Gibco®; Thermo Fisher Scientific); Potassium chloride (30076-APO; lach:ner); Potassium dihydrogen phosphate (30016-APO; lach:ner); Sodium chloride (31434-5KG-R; Sigma Aldrich); Disodium phosphate dodecahydrate (30061-APO; lach:ner); Triton X-100 (3051.2; ROTH); VECTASHIELD® Mounting Medium (H-1000, Vector Laboratories)

III. Procedures



Scheme 14.1.

Procedure

Note: The following protocol is standardized for immunofluorescent quantitative analysis of proteins from adherent cells. Non adherent cells can be adhered to the slide by using polylysine, as described by the manufacturer Sigma Aldrich (<https://www.sigmaaldrich.com/technical-documents/articles/biofiles/poly-lysine-product.html>)

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

1. Add about 0.5 liter of distilled water, adjust pH at 7.4-7.5 and add distilled water up to a volume of 1 liter; store at 4 °C ÷ 25 °C.
2. Prepare 1x concentrated PBS by adding 100 ml of 10x concentrated PBS and 900 ml of distilled water.
3. 0.5% Triton X-100 diluted in 1x PBS
4. Add 1.25 ml of Triton X-100 to 250 ml of 1x concentrated PBS, store at 4 °C ÷ 25 °C.

Blocking medium

Mix 50 ml of FBS with 450 ml DMEM medium or 1x concentrated PBS. Add 100 mg of sodium azide (0.02%).

Sample preparation

Procedure is preceded by cultivation of cells. Adherent cells are cultured on coverslips or plastic inserts, alternatively inside specialized cultivation chambers which are compatible with detailed analysis using fluorescent microscope (e.g. multi-well plates with glass bottom). In case of suspension cells, cytopspin procedure must precede the fixation process.

1. Wash cells with 1x PBS.
2. Fix cells with 4% formaldehyde cooled at 4 °C for 15 min.
3. Wash cells with 1x PBS.
4. Permeabilize cell by adding of 0.5% Triton X-100 diluted in PBS for 5 min.
5. Wash cells with 1x PBS.
6. Incubate with blocking medium for 5 min.
7. Add a primary antibody to the blocking medium used in previous step. Incubate the solution with cells at 4 °C overnight inside a humidity chamber.

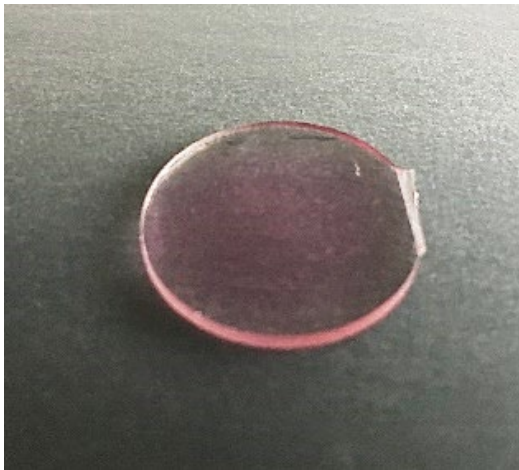


Figure 14.6: Insert covered by medium with primary antibody.

8. Wash cells with 1x PBS for 5 min three times.
9. Incubate samples in a fluorescein-conjugated secondary antibody in a dark for 1 hour.
10. Wash cells with 1x PBS for 5 min three times.
11. 11. Stain with DAPI diluted in distilled water for 5 min.
12. Wash cells quickly with distilled water.

13. Air dry.
14. Mount sample by mounting medium.
15. Cover the sample by a coverslip.

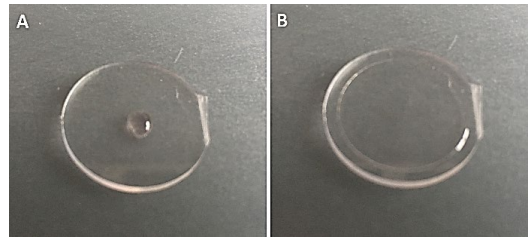


Figure 14.7. Insert covered by mounting medium B and covered by a coverslip.

16. Fix the sample at a microscopic glass slide with a glue or nail polish.



Figure 14.8. Prepared sample.

17. Set up and run a quantitative fluorescence microscopy.

Image acquisition

1. Put the sample inside the microscope.
2. Set up microscope according to one's own requirement: resolution, fluorescence filters, exposure time, number of images, magnification, etc.
3. Acquire multiple images. Keep all the set parameters constant and avoid long expositions of the sample to the excitation light.

Image analysis

1. Open the images with analysing software.
2. Set up segmentation for structure and/or objects detection. Set up background subtraction if necessary.
3. Run the analysis such of desired parameters (e.g. area, shape, intensity etc.)
4. Option: When the analysis is finished, export numerical data and analyse test by proper statistical routines.

IV. Comments

- There are multiple options, how to fix the cells. Formaldehyde can be substituted by other reagents such as ice cold methanol, ice cold ethanol or ice cold methanol/acetone 1:1 mixture. Formaldehyde is the

most common crosslinking agent, whereas methanol and acetone belong to coagulating reagents. Methanol/acetone mixture do not need permeabilization step. All alcohol-based fixative washes-off most of the diffuse proteins which might be wanted feature. Some antibodies recognize proteins fixed only by one of the approaches.

- Permeabilization is essential for the disruption of membrane structures to make better access of the antibodies into the inner cell space.
- Antibodies of various species origin need to be used if more than one target is examined. Corresponding secondary antibodies are needed for the next steps.
- For staining DNA and specific cellular structures exist multiple fluorescent dyes which can be combined with the immunofluorescence.

V. Pitfalls

Problem	Cause and cure
Weak or none staining	• The antibodies have not penetrated to the sample: permeabilize before antibody addition. • The sample has been treated with low concentration of antibodies: increase the concentration of antibodies in blocking medium. • Fluorophore bleaching has happened: protect the sample from excessive exposure to light. • The microscope has been set incorrectly: set filters to the corresponding wavelength of excitation and emission. Increase gain and time exposure. • The antibody has not been suitable for immunofluorescence staining: choose different antibody which has been verified by the manufacturer or in a publication. • The sample has not been pre-extracted: pre-extract with 0.5% Triton X-100 for 1 min before fixation to improve staining and to unmask some epitopes of immobilized proteins. • The targets have not been recognized: Try to choose different fixation procedure.

High background

- The sample has high autofluorescence: choose secondary antibody with a different fluorophore (emission of a different wavelength).
- The sample was not blocked for antibody cross-reactions: use blocking medium.
- The sample has not been washed properly: prolong washing times and washing repetitions.
- The microscope has been set incorrectly.
- The dilution of the antibodies was too low: decrease concentration of antibodies.

Sample degradation

- The samples have been contaminated by bacteria during storage: store the sample in PBS with added sodium azide in 4 °C maximum two weeks before staining.

Incorrect result

- The spectra of two different fluorophores overlapped (bleed-through): choose secondary antibodies with different fluorophores or different filter set.
 - The used antibodies are promiscuous and may detect off targets: use different antibodies.
 - Image has not been segmented properly or has been oversegmented: Change the setup of the segmentation algorithm.
-

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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ISH technology, FISH probes for DNA detection

Radek Trojanec and Zuzana Šporiková

I. Introduction

Fluorescence *in situ* hybridization (FISH) is a molecular-cytogenetic technique based on DNA complementarity of fluorescently labeled probes. This method was invented in the 1980s to detect and localize the presence or absence of specific DNA sequences on chromosomes (1). Main advantage of FISH assay is its high specificity and sensitivity, as well as a versatility regarding range of biological samples examined. FISH can be used for biological samples such as tissue prints, cytopins, and paraffin sections (formalin – fixed paraffin - embedded tissues, blood smears, mitotic preparations, cell lines etc.).

Fixation and paraffinization of the tissue section is a very frequent and well-established way of tissue preservation for further examination after a long time. Formalin-fixed and paraffin-embedded tissue (FFPE) sections allow the analysis of the genetic status of the cells and also the topographic distribution of abnormal cells and the relationships between the morphological characteristics, immunophenotypic characteristics, and the genetic changes within cells.

FISH probes are divided into three groups: (A)

Locus Specific Identifier (LSI) probes hybridizing on the selected locus (gene or parts of the chromosome); (B) Alpha-satellite Centromeric Enumeration Probes (CEP) and Telomeric Probes, binding tandem repeats (consisting from identical or almost identical DNA units); (C) Whole Chromosome Painting (WCP) probes hybridizing to the entire chromosome or chromosome arms.

The DNA of potential probes is usually cloned into a vector – most commonly used is *Escherichia coli* plasmid. Cloned DNA is extremely versatile, inserts ensures isolation in large quantities of high purity and fidelity to original sequence. The length of the insert for LSI probe is commonly 100÷500 kb.

The principle of FISH method is based on DNA and probe denaturation under specific conditions (heat, pH, or presence of formamide in buffers) followed by hybridisation (incorporation) of the fluorescently labelled probe to the DNA, according complementarity rules. Next step is washing away the unbound probes and covering the sample with mounting medium with DAPI. DAPI is a fluorescent dye that binds selectively

to double-stranded DNA and forms fluorescent DNA-DAPI complexes with high specificity and, thus, it helps to localize the chromosomes. Such a sample is ready for visualization under the fluorescent microscope.

By FISH assay number of genes, chromosomal parts, number of chromosomes or genes/chromosomes translocations can be found. In medicine, this method is the most commonly used to determine cytogenetic changes in prenatal screening (identification of developmental defects and fetal syndromes) or oncogenetics (the determination of cytogenetic aberrations within the tumor that may be relevant for the prediction or prognosis of the disease). For example, in prenatal/postnatal medicine diseases such as Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblastic leukemia, Cri-du-chat, Velocardiofacial syndrome, or Down syndrome can be revealed by FISH. In oncology, wide spectrum of predictive/prognostic markers are detected: *ALK* or *ROS1* translocation, status of *ERBB2* (*Her-2/neu*), *TOP2A*, *MYCN* (*n-MYC*),

MET (*c-MET*), and *TP53* genes are often detected.

An alternative to the FISH assay is chromogenic *in situ* hybridization (CISH) (2). In the detection step, visualization is achieved not by the fluorophore but by using peroxidase or alkaline phosphatase to convert chromogenic substrates. According to special types of sample or requirements, different permutations of FISH assay were developed: Fiber-FISH (where biological material is stretched out in a straight line and thus allows better resolution), RNA-FISH or single-molecule RNA FISH (for detecting and quantifying mRNA and other long RNA molecules by fluorescence microscopy imaging), Q-FISH (combining FISH with artificially synthesized polymer peptide similar to DNA or RNA and computer software to quantify fluorescence intensity – often used for telomere length detection), Flow-FISH (hybridizing in the cells in suspension and measuring by flow cytometry) or MA-FISH (using a microfluidic flow to increase DNA hybridization efficiency) (3, 4).

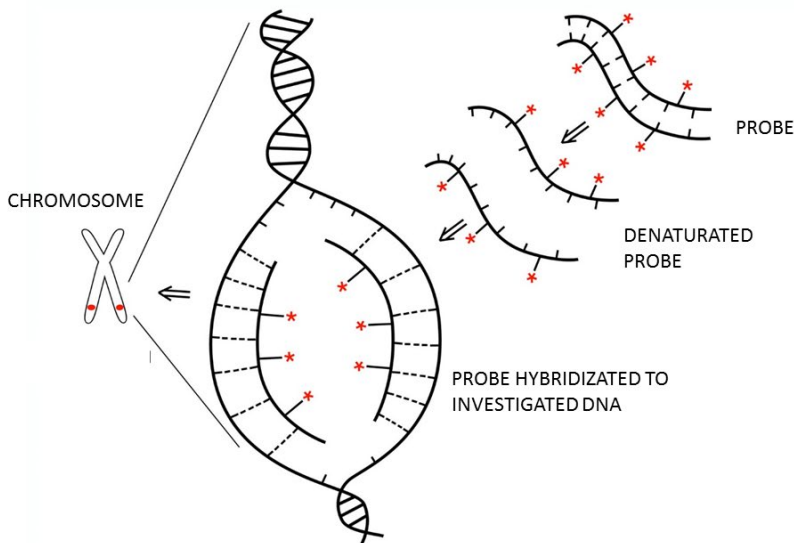


Figure 5.1. The principle of FISH method: The DNA is denatured and during renaturation the probes are complementary hybridized to the examined DNA.

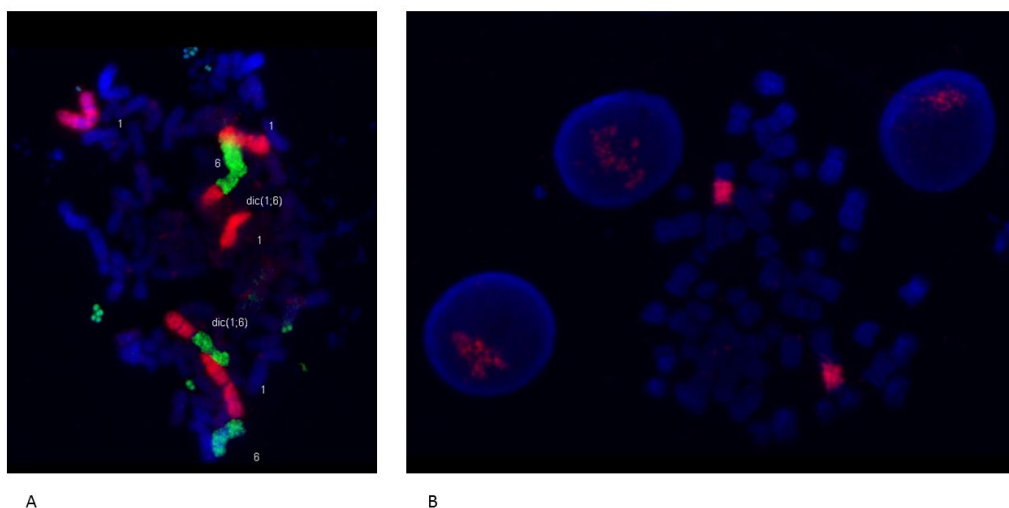


Figure 5.2: FISH assay:

- a) With WCP probes capturing translocation among chromosome 1 and 6.
- b) With LSI probe for C-MYC detection. The interphase nuclei and chromosomes harbouring C-MYC amplification.

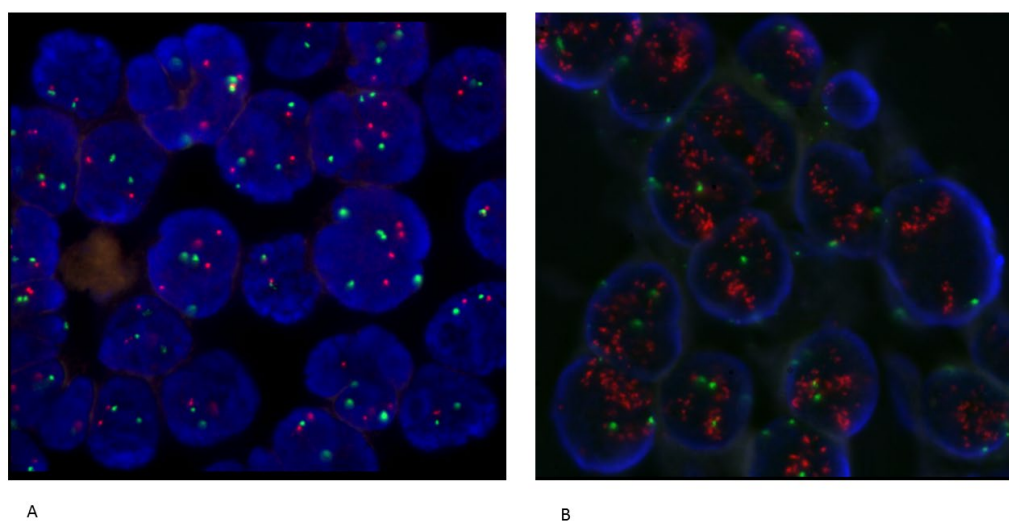


Figure 5.3: Detection of the chromosome 17 (CEP17 probe, green) and Her-2/neu gene (LSI-probe, red.): (A) physiological status and (B) amplification of Her-2/neu gene.

II. Materials and instrumentation

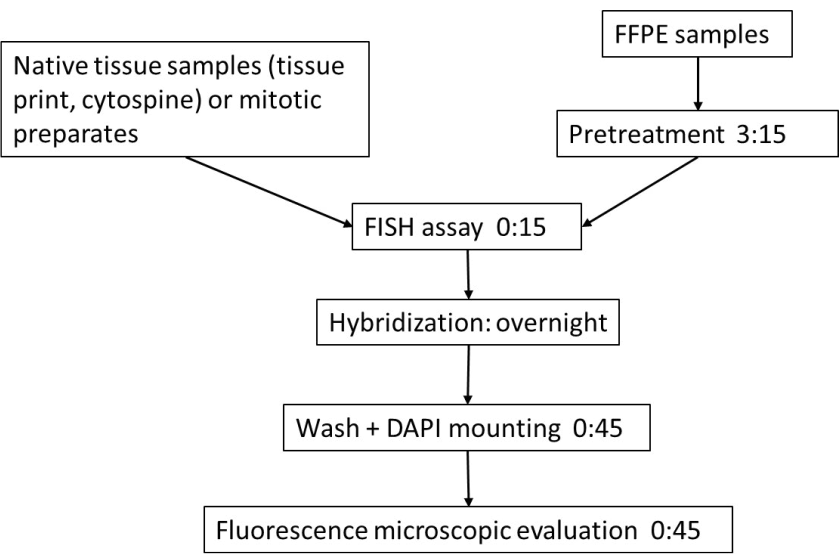
Instrumentation

Water bath, pH meter, thermostat, microcentrifuge, heating plate (thermoblock), wet chamber or hybridizer, fluorescence microscope with appropriate filters (according to a probe used), and immersion lens according to manufacturer's probe protocol.

Materials

Pepsin from porcine gastric mucosa (P7012, MERCK), sodium thiocyanate (NaSCN, S7757,

MERCK), sodium citrate dihydrate (S 4641, MERCK), formamid (AM9342, Ambion), counterstain DAPI (LK-096A, Kreatech), probes (according to the selected marker: IntellMed, Kreatech, Vysis, Cytocell, Empire Genomics, Abnova, Metasystems, Agilent etc.). Other commonly used chemicals (at least in p.a quality): NaCl, KCl, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, KH_2PO_4 , formaldehyde, physiological saline, xylene, 96% ethanol, immersion oil, purified water (deionized or distilled), and HCl/NaOH to adjust pH of the solutions. Cover glasses, rubber cement, pipettes, microcentrifuge tubes, dye vessels, thermometer, and pulp or filter paper.



Scheme 15.1

III. Procedures

There are more pretreatment procedures with basically the same principle in the literature. The difference is mainly in the duration of digestion or washing time and the procedure is adjusted according to the laboratory conditions.

Procedure mentioned below is optimized for deparaffinization of 6 samples. Unless otherwise noted, solutions are used at room temperature and the whole procedure takes 2 days (5-7).

Solutions

20x concentrated SSC stock

- 175 g of NaCl
- 88 g of sodium citrate dihydrate

Add deionized water up to 1 liter and adjust pH to 5.3; store at 4 °C ÷ 25 °C.

10x PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Add distilled water up to 1 liter; store at 4 °C ÷ 25 °C.

Rinse buffer (2 x SSC, pH 7.0).

Prepare by diluting and adjusting the pH from a 20x concentrated SSC stock.

Store at 4 °C ÷ 25 °C.

Pretreatment Solution

- 1 M sodium thiocyanate - NaSCN

Store at 4 °C ÷ 25 °C.

Prewarm before use to 80 °C ± 1 °C.

Protease buffer

- Physiological solution (0.9% NaCl), with HCl adjusted to pH 2.0; store at 4 °C ÷ 25 °C.

Check the pH of the solution before use.

Protease

- 25 mg of pepsin (catalytic activity 2500-3000 U / mg)

Dissolve immediately before use in 50 ml of protease buffer heated to 37 °C ± 1 °C.

Washing solution I (0.4x SSC / 0.3% NP-40)

Prepare by mixing 20 ml of 20xSSC, 3 ml of NP-40, add up to 1 liter of purified water, adjust the pH to 7.0-7.5, prewarm to 58 °C ± 1 °C before use.

Washing solution II (2x SSC / 0.1% NP-40)

Prepare by mixing 100 ml of 20xSSC, 1 ml of NP-40, add up to 1 liter of purified water, adjust the pH to 7.0 .

FFPE samples pre-treatment and FISH method

Deparaffinization of the samples

1. Incubate paraffin slides, preferably on positively charged glass, at 56 °C overnight. The aim of this step is to achieve full adhesion of the tissue to the microscopic glass.
2. Incubate the glass in xylene at room temperature for 3 min. Repeat incubation in 2 another jars with xylene. After removal from the xylene, dry slightly by pressing the edge of the glass on the absorbent pad.
3. Incubate the samples in 96% ethanol at room temperature for 5 min. Repeat this step once more in another jar with 96% ethanol.
4. Dry the samples on the heated plates at 45 °C ÷ 50 °C for 2 min ÷ 5 min.

Pretreatment

Incubate the samples in the following solutions:

- 0.2 M HCl, 20 min.
- Deionized water, 3 min.
- Rinse buffer, 3 min.
- NaSCN solution, at 80 °C, 30 min.
- Deionized water, 1 min.
- Rinse buffer, 5 min, repeat this step once more with a new buffer solution.
- After the glass removal from the 2nd cuvette with the rinse buffer, drain the samples by pressing the edge of the glass on the absorbent pad.
- Incubate in solution of pepsin in protease buffer at 37 °C for 40 min ÷ 45 min (depending on tissue type).
- Wash the samples in rinsing buffers, 2 x 5 min.
- Dry on a heated plate at 45 °C ÷ 50 °C, 2 min ÷ 5 min.
- Incubate the samples in 10% buffered formalin for 10 min.
- Wash in rinse buffer, 2 x 5 min.
- Dry on the thermoblock at 45 °C ÷ 50 °C, 2 min ÷ 5 min

After completely drying out, samples are prepared for subsequent steps.

The processed paraffin sections are checked in a light microscope. Properly prepared samples contain well-digested tissue; cell nuclei are not too disturbed.

Denaturation and hybridization

1. Incubate the samples in denaturing buffer placed in a water bath, for 5 minutes at $73\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$.
2. Immediately after removal, place the samples in a solution of 70% ethanol for 1 minute, then for 1 minute to 85% ethanol and finally to 96% ethanol, where the preparations can be leaved until the probes are applied (at least one minute).
3. In the meantime, prepare a hybridization buffer probe mix:
4. In a 0.2 ml test tube, mix 7 μl of hybridization buffer and 3 μl of probe. Centrifuge for $1\text{ s} \div 3\text{ s}$, vortex and centrifuge for $1\text{ s} \div 3\text{ s}$. Place in a water bath at $73\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ for 5 minutes. Then place it in a $45\text{ }^{\circ}\text{C} \div 50\text{ }^{\circ}\text{C}$ thermoblock until use. If the probe is already prepared, remove the preparations from ethanol, slightly dry by pressing the glass edge on the absorbent pad, place it on a $45\text{ }^{\circ}\text{C} \div 50\text{ }^{\circ}\text{C}$ thermoblock and let the remaining ethanol evaporate ($3\text{ min} \div 5\text{ min}$).
5. Apply the probe solution on the sample and cover it with a cover glass. We recommend to seal the cover glass with a rubber cement (e.g. Fixogum).
6. Incubate overnight at $37\text{ }^{\circ}\text{C}$ in a humid chamber.

The steps 1 $\div$ 5 can be replaced by hybridization station (programmable heat plate) by so-called co-denaturation method. In this procedure, add a mixture of the buffer and probe (some probes are available as ready-to-use, already mixed with the buffer) on the deparaffinized and pretreated sample, cover it with the cover glass. The setting is temperature elevation to $85\text{ }^{\circ}\text{C}$ for 1 min and after than overnight incubation for $37\text{ }^{\circ}\text{C}$.

Washing of unbound probe

1. Remove the cover slide and immerse the preparations in Washing Solution I (0.4x SSC / 0.3% NP-40), heated to $58\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Shake vigorously the dye vessel for $3\text{ s} \div 5\text{ s}$ and then incubate for 4 min.
2. Transfer samples to Washing Solution II (2x SSC / 0.1% NP-40), shake vigorously and incubate 30 s.

3. Gently dry by pressing the edge of the glass on the absorbent pad and let it air dry in the dark (30 min).
4. Mount slides with adding 10 μl of DAPI and cover it with a cover glass.

IV. Comments

- The samples are evaluated in a fluorescence microscope using the appropriate filter and immersion objective. The signals are counted in $100 \div 200$ nuclei followed by calculation of average number of genes and chromosomes (using centromeric probes simultaneously). We recommend archiving parts of the specimen using digital image analysis (MetaSystem, Lucie Capturing systems, etc.).
- Up to three probes (with different fluorophores) can be used simultaneously. Higher number of probes included in one reaction is not recommended due to the difficulties with the evaluation.
- DAPI is commercially available from variety of producers. For example, DAPI I, II, III (Vysis) buffered glycerol solution differ in decreasing dye concentration. DAPI II is often used in FISH for paraffin sections, DAPI III is more suitable when weak signals occur. You can also prepare DAPI mounting solution from concentrated DAPI and mounting buffer (e.g. Kreatech, Cytocell) offered separately or as a DAPI antifade mounting buffer mixture (e.g. Kreatech, Cytocel).
- The probes are sensitive to light. During the work minimize exposure to light.

V. Pitfalls

Problem	Cause and cure
Tissue loss	• Inappropriate tissue (containing parts of skin, ligaments, or too much of the fat): use another part of the sample. • The glass has not been positively charged: use positively charged or poly-lysine slides. • Paraffin cut is too thick: we recommend using slices with a thickness of $4\text{ }\mu\text{m} \div 6\text{ }\mu\text{m}$. • The slides were not incubated at $56\text{ }^{\circ}\text{C}$: bake the slides at $56\text{ }^{\circ}\text{C}$ overnight. • Loss after transfer from NaSCN solution to water: too high temperature difference. After removing from NaSCN, let the slides hold in the air for at least a minute. • Loss after transfer from pepsin: too long incubation. Decrease the time of the incubation.
Bad tissue morphology	• Too long/short incubation in pepsin: adjust the incubation time. • Careless removal of cover slide: do not move with the cover slide, fold it up.
Poor or no hybridization signals	• Unappropriate digestion of the sample: adjust time of the digestion in pepsin, check the temperature and the pH of the buffer. • Bleaching of the sample: avoid of long enlightenment during the procedure. • Bad probe concentration or the bad probe: check the probe on other sample. • Long incubation in formalin: ensure that before paraffinisation the tissue was incubated in formaline no longer than 3 days. • Bad tissue, necrotic sample: no solution – use another sample. • Short hybridization: increase the time of the hybridization to 24 hours. • Unappropriate hybridization: increase temperature of hybridization ($+ 2\text{ }^{\circ}\text{C}$). • Incorrect or old fluorescence filters, weak fluorescence lamp: check filters and/or change the lamp. • Keeping the procedure or the quality of the solutions: check that all the procedure steps were performed according to this protocol.

High unspecific background

- Inappropriate incubation in NaSCN: check the time and temperature of the incubation.
 - Damaged tissue: necrotic or poorly/badly fixed sample show high unspecific background and weak signals. Use another sample. Ensure, that samples are fixed minimally 8 hours in well buffered formaline and that the exposition to formaline does not exceed 72 hours (after 72 hours only 50% are possible to investigate by FISH). Do not use accelerated fixation by flame.
 - Washing: Check the incubation time, pH, and temperature of washing buffers.
-

VI. Acknowledgment

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Immunofluorescence staining of intracellular protein targets

Jiří Řehulka

I. Introduction

Immunofluorescence is a method for the visualization of cellular antigens using immunochemistry and microscopy. It reveals subcellular localization of the particular protein in cell compartments or organelles as well as its participation in cellular structures (Fig. 16.1, 16.2). Direct immunofluorescence is based on antigen-specific binding of the primary antibody conjugated to a fluorescent label. Indirect immunofluorescence relies on non-labeled primary antibody and secondary antibody with a fluorophore. Secondary antibody amplifies the signal of less abundant antigens and, in addition, enables the combination of fluorophores for more proteins of interest (double or triple immunofluorescence).

The preparation of a specimen for immunochemistry requires fixation of proteins by a conformational change of their tertiary structure. The most used fixatives are paraformaldehyde (PFA), methanol, acetone or acetone/methanol (1:1). Each fixative provides a different degree of protein denaturation and cell membrane porosity, therefore the choice of the fixative is dependent on the particular protein of

interest (1). PFA is recommended as a universal fixative for the preservation of native structures such as cytoskeletal filaments whereas methanol-based fixatives cause protein denaturation and disruption of membranes. In contrast to methanol and acetone, PFA preserves cell-surface proteins and markers and it is compatible with over-expressed fluorescent proteins (2). Section A of this protocol describes a method for preparation of single glass slide for widefield or confocal microscopy. The microscope slide is finalized by mounting with mounting medium that protects the specimen and increases the clarity of the microscopic image. Another method, described in section B, is a preparation of 384-well plate for automated microscopy suitable for high-content screening. It allows fast acquisition of many fields of view for each well and facilitates image analysis as well as data management.

II. Materials and instrumentation

A. Microscope slides

Round or rectangle cover glasses with thickness

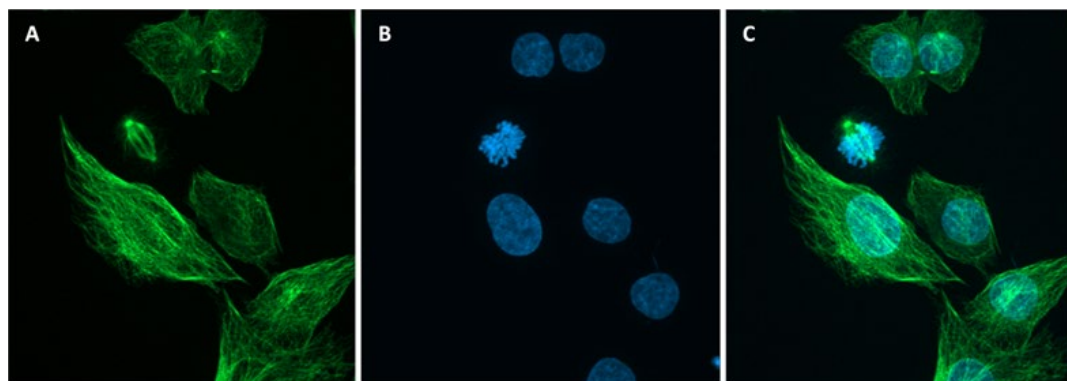


Figure 16.1 Immunofluorescence and confocal microscopy (63x objective) of α -tubulin in U2OS cells. Cells were fixed with 4% PFA in the cytoskeleton buffer, then α -tubulin was visualized by monoclonal mouse antibody (Cat. No. T9026, Sigma Aldrich) and Alexa Fluor 488-conjugated secondary antibody (A). Nuclei were stained with DAPI (B) and image overlay was created using ZEN software (C).

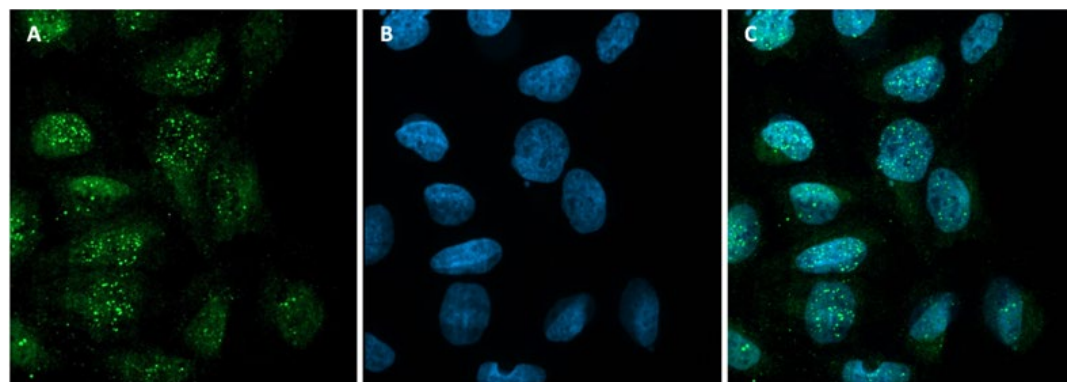


Figure 16.2 Immunofluorescence and confocal microscopy (63x objective) of ubiquitin in U2OS cells. Cells were fixed with 4% PFA and the protein visualized using anti-ubiquitin primary antibody (Cat. No. 3936, Cell Signaling Technology) and FITC-conjugated secondary antibody (A). Nuclei were stained with DAPI (B) and image overlay was created using ZEN software (C).

0.17 mm (474030-9000-000, Carl Zeiss), microscopy glass slides, sterile culture dish, 6-well or 12-well plate, shaker, humid chamber (large Petri dish with a wet filter paper and layer of parafilm on the top), forceps, primary antibody recognizing particular antigen, secondary antibody conjugated with Alexa Fluor dye (Thermo Fisher Scientific).

Solutions

- 1× Phosphate Buffered Saline (PBS)
- Triton X-100 stock solution 10% (v/v) in PBS
- Permeabilization and blocking solution: 5% normal goat or donkey serum (serum of the animal in which secondary antibody was raised in) with 0.3% Triton X-100 in PBS. Alternatively 1% bovine serum albumin in PBS and 0.3% Triton X-100.
- Antibody dilution buffer: 1% bovine serum albumin and 0.3% Triton X-100 in PBS.
- 4% paraformaldehyde in PBS (4% PFA): Dissolve 20 g paraformaldehyde in 500 ml PBS using a magnetic stirrer with heating to 60 °C. Always work in the laboratory fume hood. Add a few drops of 1M NaOH to facilitate the process of dissolving. Adjust pH to 6.9 with HCl and filter through a cell culture filter. Store at room temperature or freeze the aliquots.
- Methanol p.a. (Penta)
- Acetone/methanol 1:1 (v/v)
- Vectashield antifade mounting medium with DAPI (H-1200, Vector Laboratories).
- Clear nail varnish
- Optional: Cytoskeleton buffer 10 mM MES, 150 mM NaCl, 5 mM EGTA, 5 mM MgCl₂, 5 mM glucose; pH 6.1, RT. For fixation use 4% solution of PFA in the same buffer (3).

B. 384-well plate for automated microscopy

Black plate with an optically clear bottom and tissue culture quality (CellCarrier, PerkinElmer, Cat. No. 6007550), primary antibody recognizing particular antigen, secondary antibody conjugated with Alexa Fluor dye (Thermo Fisher Scientific).

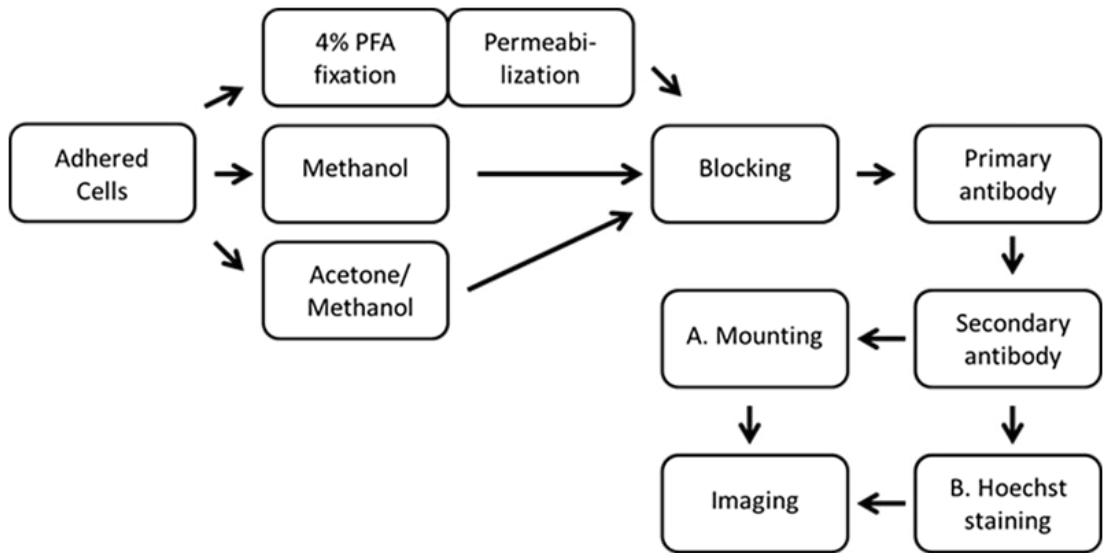
Solutions

- 1× Phosphate Buffered Saline(PBS)
- 4% paraformaldehyde in PBS (4% PFA)
- Triton X-100 stock solution 10% (v/v) in PBS
- Permeabilization and blocking solution: 5% normal goat or donkey serum (serum of the animal the secondary antibody was raised in) with 0.3% Triton X-100 in PBS. Alternatively 1% bovine serum albumin in PBS and 0.3% Triton X-100.
- Hoechst 33342 (Thermo Fisher Scientific, Cat. No. H1399) *stock solution 10 mg/ml in PBS*
- Secondary antibody dilution buffer: 1% bovine serum albumin and 0.3% Triton X-100 in PBS with 1 µg/ml Hoechst 33342 (10,000-fold dilution of 10 mg/ml stock solution).

TABLE 16.1 Example of Thermo Fisher Scientific secondary antibodies suitable for double immunofluorescence.

Colour	Fluorophore	Max Exc. /Emiss.	Anti-Mouse	Anti-Rabbit	Anti-Goat
Green	Alexa Fluor 488	496/519	Cat. No. A-11001	Cat. No. A-11070	Cat. No. A-11055
Red	Alexa Fluor 568	578/603	Cat. No. A-10037	Cat. No. A-1004	-

III. Procedures



Scheme 16.1

A. Preparation of individual microscope slides

1. Sterilize the coverslips in the dish by exposure to UV in a flow hood. Alternatively, immerse the coverslips in 98 % ethanol, take out individual coverslips using forceps and burn the remaining ethanol on the coverslip. Place the coverslip into the suitable laboratory plastic (12-well plate, 6-well plate, or petri -ish).
2. Seed the adherent cell line into the plate with the coverslips. Initial cell number is dependent on the cell line and time of cultivation on treatment. The untreated control cells should not exceed 80 % confluency at the time of fixation.
3. Following the overnight incubation, the coverslips with attached cells can be fixed or subjected to treatment.
4. Aspirate medium and wash with PBS.
5. Add one of the following fixative solutions (4):
 - a) v4% PFA for 15 minutes at RT
 - b) pure methanol 15 minutes at -20 °C
 - c) acetone/methanol 1:1 for 20 minutes at -20 °C.
6. Aspirate fixative solution.
7. Add 0.3% Triton X-100 and incubate for 3 minutes at RT to permeabilize the cell membranes. This step can be omitted when using permeabilization and blocking solution containing 0.3% Triton X-100. When using methanol or acetone based fixatives the permeabilization with 0.3% Triton X-100 is not required. When detecting cell-surface proteins, avoid the use of Triton X-100 in all solutions and buffers.
8. Wash 3 x 5 min with PBS.
9. Block the specimen in blocking solution for 1 hour.
10. Transfer coverslips into the humid chamber (cell side up).
11. Apply diluted primary antibody on the coverslips (1:100-1:2000, optimal dilution can be determined experimentally).
12. Incubate 1 hour at RT or at 4 °C overnight.
13. Place coverslips back into the plate (cell side up).
14. Wash 3 x 5 min with PBS and with gentle shaking.
15. Again transfer coverslips on a new parafilm (cell side up).
16. Apply diluted secondary antibody (dilution

1:50 - 1:1000, optimal dilution can be determined experimentally).

17. Incubate in the dark for 60 min at RT.
18. Place coverslips back into the plate (cell side up).
19. Wash 3 x 5 min with PBS and with gentle shaking. Protect from light.
20. Apply approximately 10 µl of Vectashield mounting medium on a clean glass slide and lay the coverslip on the drop (cell side down). Prevent formation of the bubbles.
21. Wipe excess of mounting medium by Kimwipe.
22. Seal the edges of the coverslip with nail varnish.
23. Clean the glass slide and coverslip by ethanol and examine under the microscope.
24. Store the slides in dark at 4 °C.

B. Preparation of 384-well plate for immunofluorescence

1. Seed the adherent cell lines into the 384-well plate. Initial cell number is dependent on cell line and time of cultivation on treatment. The untreated control cells should not exceed 80 % confluency at the time of fixation.
2. Aspirate medium.
3. Immediately add 50 µl 4% PFA in PBS/well.
4. Incubate 20 minutes at RT.
5. Aspirate and wash twice with 50 µl PBS.
6. Add 50 µl of permeabilization and blocking solution.
7. Incubate 20 minutes at RT.
8. Aspirate and add 25 µl of primary antibody diluted in permeabilization and blocking solution.
9. Incubate for 60 minutes at RT.
10. Aspirate and wash with 50 µl PBS.
11. Add 25 µl of secondary antibody diluted in secondary antibody dilution buffer.
12. Incubate in the dark for 60 minutes at RT.
13. Aspirate and wash 4 times with 50 µl PBS. After the last wash leave 50 µl of PBS/well.
14. Store the plate in dark at 4 °C (5).

IV. Comments

- Solutions containing PFA, methanol or acetone are toxic. They must be used in the hood and discarded into toxic waste.
- The primary antibody should be raised in species different from the species of the sample to prevent the cross-reactivity of the secondary antibody. The secondary antibody should be developed against the host species of the primary antibody.
- The secondary antibody fluorescent conjugates should be compatible with the filters of your microscope. They should not overlap in their emission wavelength when performing double or triple immunofluorescence. In addition, they should not interfere with signals of DAPI and Hoechst 33342 (excitation 405 nm/emission 450-460 nm).
- When using an oil immersion make sure the mounting medium in the sample has solidified. After finishing the work clean the objective. Follow manufacturer's recommendation (6) and always use only lens cleaning tissue (Thorlabs).
- Filaments of the cytoskeleton can be preserved using cytoskeleton buffer instead of PBS for washing steps prior to the fixation (3). Microtubules can also be fixed by cold methanol for 3 min (7).

V. Pitfalls

Problem	Cause and cure
No signal	• Degraded primary or secondary antibody. Use new antibody. • Poor fixation or extraction of the antigen by the fixative. Make sure that the cell side of coverslip was exposed to solutions. • Antigen not present. Use positive control. • Wrong antibody dilution. Optimize primary and secondary antibody dilution.
Low signal	• Insufficient washing or blocking. Optimize dilution of secondary antibody.
High background	• Filter 4% PFA and PBS solutions
High number of artefacts	• Wrong antibody dilution. Optimize primary and secondary antibody dilution. • Insufficient washing or blocking. Optimize dilution of secondary antibody.

VI. Acknowledgment

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***In situ* detection of replicated DNA in mammalian cell nuclei**

Anna Ligasová and Karel Koberna

I. Introduction

Various techniques are used for the detection of the sites of DNA synthesis in cell nuclei. Most of them are based on the labeling of the replicated DNA using modified nucleosides. These nucleosides are incorporated into DNA and in the following steps are detected by various ways depending on the type of the modification. At the beginning, isotopically labeled nucleoside, ³H-thymidine, was used for the staining of replicated DNA followed by autoradiography detection (1, 2). Although this method allowed to describe many interesting findings concerning DNA synthesis, due to its low resolution, the relatively long processing time and the necessity to work with radioactive material, it was gradually substituted by other techniques. Currently, the techniques based on halogenated derivatives of thymidine (HDT) and 5-ethynyl-2'-deoxyuridine (EdU) are most commonly used (1).

HDT are modified in the 5 position of the thymine ring by halogen atoms (bromine, fluorine, chlorine and iodine) (1). All the mentioned HDT can be detected by the specific antibodies. However, as HDT are inaccessible

in double-stranded DNA for a reaction with an antibody, it is necessary to use special treatments to make them accessible for such reaction. In this respect, various technologies were developed. Probably the most known and the most frequently used technology is based on the use of strong acids, mainly hydrochloric acid (1, 3-6). The less used approaches for HDT revelation rely on the use of alkali (5, 7), nucleases (3, 5, 8-10), combination of low concentrated HCl and exonuclease III (11), the cleavage of DNA using monovalent copper ions (12), photolysis of HDT-labeled DNA (13) or heat denaturation (5). The most frequently used representative of HDT is 5-bromo-2'-deoxyuridine (BrdU).

Contrary to HDT, incorporated EdU (14) is detected by a click reaction – a reaction when the terminal alkyne group of EdU reacts with azides. As these azides are usually fluorescently labeled (14-16), the click reaction is highly specific and does not require DNA denaturation, the detection is fast and the destruction of other cellular components is lower than in the most methods based on the detection of HDT. On the other hand, EdU is highly toxic compound and therefore, it is not suitable for the long-term

experiments (17-21). Moreover, the click reaction results in the destruction of the fluorescence of the proteins such as GFP or R-phycoerythrin (22). Therefore, special protocols preventing the degradation of fluorescent proteins have been developed (23, 24).

When choosing the correct protocol for the detection of DNA replication, one should take into account several aspects. They mainly include the length of the incubation time with the modified nucleoside, the susceptibility of the cell line to the used analog and whether the co-localization study will be performed. In the case of BrdU and also other HDT, it is also necessary to choose the right antibody clone, as presently many clones are available on the market, but with the different affinities to the particular HDT (25). If synchronization protocol using elevated concentrations of thymidine will be used or if the pulse with EdU should be performed immediately after the BrdU pulse, EdU is not suitable marker nucleoside as it results in the nearly complete omission of the EdU signal or its significant decrease (1). Concerning the double labeling experiments with BrdU and EdU, it should be also taken into account that many of anti-BrdU antibodies cross-react with EdU (26).

In this chapter, we will describe two protocols for the detection of replicated DNA in adherent cells. The first one is based on the BrdU labeling and its detection using DNase I and exonuclease III. This protocol enables co-localization studies including the co-localization with the fluorescent proteins. The second protocol is based on EdU and click reaction. This protocol is relatively inexpensive and fast, and is convenient for co-localization studies with the exception of fluorescent proteins. It is also not convenient if the synchronization protocol using elevated concentrations of thymidine is used as it results in the omission or progressive lowering of the signal. In that case, the protocol based on BrdU should be used.

II. Materials and instrumentation

Instruments

- Vortex Lab Dancer (0003365000, IKA)
- Heating magnetic stirrer ARE (F20500162, Velp Scientifica, Inc.)

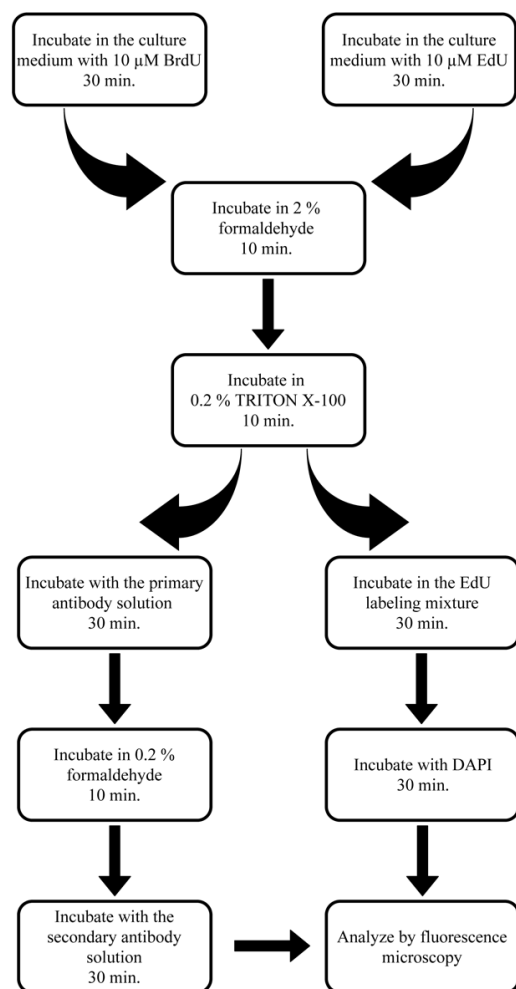
- Laboratory shaker Vortex Wizard (X000102, Velp Scientifica, Inc.)
- Laboratory shaker ThermoMixer® C (5382000015, Eppendorf)
- The positive displacement pipette for the pipetting of viscous liquids (for example Microman ME100, FD10004, Gilson)
- pH meter (pH 8, XS Instruments)
- Fluorescence microscope (for example Olympus IX83, Olympus)

Materials

Sodium chloride (NaCl; 31434, Sigma Aldrich), potassium chloride (KCl; 60130, Sigma Aldrich), sodium phosphate dibasic (Na_2HPO_4 ; S5136, Sigma Aldrich), potassium dihydrogen phosphate (KH_2PO_4 ; P5655, Sigma Aldrich), sodium hydroxide (NaOH; 06203, Sigma Aldrich), paraformaldehyde (PFA; 818715, Merck), formaldehyde solution, 35 wt. %, with 10 % methanol as stabilizer (15513, Sigma Aldrich), Triton X-100 (X100, Sigma Aldrich), ethanol (100983, Merck), Tris base (T6066, Sigma Aldrich), HCl (10033-A35, Lach-Ner), DAPI (D9542, Sigma Aldrich), glycerol (G7893, Sigma Aldrich), 1,4- diazabicyclo[2.2.2]octane (DABCO; D2522, Sigma Aldrich), 5-bromo-2'-deoxyuridine (BrdU; NB06315, Carbosynth), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; M2393, Sigma Aldrich), calcium chloride anhydrous (CaCl_2 ; 30974, Lach-Ner), anti-BrdU antibody (for example mouse anti-BrdU clone B44; 347580, Becton Dickinson or mouse anti-BrdU clone Bu20a; 11-682, Exbio Praha), exonuclease III (EN0191, Thermo Fisher Scientific), DNase I (EN0525, Thermo Fisher Scientific), antibody against anti-BrdU antibody conjugated with fluorescent dye (for example AffiniPure Goat Anti-Mouse IgG (H+L) conjugated with Alexa Fluor® 488; 115-545-146, Jackson ImmunoResearch), 5-ethynyl-2'-deoxyuridine (EdU; NE08701, Carbosynth), copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; C8027; Sigma Aldrich), sodium ascorbate (11140; Sigma Aldrich), glycine (G8898, Sigma Aldrich), azide dye (for example FAM azide, 5-isomer; C4130, Lumiprobe), dimethyl sulfoxide (DMSO; 34943, Sigma Aldrich), 5-chloro-2'-deoxyuridine (CldU; C6891, Sigma Aldrich), 5-iodo-2'-deoxyuridine (IdU; I7125, Sigma Aldrich) or 5-trifluoromethyl-2'-deoxyuridine (TFdU; T2511, Tokyo Chemical Industry).

Other commonly used material such as deionized water (deionized or distilled), nailpolish, cover glasses, tweezers, parafilm, Petri dishes, pipettes and tips, microtubes, centrifugation tubes, filter paper, and immersion oil.

III. Procedures



Scheme 17.1.

Scheme of the procedure with the formaldehyde fixation (excluding the washing steps and embedding into the mounting medium). If necessary, formaldehyde fixation can be replaced by ethanol fixation. All steps are performed at room temperature if not stated otherwise.

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Dissolve appropriate amounts of the above-mentioned chemicals in deionized water (eight tenth of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Sterilize in an autoclave. Store at room temperature; after opening, store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

2 % formaldehyde in 1× PBS buffer, pH 7.4 (working solution)

Dissolve the paraformaldehyde in deionized water (one-half of the final volume) in a beaker and heat to 60 °C on a heating magnetic stirrer. When moisture forms on the sides of the beaker, add NaOH (ca 8 drops of 1 M solution per 0.5 l), and stir until the solution clears. Cool the prepared solution under the faucet. Add 10× PBS (one tenth of the final volume). If necessary, adjust the pH to 7.4. Add deionized water to the final volume, aliquot and store at -20 °C. **Formaldehyde is toxic, use protective means during its preparation and use, and work in a fume hood!**

0.2 % formaldehyde in 1× PBS buffer, pH 7.4 (working solution)

Dilute a methanol-stabilized stock solution of formaldehyde or a solution prepared from paraformaldehyde in 1× PBS to the desired concentration in 1× PBS. Prepare the solution just before the use.

0.2 % Triton X-100 in 1× PBS, pH 7.4 (working solution)

Warm up 1× PBS to the room temperature. Add 100 % Triton X-100 to the pre-warmed buffer. As Triton X-100 is highly viscous, use the positive displacement pipette for the pipetting of viscous liquids or cut the end of the common tip. Mix the prepared solution for ca 10 minutes on the laboratory

shaker. Prepare the solution just before the use.

70 % ethanol in deionized water (working solution)

Mix seven parts of 100 % ethanol and three parts of deionized water. Store at -20 °C.

1 M NaCl (stock solution)

Dissolve NaCl in deionized water (eight tenth of the final volume), then add deionized water to the final volume and store at 4 °C.

1 M Tris-HCl, pH 7.5 (stock solution)

Dissolve Tris base in deionized water (eight tenth of the final volume) and adjust pH to 7.5 with the concentrated HCl. Add deionized water to the final volume. Store at 4 °C.

25 mM Tris-HCl, pH 7.5 + 150 mM NaCl (working solution)

Mix 25 parts (e.g. 25 ml) of 1 M Tris-HCl, pH 7.5, 150 parts (e.g. 150 ml) of 1 M NaCl and fill to 1,000 parts (e.g. 1,000 ml) with deionized water. Store at 4 °C.

10 mM DAPI (stock solution; optional, used for staining of cellular DNA)

Dissolve DAPI in deionized water. Aliquot and store at -20 °C.

1 M Tris-HCl, pH 8 (stock solution)

Dissolve Tris base in deionized water (eight tenth of the final volume) and adjust pH to 8 with the concentrated HCl. Add deionized water to the final volume. Store at 4 °C.

Mounting medium (working solution)

- 90 % glycerol
- 50 mM Tris-HCl, pH 8.0
- 2.5 % DABCO

Mix 100 % glycerol (90 % of the final volume) and 1 M Tris-HCl, pH 8 (5 % of the final volume) with the deionized water (2.5 % of the final volume). If possible, use the positive displacement pipette for the pipetting of viscous liquids or cut the end of the common tip. Add DABCO to the final concentration of 2.5 % to the prepared solution and mix until dissolved. Aliquot and store at -20 °C.

Solutions used for the detection of DNA replication using BrdU

10 mM BrdU (stock solution)

Dissolve BrdU in deionized water. Aliquot and store at -20 °C.

1 M MgCl₂ (stock solution)

Dissolve MgCl₂·6H₂O in deionized water (eight tenth of the final volume). Add deionized water to the final volume. If necessary, sterilize by autoclaving. Aliquot and store at -20 °C.

1 M CaCl₂ (stock solution)

Dissolve CaCl₂ in deionized water (eight tenth of the final volume). Add deionized water to the final volume. If necessary, sterilize by filtering through the 0.2 µm filter. Do not autoclave. Aliquot and store at -20 °C.

10× DNA cleavage buffer (stock solution)

- 660 mM Tris-HCl, pH 8 at 30 °C
- 6.6 mM MgCl₂
- 10 mM CaCl₂

Mix 1 M stock solutions of Tris-HCl, pH 8 (66 % of the final volume), MgCl₂ (6.6 % of the final volume) and CaCl₂ (1 % of the final volume) and fill with the deionized water to the final volume. Aliquot and store at -20 °C.

Primary antibody solution (working solution)

- Anti-BrdU antibody (use the dilution of the antibody recommended by the manufacturer)
- Exonuclease III (final concentration: 0.4 U/µl)
- DNase I (final concentration: 10 U/ml in the case of formaldehyde-fixed cells, 5 U/ml in the case of ethanol-fixed cells)
- 1× DNA cleavage buffer

Add the above-mentioned components to deionized water. Add the 10× DNA cleavage buffer first. Mix the final solution shortly using vortex. Prepare the primary antibody solution just before the use.

Secondary antibody solution (working solution)

Add antibody against anti-BrdU antibody conjugated with the fluorescent dye to 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (use the dilution of the antibody recommended by the manufacturer). For

the detection of cellular DNA, add 10 mM DAPI (dilute 1:10,000, the final concentration is 1 μ M). Mix the final solution shortly using vortex. Prepare the secondary antibody solution just before the use.

Solutions used for the detection of DNA replication using EdU

10 mM EdU (stock solution)

Dissolve EdU in DMSO. Aliquot and store at -20 °C.

Solution A (20 mM CuSO₄, 500 mM NaCl, stock solution)

Dissolve CuSO₄·5H₂O and NaCl in deionized water (eight tenth of the final volume). Add deionized water to the final volume. Store at 4 °C.

Solution B (50 mM sodium ascorbate and 100 mM glycine, stock solution)

Dissolve sodium ascorbate and glycine in deionized water (eight tenth of the final volume). Add deionized water to the final volume. Aliquot into 2-ml microtubes and seal with parafilm. Store at -20 °C.

10 mM azide dye solution

Dissolve azide dye in DMSO. Aliquot and store at -20 °C.

1 μ M DAPI solution (working solution, optional , for staining of cellular DNA)

Add 10 mM DAPI to 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (dilute 1:10,000, the final concentration is 1 μ M). Mix the final solution shortly using vortex. Prepare DAPI solution just before the use.

A. Detection of nuclear DNA replication using BrdU

1. Incubate the adherent cells in the culture medium supplemented with 10 μ M BrdU for 30 minutes.
2. Rinse the samples three times with 1 $\times$ PBS.
3. Fix the cells either by formaldehyde (a) or

ethanol (b).

- a. Incubate the cells in 2 % formaldehyde in 1 $\times$ PBS (10 minutes). Wash the samples in 1 $\times$ PBS (3 $\times$). Permeabilize the cells by 10-minute incubation in 0.2 % Triton X-100 in 1 $\times$ PBS. Wash the samples with 1 $\times$ PBS (3 $\times$, 1 h in total, at room temperature).
 - b. Incubate the cells in the pre-cooled 70 % ethanol (-20 °C) – put the samples into the freezer and incubate them for at least 30 minutes. Wash in 1 $\times$ PBS (3 $\times$).
4. Wash with 1 $\times$ DNA cleavage buffer (3 $\times$).
In the case of cells grown on coverslips, this and all additional steps can be performed on drops of solutions. In this respect, the circular coverslips with a diameter of 12 mm provide enough cells for the analysis of the cell cycle using image cytometry.
 5. Incubate the samples with the primary antibody solution for 30 minutes at 37°C.
 6. Wash with 1 $\times$ PBS (2 $\times$, 1 minute in total) and then incubate with 0.2 % formaldehyde in 1 $\times$ PBS (10 minutes).
 7. Wash the samples in 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (3 $\times$ 5 minutes).
 8. Incubate the samples with the prepared secondary antibody solution for 30 minutes at room temperature.
 9. Wash the samples four times in 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl.
 10. If coverslips are used, remove the excess of the buffer by touching the edge of the coverslips on the filter paper. Apply drops of the mounting medium on the glass slide (3 - 5 μ l) and carefully lay the coverslips onto the mounting medium with cells facing the mounting medium. Avoid the creation of air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 μ l of the mounting media per well.
 11. Use the nail polish to seal the coverslips and to protect the medium from drying. Evaluate the sample(s) (Fig. 17.1). The well plates and coverslips can be stored at -20 °C. In the case of well plates, seal them with parafilm to protect the medium from drying.

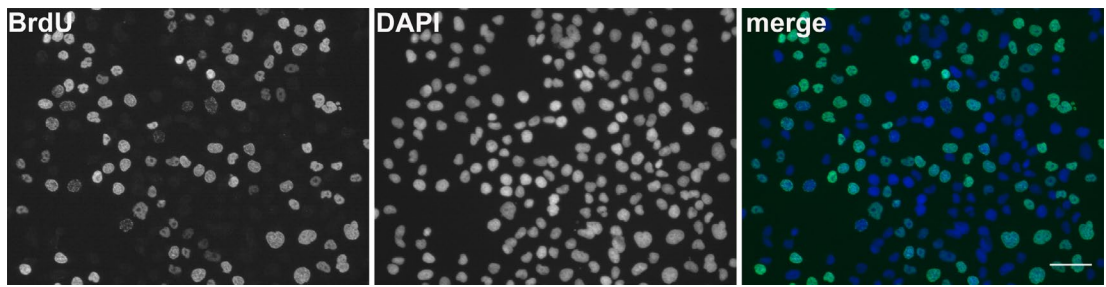


Figure 17.1. The detection of BrdU using DNase I and exonuclease III.

Examples of microscopic images of cell nuclei labeled for 5 minutes with BrdU and fixed with formaldehyde. The DNA was labeled by DAPI. The scale bar: 50 μ m. Adapted from Ligasová et al., 2017, Looking for ugly ducklings: The role of the stability of the BrdU-antibody complex and the improved method of the detection of DNA replication. PLoS ONE 12(3): e0174893 (10).

B. Detection of nuclear DNA replication using EdU

1. Incubate the adherent cells in the culture medium supplemented with 10 μ M EdU for 30 minutes.
2. Rinse the samples three times with 1 $\times$ PBS.
3. Fix the cells either by formaldehyde (a) or ethanol (b).
 - a. Incubate the cells in 2 % formaldehyde in 1 $\times$ PBS (10 minutes). Wash in 1 $\times$ PBS (3 $\times$). Permeabilize the cells by 10-minute incubation in 0.2 % Triton X-100 in 1 $\times$ PBS. Wash the samples with 1 $\times$ PBS (3 $\times$ 5 minutes).
 - b. Incubate the cells in the pre-cooled 70 % ethanol (-20 $^{\circ}$ C) – put the samples into the freezer and incubate them for at least 30 minutes. Wash in 1 $\times$ PBS (3 $\times$).
4. Prepare the fresh EdU-labeling solution from one part of the stock solution A, one part of the stock solution B and three parts of deionized water. Add 10 mM azide dye solution (dilute 1:1,000, the final concentration is 10 μ M). Mix on vortex for 5 seconds.
5. Remove the buffer from the samples and immediately add the prepared EdU-labeling mixture. In the case of cells grown on coverslips, this and all additional steps can be performed on drops of solutions.
6. Incubate the samples 30 minutes at room temperature.
7. Wash the samples with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (3 $\times$, 15 minutes in total).
8. Incubate the samples with 1 μ M DAPI solution (30 minutes).
9. Wash the samples four times in 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (15 minutes in total).
10. If coverslips are used, remove the excess of the buffer by touching the edge of the coverslips on the filter paper. Apply drops of the mounting medium on the glass slide (3 - 5 μ l) and carefully lay the coverslips onto the mounting medium with cells facing the mounting medium. Avoid the creation of air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 μ l of the mounting media per well.
11. Use the nail polish to seal the coverslips and to protect the medium from drying. Evaluate the sample(s). The well plates and coverslips can be stored at -20 $^{\circ}$ C. In the case of well plates, seal them with parafilm to protect the medium from drying.

IV. Comments

- The mentioned manufacturers of the listed instruments and materials were used during

the protocol testing or development.

- The pH values of Tris buffer solutions are temperature and concentration-dependent. Between 5 °C and 25 °C, the pH value increases of 0.03 pH units for each °C decrease in temperature. As the buffer temperature increases from 25 °C to 37 °C, the pH value decreases an average of 0.025 pH units per °C. Increasing the concentration of Tris from 0.05 to 0.5 M will increase the pH value by about 0.05 units. Decreasing the concentration from 0.05 to 0.005 M will decrease the pH value by about 0.05 units. As the effect of the temperature is relatively high and the temperature of the Tris buffer increases during the addition of HCl, allow the solution to cool to room temperature before making the final adjustments of the pH (27).
- MgCl_2 is extremely hygroscopic. Buy only small amount of the chemical. Do not store opened bottles for long time (28).
- For the anti-BrdU antibody clone B44, the optimal concentration is 0.25 - 0.5 $\mu\text{g}/\text{ml}$, for Bu20a, 2.5 - 5 $\mu\text{g}/\text{ml}$. For other anti-BrdU antibodies, test the optimal concentration.
- If necessary, other halogenated nucleosides can be used instead of BrdU such as CldU, IdU, or TFdU. Dilute the mentioned HDT in DMSO to 10 mM stock solution, aliquot and store at -20 °C. Use anti-BrdU antibody clones reacting with these nucleosides for their detection, for example mouse anti-BrdU clone B44.
- Generally, a longer washing step after the permeabilization (point A3a.) is not critical, but its shortening leads to a decrease of the signal.
- If RNA has to be removed (e.g. if propidium iodide is used for DNA labeling instead of DAPI), add RNase A (100 $\mu\text{g}/\text{ml}$) to the primary antibody solution.
- Use 30 μl of antibody solution per one circular coverslip with a diameter of 12 mm or 75 μl per well in the case of 96-well plates.
- In the case of the detection of additional cellular components using specific antibodies, add the required primary antibody to the primary antibody solution.
- The step with post-fixation of samples with 0.2 % formaldehyde serves the stabilization of an antibody with incorporated BrdU,

otherwise, the antibody-BrdU complex can dissociate quickly (10). The quick wash followed by the stabilization step is important in the ethanol-fixed cells only in the case of some antibodies. This step is necessary e.g. for antibody clone Bu20a; however, not for clone B44. The data about some other clones are available in the above-mentioned paper. On the contrary, for the formaldehyde-fixed cells, this step is important for the majority of the antibodies tested (10).

- If the stabilization step is not used, the signal can be improved by a quick reaction with the secondary antibody although the stabilization step usually allows faster and more effective stabilization of the antibody.
- In the case of EdU labeling, use the incubation time at least 10 minutes as the shorter incubation times can lead to the underestimation of the replicated cells (10, 11).
- Pre-warm all solutions necessary for the preparation of EdU-labeling solution to 25 °C before use.
- 1 ml of the prepared EdU-labeling solution is enough for Petri dish with diameter 3.5 cm or use 350 μl of the mixture per one circular coverslip with a diameter of 12 mm or 100 μl per well in the case of 96-well plates.

V. Pitfalls

Problem	Cause and cure
BrdU detection	
Low signal of DNA dyes	• Too long incubation or too high activity of DNase I in the primary antibody solution was used. This can cause the lowering of DNA-derived signal after staining with DNA dyes. Shorten the incubation time or use the lower concentration of DNase I.
Low or no replicational signal	• The unsuitable anti-BrdU antibody was used or the stabilization step by formaldehyde was not performed and/or the washing step between the incubation with primary and secondary antibodies was too long resulting in the dissociation of primary antibody from BrdU epitopes. Use other clone of anti-BrdU antibody or use the stabilization step or shorten the washing step between the incubation with primary and secondary antibody.
EdU detection	
Low or no signal	• Solution B containing sodium ascorbate was old and/or vial with it was opened and sodium ascorbate oxidized. Prepare the fresh stock solution B.
Low signal/ background ratio	• The low rate of incorporation of EdU or short washing step after click reaction was used. Prolong the incubation step with EdU and/or increase its concentration. Prolong the washing step.

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***In situ* detection of overall and replicated mitochondrial DNA in mammalian cells**

Anna Ligasová and Karel Koberna

I. Introduction

The mitochondria of mammalian cells contain extremely low amount of DNA. For example, the human mitochondrial DNA (mtDNA) contains $\pm 16,500$ bp (1) in contrast to $\pm 3,000,000,000$ bp of nuclear genome. Therefore, it is not surprising that the common methods developed for the detection of nuclear DNA provide very low signal. In addition, this signal is usually masked by the signal derived from the cell nucleus (2).

Presently, several approaches are used for the detection of overall mtDNA. It includes for example the staining of mitochondrial DNA using DAPI. This dye is commonly used for the labeling of nuclear DNA. Although it can be applied also for the detection of mammalian mtDNA, its use requires sensitive cameras with an adequate dynamic range (3). Other approaches are based on the use of PicoGreen (4, 5), SYTO13 (6), ethidium bromide (7, 8), SYBR Green I (9), or GFP tagging the mitochondrial core nucleoid factor Twinkle (10). Besides these methods, overall mtDNA can be visualized by the enzymatic labeling with the modified nucleotides (2, 11), by antibodies raised against the double-

stranded DNA (7), or by *in situ* hybridization (12).

Similarly, the detection of replicated mtDNA suffers from the low content of mtDNA. The current methods rely on the incorporation of modified nucleosides and their detection by means of antibodies or click reaction. Typically, 5-bromo-2'-deoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) are used. BrdU is detected using anti-BrdU antibodies while EdU by means of click chemistry (8, 13-17). BrdU detection requires special treatment of DNA for displacement of the masking strand as BrdU is not accessible in the double stranded DNA for the reaction with antibodies. This step is usually done by strong acids (HCl) or monovalent copper ions (17, 18). In the case of EdU, no such step is necessary. However, EdU is highly toxic (19-24) and contrary to BrdU, it requires an amplification step (14, 25).

In this chapter, we will describe the protocol for the detection of overall mtDNA using the enzymatic labeling and for the detection of mtDNA synthesis using BrdU. Both methods are sensitive approaches that do not require

additional enhancement of the signal. The protocols are based on the cleavage of DNA by monovalent copper ions resulting in the formation of frequent gaps in DNA strands. The generated DNA ends are then used as origins for the enzymatic labeling of the overall mtDNA by DNA polymerase I or they are enlarged by exonuclease activity resulting in the unmasking of BrdU incorporated into DNA during mtDNA synthesis (Fig. 18.1) (2, 11, 17).

- Laboratory shaker Vortex Wizard (Velp Scientifica, Inc., X000102)
- Laboratory shaker ThermoMixer® C (Eppendorf, 5382000015)
- The positive displacement pipette for the pipetting of viscous liquids (for example Microman ME100 from Gilson, FD10004)
- pH meter (pH 8, XS Instruments)
- Fluorescence microscope (for example Olympus IX83, Olympus)

II. Materials and instrumentation

Instruments

- Vortex Lab Dancer (IKA, 0003365000)
- Heating magnetic stirrer ARE (Velp Scientifica, Inc., F20500162)

Materials

Sodium chloride (NaCl; 31434, Sigma Aldrich), potassium chloride (KCl; 60130, Sigma Aldrich), sodium phosphate dibasic (Na_2HPO_4 ; S5136, Sigma Aldrich), potassium dihydrogen phosphate (KH_2PO_4 ; P5655, Sigma Aldrich), sodium hydroxide (NaOH; 06203, Sigma Aldrich),

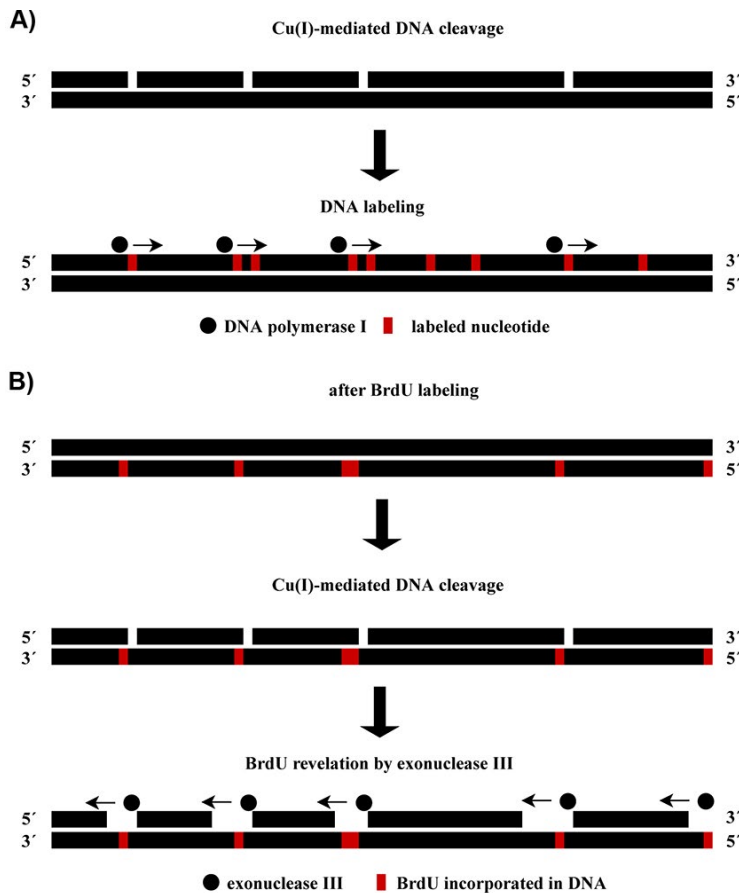
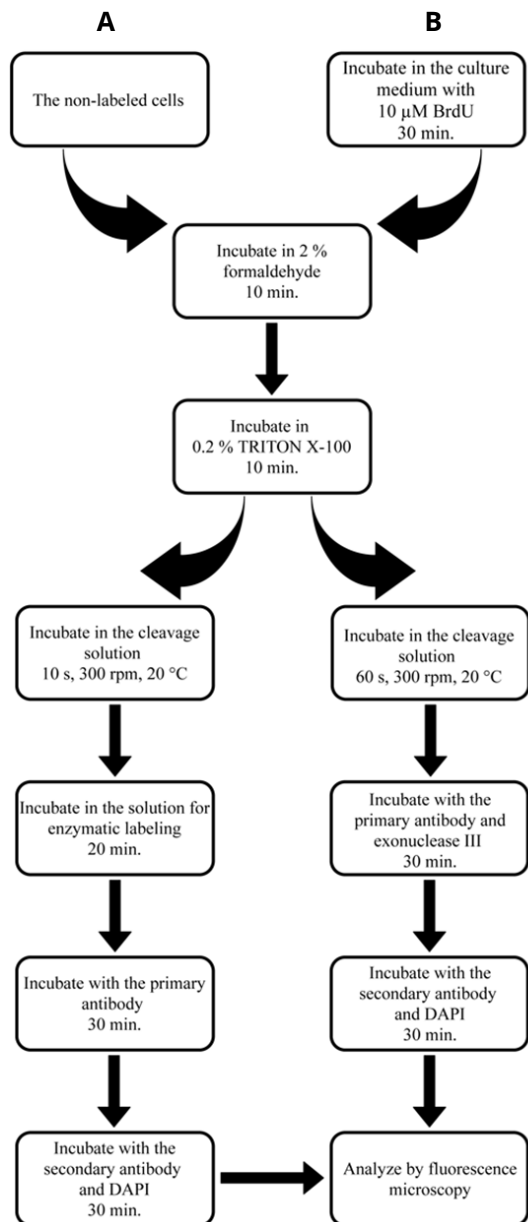


Figure 18.1. The Cu(I)-mediated DNA cleavage and the use of the generated gaps for the detection of overall mtDNA by enzymatic labeling (A) or replicated mtDNA using the strand displacement by exonuclease activity (B).

paraformaldehyde (PFA; 818715, Merck), Triton X-100 (X100, Sigma Aldrich), Hepes (H4034, Sigma Aldrich), Tris base (T6066, Sigma Aldrich), HCl (10033-A35, Lach-Ner), DAPI (D9542, Sigma Aldrich), glycerol (G7893, Sigma Aldrich), 1,4-diazabicyclo[2.2.2]octane (DABCO; D2522, Sigma Aldrich), 5-bromo-2'-deoxyuridine (BrdU; NB06315, Carbosynth), anti-BrdU antibody (for example mouse anti-BrdU clone B44; 347580, Becton Dickinson), exonuclease III (EN0191, Thermo Fisher Scientific), 10× buffer for exonuclease III (EN0191, Thermo Fisher Scientific), antibody against anti-BrdU antibody conjugated with fluorescent dye (for example AffiniPure Goat Anti-Mouse IgG (H+L) conjugated with Alexa Fluor® 488; 115-545-146, Jackson ImmunoResearch), DNA polymerase I (EP0042, Thermo Fisher Scientific), 10× buffer for DNA polymerase I (EP0042, Thermo Fisher Scientific), 2'-deoxyadenosine-5'-triphosphate (dATP; U120A, Promega), 2'-deoxyguanosine-5'-triphosphate (dGTP; U121A, Promega), 2'-deoxycytidine-5'-triphosphate (dCTP; U122A, Promega), thymidine 5'-triphosphate (dTTP; U123A, Promega), modified nucleotides: biotin-16-2'-deoxyuridine-5'-triphosphate (biotin-dUTP; NB32058, Carbosynth) or digoxigenin-11-2'-deoxyuridine-5'-triphosphate (digoxigenin-dUTP; 11093088910, Roche Diagnostics) or Alexa Fluor® 555-aha-2'-deoxyuridine-5'-triphosphate (Alexa Fluor 555-dUTP; A32762, Thermo Fisher Scientific), anti-biotin antibody (for example rabbit anti-biotin; ab53494, Abcam) or anti-digoxigenin antibody (for example mouse anti-digoxigenin; 11333062910, Roche Diagnostics), copper(II) sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; C8027; Sigma Aldrich), sodium ascorbate (11140; Sigma Aldrich), antibody against anti-biotin antibody conjugated with fluorescent dye (for example AffiniPure Goat Anti-Rabbit IgG (H+L) conjugated with Alexa Fluor® 488; 111-545-144, Jackson ImmunoResearch) or antibody against anti-digoxigenin antibody conjugated with fluorescent dye (for example AffiniPure Goat Anti-Mouse IgG (H+L) conjugated with Alexa Fluor® 488; 115-545-146, Jackson ImmunoResearch)

Other commonly used material such as deionized water (deionized or distilled), cover glasses, tweezers, parafilm, Petri dishes, pipettes and tips, microtubes, centrifugation tubes, filter paper, and immersion oil.



Scheme 18.1

A) Scheme of the detection of overall mtDNA and B) scheme of the detection of mtDNA replication (both excluding the washing steps and embedding into the mounting medium. All steps are performed at room temperature if not stated otherwise.)

III. Procedures

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Dissolve appropriate amounts of the above-mentioned chemicals in deionized water (eight tenth of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Sterilize in an autoclave. Store at room temperature; after opening, store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

2 % formaldehyde in 1× PBS buffer, pH 7.4 (working solution)

Dissolve the paraformaldehyde in deionized water (one-half of the final volume) in a beaker and heat to 60 °C on a heating magnetic stirrer. When moisture forms on the sides of the beaker, add NaOH (ca 8 drops of 1 M solution per 0.5 l), and stir until the solution clears. Cool the prepared solution under the faucet. Add 10× PBS (one tenth of the final volume). If necessary, adjust the pH to 7.4. Add deionized water to the final volume, aliquot and store at -20 °C. **Formaldehyde is toxic, use protective means during its preparation and use, and work in a fume hood!**

0.2 % Triton X-100 in 1× PBS, pH 7.4 (working solution)

Warm up 1× PBS to the room temperature. Add 100 % Triton X-100 to the pre-warmed buffer. As Triton X-100 is highly viscous, use the positive displacement pipette for the pipetting of viscous liquids or cut the end of the common tip. Mix the prepared solution for ca 10 minutes on the laboratory shaker.

1 M NaCl (stock solution)

Dissolve NaCl in deionized water (eight tenth of the final volume), then add deionized water to the final volume and store at 4 °C.

1 M Hepes, pH 7 (stock solution)

Dissolve Hepes in deionized water (eight tenth of the final volume), adjust the pH to 7 using 1 M NaOH) and add deionized water to the final volume. Store at 4 °C.

Solution A - 20 mM CuSO₄·5H₂O and 500 mM NaCl (stock solution)

Dissolve CuSO₄·5H₂O and NaCl in deionized water (eight tenth of the final volume), add 1 M NaCl (stock solution). Add the deionized water to the final volume. Store at 4 °C.

Solution B - 50 mM sodium ascorbate and 200 mM Hepes, pH 7.0 (stock solution)

Dissolve sodium ascorbate in deionized water (one-half of the final volume), add 1 M Hepes buffer, pH 7 (stock solution) and fill with the deionized water to the final volume. Aliquot into the 2-ml microtubes and store at -20 °C. Solution of sodium ascorbate is subject to quick air oxidation. Therefore, seal the tubes with parafilm.

1 M Tris-HCl, pH 7.5 (stock solution)

Dissolve Tris base in deionized water (eight tenth of the final volume) and adjust pH to 7.5 with the concentrated HCl. Add deionized water to the final volume. Store at 4 °C.

100 mM Tris-HCl pH 7.5 (working solution)

Dilute ten times the stock solution of 1 M Tris-HCl pH 7.5 in deionized water. Store at 4 °C.

25 mM Tris-HCl, pH 7.5 + 150 mM NaCl (working solution)

Mix 25 parts (e.g. 25 ml) of 1 M Tris-HCl, pH 7.5, 150 parts (e.g. 150 ml) of 1 M NaCl and fill to 1,000 parts (e.g. 1,000 ml) with deionized water. Store at 4 °C.

1 M Tris-HCl, pH 8 (stock solution)

Dissolve Tris base in deionized water (eight tenth of the final volume) and adjust pH to 8 with the concentrated HCl. Add deionized water to the final volume. Store at 4 °C.

10 mM DAPI (stock solution; optional, for staining of cellular DNA)

Dissolve DAPI in deionized water. Aliquot and store at -20 °C.

Mounting medium (working solution)

90 % glycerol
50 mM Tris-HCl, pH 8.0
2.5 % DABCO

Mix 100 % glycerol (90 % of the final volume) and 1 M Tris-HCl, pH 8 (5 % of the final volume) with the deionized water (2.5 % of the final volume). If possible, use the positive displacement pipette for the pipetting of viscous liquids or cut the end of the common tip. Add DABCO to the final concentration of 2.5 % to the prepared solution and mix until dissolved. Aliquot and store at -20 °C.

Solutions used for the detection of overall mtDNA

Solution for enzymatic labeling (working solution)

- DNA polymerase I (final concentration is 0.2 U/μl)
- 10× buffer for DNA polymerase I (final concentration is 1×)
- Mixture of 0.05 mM dATP, 0.05 mM dGTP, 0.05 mM dCTP
- Modified nucleotides: 0.05 mM biotin-dUTP or 0.05 mM digoxigenin-dUTP or 0.05 mM Alexa Fluor 555-dUTP

Add above mentioned components to deionized water. Add the 10× buffer for DNA polymerase first. Mix the final solution shortly using vortex. Prepare the fresh enzymatic solution prior the use.

Primary antibody solution (working solution)

Add anti-biotin antibody or anti-digoxigenin antibody to 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl. Use the dilution of the antibody recommended by the manufacturer. Mix the final solution shortly using vortex. Prepare the antibody solution just before the use.

Secondary antibody solution (working solution)

Add antibody against anti-biotin or anti-digoxigenin antibody conjugated with fluorescent dye to 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl. Use the dilution of the antibody recommended by the manufacturer. For the detection of cellular DNA, add 10 mM DAPI (dilute 1:10,000, the final concentration is 1 μM). Mix the final solution shortly using vortex.

Prepare the secondary antibody solution prior the use.

Solutions used for the detection of mtDNA replication

10 mM 5-bromo-2'-deoxyuridine (BrdU; stock solution)

Dissolve BrdU in deionized water. Aliquot and store at -20 °C.

Primary antibody solution (working solution)

- Anti-BrdU antibody (use the dilution of the antibody recommended by the manufacturer)
- Exonuclease III (final concentration: 1 U/μl)
- 10× buffer for exonuclease III (final concentration: 1×)

Add the above mentioned components to deionized water. Add the 10× buffer for exonuclease first. Mix the final solution shortly using vortex. Prepare the primary antibody solution just before the use.

Secondary antibody solution (working solution)

Add antibody against anti-BrdU antibody conjugated with fluorescent dye to 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (use the dilution of the antibody recommended by the manufacturer). For the detection of cellular DNA, add 10 mM DAPI (dilute 1:10,000, the final concentration is 1 μM). Mix the final solution shortly using vortex. Prepare the secondary antibody solution just before the use.

A. Detection of mitochondrial DNA using copper ions

1. Rinse the cells three times with 1× PBS.
2. Fix the cells in 2 % formaldehyde in 1× PBS (10 minutes).
3. Wash the samples in 1× PBS (3×).
4. Permeabilize the cells by 10-minute incubation in 0.2 % Triton X-100 in 1× PBS.
5. Wash the samples in 1× PBS (3×).
6. Prepare the fresh cleavage mixture. Add one volume of the solution A (stock solution) and one volume of the solution B (stock solution) to twenty-three volumes of deionized water just prior to the DNA

- cleavage. Mix on vortex for 5 second.
7. Remove 1× PBS from the samples and immediately add the prepared cleavage mixture.
 8. Put the samples on the shaker for 10 seconds at 300 rpm.
 9. Remove the working cleavage solution.
 10. Wash the samples with 100 mM Tris-HCl, pH 7.5 (3×).
 11. Prepare the fresh solution for enzymatic labeling of mtDNA.
 12. Incubate the cells in the solution for enzymatic labeling for 20 minutes.
 13. Wash the cells with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (3×).
 14. If **Alexa Fluor 555-dUTP** was used, continue with step 17. If **biotin-dUTP** or **digoxigenin-dUTP** were used, incubate the samples with the primary antibody solution for 30 minutes.
 15. Wash the samples four times with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl.
 16. Incubate the samples with the prepared secondary antibody solution and incubate for 30 minutes.
 17. Wash the samples four times with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl.
 18. If coverslips are used, remove the excess of the buffer by touching the edge of the coverslips on the filter paper. Apply drops of the mounting medium on the glass slide (3 – 5 µl) and carefully lay the coverslips onto the mounting medium with cells facing the mounting medium. Avoid the creation of air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 µl of the mounting media per well.
 19. Use the nail polish to seal the coverslips and to protect the medium from drying. Evaluate the sample(s) (Fig. 18.2). The well plates and coverslips can be stored at -20 °C. In the case of well plates, seal them with parafilm to protect the medium from drying.

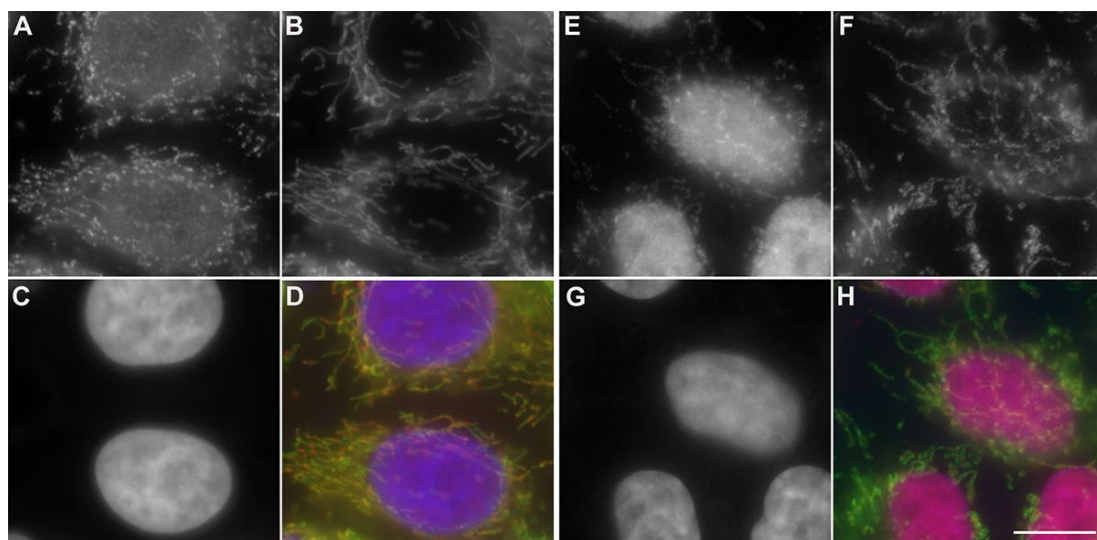


Figure 18.2. The detection of the mitochondrial DNA.

The detection of the mitochondrial DNA by a 10-second cleavage in a solution containing copper(I) ions followed by the labeling of mitochondrial DNA by DNA polymerase I and biotin-dUTP (A, D; red in the color image) or Alexa 555-dUTP (E, H; red in the color image). The anti-mitochondrial antibody MTCO2 (B, D, F, H; green in the color images) was used for the identification of mitochondria. DNA was stained by DAPI (C, D, G, H; blue in the color images). Bar: 10 µm. Adapted from Ligasová et al., 2013, A New Method of the Visualization of the Double-Stranded Mitochondrial and Nuclear DNA. PLoS ONE 8(6): e66864 (2).

B. Detection of mitochondrial DNA replication

1. Incubate the adherent cells in the culture medium supplemented with 10 μ M BrdU for 60 minutes.
2. Rinse the samples three times with 1 $\times$ PBS.
3. Fix the cells with 2 % formaldehyde in 1 $\times$ PBS (10 minutes).
4. Wash the cells in 1 $\times$ PBS (3 $\times$).
5. Permeabilize the cells by 10-minute incubation in 0.2 % Triton X-100 in 1 $\times$ PBS.
6. Wash the cells in 1 $\times$ PBS (3 $\times$).
7. Prepare the fresh working cleavage mixture from the stock solutions. Add one volume of the solution A and one volume of the solution B to three volumes of deionized water just prior to DNA cleavage. Mix on vortex for 5 seconds.
8. Remove 1 $\times$ PBS from the samples and immediately add the prepared cleavage mixture.
9. Put the samples on the laboratory shaker for 60 seconds, 300 rpm.
10. Remove the working cleavage solution.
11. Wash the cells with 100 mM Tris-HCl, pH 7.5 (3 $\times$, 20 minutes in total).
12. Rinse the cells with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl (3 $\times$).
13. Incubate the cells with the primary antibody solution for 30 minutes.
14. Wash the samples four times with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl.
15. Incubate the samples with the prepared secondary antibody solution for 30 minutes.
16. Wash the samples four times with 25 mM Tris-HCl, pH 7.5 and 150 mM NaCl.
17. If coverslips are used, remove the excess of the buffer by touching the edge of the coverslips on the filter paper. Apply drops of the mounting medium on the glass slide (3 -5 μ l) and carefully lay the coverslips onto the mounting medium with cells facing the mounting medium. Avoid the creation of air bubbles. In the case of 96-well plates, aspirate the buffer and add 100 μ l of the mounting media per well.
18. Use the nail polish to seal the coverslips and to protect the medium from drying. Evaluate the sample(s) (Fig. 18.3). The well plates and coverslips can be stored at -20 $^{\circ}$ C. In the case of well plates, seal them with parafilm to protect the medium from drying.

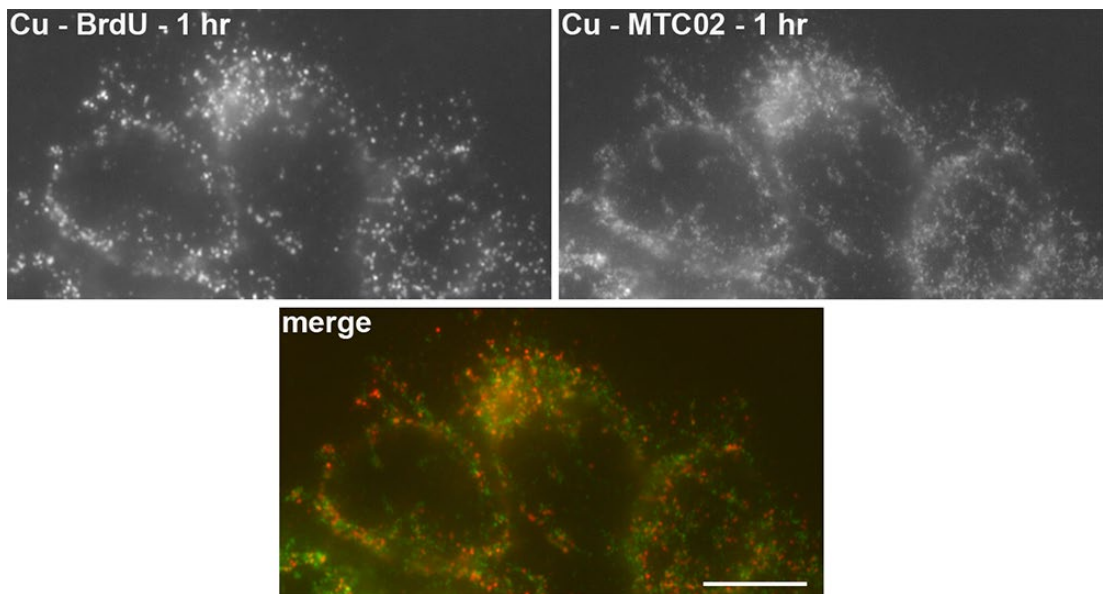


Figure 18.3. The detection of the BrdU-labeled DNA in mitochondria.

The detection of the BrdU-labeled mtDNA (red in the color image) after a 1-hour BrdU pulse in cells treated with 4 mM copper(I) for 60 seconds followed by exonuclease III cleavage is shown. A simultaneous co-localization with the mitochondrial marker MTC02 has been performed (green in the color image). Bar: 10 μ m. Adapted from Ligasová et al., 2012, Atomic Scissors: a new method of tracking the 5-bromo-2'-deoxyuridine-labeled DNA in situ. *PLoS ONE* 7(12): e52584 (17).

IV. Comments

- The mentioned manufacturers of the listed instruments and materials were used during the protocol testing or development.
- The pH values of Tris buffer solutions are temperature and concentration-dependent. Between 5 and 25 °C, the pH value increases of 0.03 pH units for each °C decrease in temperature. As the buffer temperature increases from 25 to 37 °C, the pH value decreases an average of 0.025 pH units per °C. Increasing the concentration of Tris from 0.05 to 0.5 M will increase the pH value by about 0.05 units. Decreasing the concentration from 0.05 to 0.005 M will decrease the pH value by about 0.05 units. As the effect of the temperature is relatively high and the temperature of the Tris buffer increases during the addition of HCl, allow the solution to cool to room temperature before making the final adjustments of the pH (11).
- Pre-warm solution A, solution B and deionized water to 20 °C - 25 °C before preparation of the working cleavage solution.
- In the case of cells grown on coverslips, perform the incubation of samples in the cleavage solution in a Petri dish. Do not perform it on drops of the prepared mixture as it protects the sample from oxygen. 1 ml of the mixture is enough for a Petri dish with the diameter of 3.5 cm (11). Use one coverslip per one Petri dish; otherwise, it is necessary to check the position of the coverslips during the next step to protect their mutual sliding resulting in stacking (11).
- DNA cleavage using copper ions is a critical step. The extent of the cleavage has to be controlled as high DNA cleavage can result in the loss of DNA, proteins bound to DNA or even to the loss of other cellular components. On the other hand, low cleavage does not provide a sufficient amount of gaps. The cleavage intensity can be monitored, e.g. by DAPI staining of cell nuclei. A substantial decrease of DAPI signal clearly indicates the progressive cleavage of DNA (11).
- The cleavage is influenced by several factors.

The cleavage is directly proportional to the temperature, incubation time, copper(I) ions, and oxygen concentration. If DNA cleavage is high (a substantial decrease of DAPI signal is observed) or low (no or low mtDNA- or BrdU-derived signal is observed), it is necessary to adjust at least some of these parameters. We recommend to change the concentration of the copper(II) sulfate and the concentration of sodium ascorbate first. The concentration of the copper(II) sulfate, sodium ascorbate, and Hepes buffer should be changed proportionally. The ratio between the concentration of the copper(II) sulfate, sodium ascorbate, and Hepes buffer should be 1:2.5:50. The effect of NaCl on cleavage is relatively low; however, it should not be substantially above the concentration of 100 mM in the final mixture (11).

- In the case of cells grown on coverslips, incubation step in the solution for enzymatic labeling or in primary antibody solution and all the following steps can be performed on drops of solutions. Use 30 µl per circular coverslip with a diameter of 12 mm or 50 µl per well in the case of 96-well plates.
- When biotin-dUTP and digoxigenin-dUTP are used, add 0.05 mM dTTP to the solution for enzymatic labelling. This usually results in an increase of the signal. Do not add dTTP if Alexa Fluor 555-dUTP is used for the detection of mtDNA. The addition of dTTP can completely eliminate the signal (11).
- In the case of the detection of additional cellular components using specific antibodies, add the required antibody to the antibody solution.
- We tested several anti-BrdU antibodies. The highest signal provided anti-BrdU antibody clone B44 and chicken polyclonal antibody. If there is need for other antibody clone that provides low signal, you can test protocol based on the stabilization of BrdU-antibody complex by formaldehyde (26). In that case, wash samples immediately after incubation with antibody with 1× PBS (2x, 1 min or less in total) and incubate with 0.2 % formaldehyde in 1× PBS (10 min, room temperature) (11).

V. Pitfalls

Problem	Cause and cure
Overall mtDNA	
Low or no signal	If the signal is low and biotin-dUTP or digoxigenin-dUTP are used, add 0.05 mM dTTP to the solution, it can improve the signal. Do not add dTTP if Alexa Fluor 555-dUTP is used as it can completely eliminate the signal.
mtDNA replication	
Low or no signal	- Too long incubation in the solution containing copper ions. This can cause the complete cleavage of DNA. Shorten the incubation time. - Alternatively, it can be caused by the use of inappropriate clone of anti-BrdU antibody. Use another antibody clone such as mouse anti BrdU clone B44 (Becton Dickinson) or chicken polyclonal anti-BrdU antibody (Abcam).

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Reporters for subcellular localization and image analysis

Jarmila Stanková

I. Introduction

Cellular organelles and compartments are evolutionary benefits to eukaryotic cells and have been developed to ensure that complex biological processes occur in an organized and efficient manner. The dynamic recruitment of macromolecules to specific subcellular locations is crucial for both membrane defined organelles and compartments that lack a membranous border (1, 2). Most subcellular structures are dynamic, with respect to their organization and function. Cellular processes and their regulation at the subcellular level are highly important under special condition induced by environmental or disease-related stress (3).

Fluorescent microscopy combined with image analysis offers an effective tool to analyse the organization and function of cellular organelles and compartments (2). Various methods are used for identification of such subcellular structures. Usually, they rely on the visualization of specific molecules, features (e.g. pH values) or enzyme activities using various probes. The right choice of the marker is critical for this step. Markers that are visualized by fluorescent microscopy should

be reliable and simple (1). Examples of molecular markers of some organelles and compartments are summarized in table (Tab. 19.1).

In this chapter, the identification of mitochondria using the MitoTracker®, and cell nuclei using DNA dyes, and the simple analysis of the acquired data providing the averaged mitochondrial area per cell using ImageJ software (5, 6) will be described. Obtained data can be also used for the calculation of the averaged mitochondrial/nuclear area per cell. The protocol for development of cell line(s) with the fluorescent fusion proteins is described in chapter 9, and the use of antibodies against the compartment marker proteins is described in chapter 16.

II. Materials and instrumentation

Instruments

- Vi-CELL XR Cell Viability Analyzer (Beckman Coulter)
- Echo® 550 (Labcyte)

TABLE 19.1. Often used markers for subcellular structures

Subcellular structure	Target/feature/activity	Probe/tag
Cytoskeleton	Tubulin	GFP, RFP, and mCherry
Multiple	Antibody against compartment marker	Fluorescent primary or usually secondary antibodies
Mitochondria	Mitochondrial membrane potential	Cationic fluorophores (MitoTracker®)
Lysosomes	Acidic pH	LysoTracker®
Nucleus	DNA	Fluorescent DNA dyes (DAPI, Hoechst, NucRed®, and propidium iodide)
Nucleoli	Nucleolin, fibrillarin, cellular apoptosis susceptibility protein	Specific antibodies/fluorochrome or fluorochrome-tagged secondary antibodies

- CellVoyager CV7000 High-Content Screening System (Yokogawa)

Materials

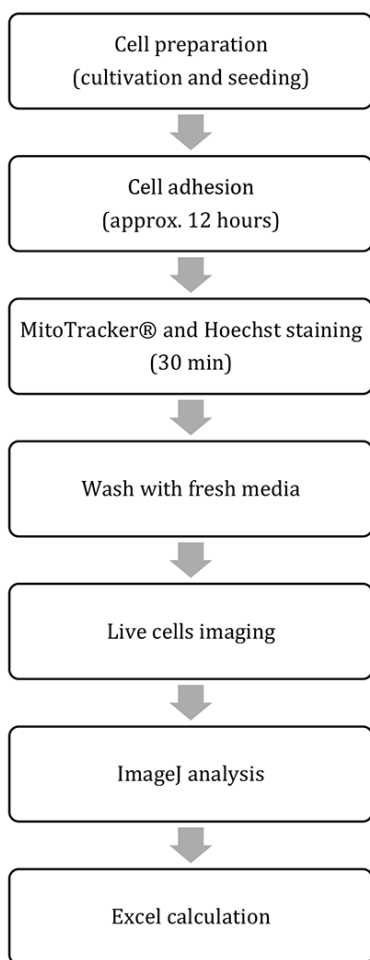
MitoTracker® Red CMXRos (MitoTracker®; M7512, Invitrogen™), Hoechst 33342, Trihydrochloride, Trihydrate (Hoechst; H1399, Invitrogen™), McCoy's 5A (Modified) Medium (16600082, Gibco), dimethyl sulfoxide cell culture grade (DMSO; A3672, AppliChem), TrypLE™ Express Enzyme (Tryple; 12604054, Gibco), tissue culture flasks 150 cm² (90151, TPP), LDV microplate (LP-0200, LabCyte), CellCarrier-384 Black, Optically Clear Bottom well plates (CellCarrier-384; 6007550, PerkinElmer), fetal

bovine serum (FBS; F2442, Sigma Aldrich), sodium chloride (NaCl; 31434, Sigma Aldrich), potassium chloride (KCl; 60130, Sigma Aldrich), sodium phosphate dibasic (Na₂HPO₄; S5136, Sigma Aldrich), potassium dihydrogen phosphate (KH₂PO₄, P5655, Sigma Aldrich), sodium hydroxide (NaOH; 06203, Sigma Aldrich). Other commonly used material such as deionized/distilled water (sterile), pipettes and tips, microtubes, and centrifugation tubes.

Reference cell line (U-2 OS cells, ATCC® HTB-96™, and ATCC).

- CellVoyager CV7000 High-Content Screening System (Yokogawa)

III. Procedures



Scheme 19.1

Scheme of the procedure with all the steps. Incubation times can be adjusted.

Solutions

Culture medium

- McCoy's 5A (Modified) Medium
- 10% FBS

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Dissolve all chemicals in deionized water, adjust pH to 6.8 with 1 M NaOH and add deionized water to the final volume

(1, 000 ml). Sterilize by autoclaving. Store at room temperature; after opening, store at 4 °C. Use as a diluted 1× solution.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

MitoTracker®

Prepare a stock solution by dissolving MitoTracker® product in DMSO to a final concentration of 1 mM, store at -20 °C. Use a working concentration of 150 nM.

Hoechst solution

Prepare a stock solution by dissolving Hoechst 33342 dye in water to a final concentration of 10 mg ml⁻¹.

A. Cell preparation and staining

1. Rinse the cells in the culture flask (the optimal cell confluence is ca 80%) with 1× PBS buffer.
2. Add 2 ml of Tryple solution (150 cm² culture flask) and incubate cells 5 - 10 minutes to detach (37° C, 5 % CO₂).
3. Add 5 ml of culture media and prepare a single cell suspension by gently pipetting the cells up and down.
4. Count the cells using Vi-Cell Viability Analyzer.
5. Dilute the cell suspension to the 100,000 cells per 1 ml with the fresh media.
6. Add 30 µl of the diluted cell suspension containing 3,000 cells to per wells of a 384-well plate (CellCarrier-384 plate).
7. Allow cells to adhere (12 hours or more).
8. Prepare a LDV microplate with MitoTracker® stock solution by adding 11 µl of MitoTracker® stock solution.
9. Add 4.5 nl of the stock solution from the LDV microplate to the CellCarrier-384 plate with cells using Echo® 550. Incubate cell with Mitotracker for 10 minutes at 37 °C and 5 % CO₂.
10. Stain the cells with Hoechst solution by adding 3 µl of Hoechst stock solution per well using a multichannel pipette. Incubate cells for 20 minutes at 37 °C and 5 % CO₂.
11. Wash the cells with a fresh media.

B. Live cells imaging and image analysis

1. Start the CV7000 High-Content Screening System and turn on the incubator. Adjust the temperature, CO₂ level and water supply for humidity for live-cell imaging.
2. Use a 60× objective and select appropriate lasers and channels for Hoechst (blue) and MitoTracker® (red) according to the fluorescent intensity of dyes. Use as lowest exposition time to avoid a phototoxicity.
3. For image analysis of cell count and mitochondrial area, use ImageJ program. Use a red channel for area analysis of mitochondria and a blue channel for cell counting.
4. Load the image in the ImageJ program by dragging and dropping the images or by this path “File → Open”. Cell counting and area analysis work only with a single channel image. If the source image is stacked, then use “Image → Stacks → Stack to Images” to convert a .tiff stack to single channel .tiff file.
5. Change the image to 8-bit version, using Image → Type → 8-bit.
6. Adjust the threshold using “Image → Adjust → Threshold → Set a minimum and a maximum → Set”.
7. Analyse area: “Analyse → Analyse particles → Set pixel size 50-Infinity”, Select Show Outline and check the Display Results and Clear Results options from the drop-down menu. Copy the data to Microsoft Excel, and save the Excel file. An example of image analysis using ImageJ is shown in Fig 19.1.
8. Count the cells following the same approach using a blue channel (Hoechst).
9. Calculate average mitochondrial area per cell in Excel by dividing the sum of mean values of mitochondrial area of all objects (Red channel) by the cell count (Blue channel).

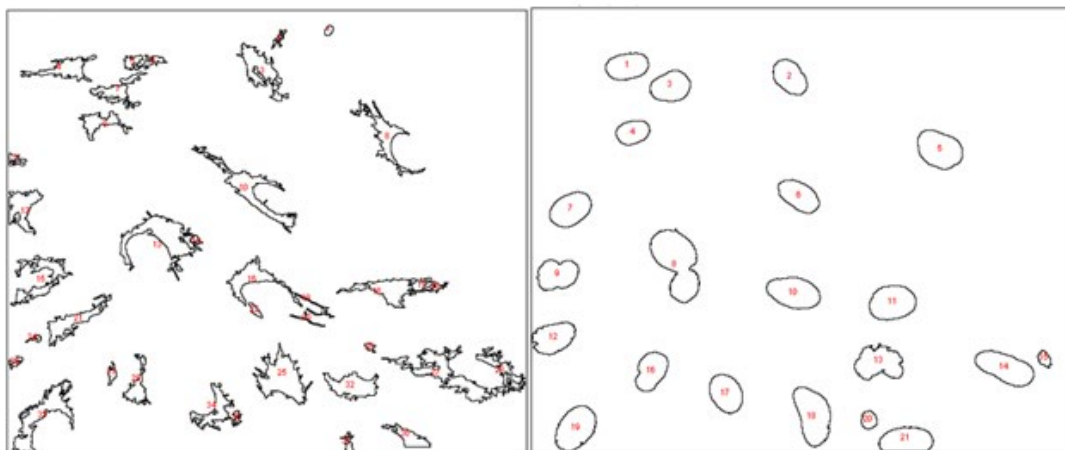


Figure 19.1. Examples showing area analysis (left) and cell count (right).

IV. Comments

- The described procedure can be used for compound activity assessment, especially the compounds that affect the structure of mitochondrion. Notice that live cell imaging

in combination with MitoTracker® is most applicable only for short-term imaging experiments, a few hours lasting (4). Example images of U-2 OS cells stained with Hoechst and MitoTracker® are shown in Fig. 19.2.

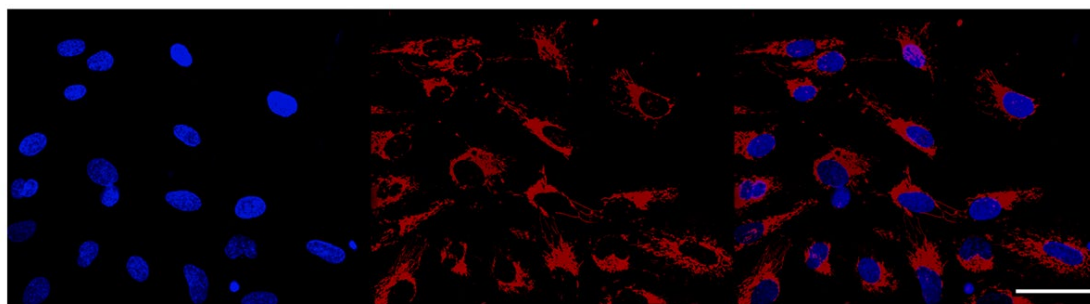


Figure 19.2. *U-2 OS cell line stained with Hoechst (blue channel) and MitoTracker® (red channel). Scale bar: 240 μm .*

V. Pitfalls

Problem	Cause and cure
Hoechst staining	
No or very low signal	An old working solution of Hoechst was used for staining. Prepare a fresh solution and repeat the staining.
Highly irregular shaped nuclei	The cells were not in a good condition. Use cells from cultures that are 80-85% confluent and in a good condition.
MitoTracker® staining	
No or low signal	Low concentration of MitoTracker® dye was used. Increase the concentration and adjust the incubation time.
Very high signal High background	- The time of incubation was too long. Decrease the incubation time. - The washing step was not enough. Increase the number of washing steps or the time of washing.

VI. Acknowledgement

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 – Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Reporters based on luminescence or fluorescence detection

Ivo Frydrych

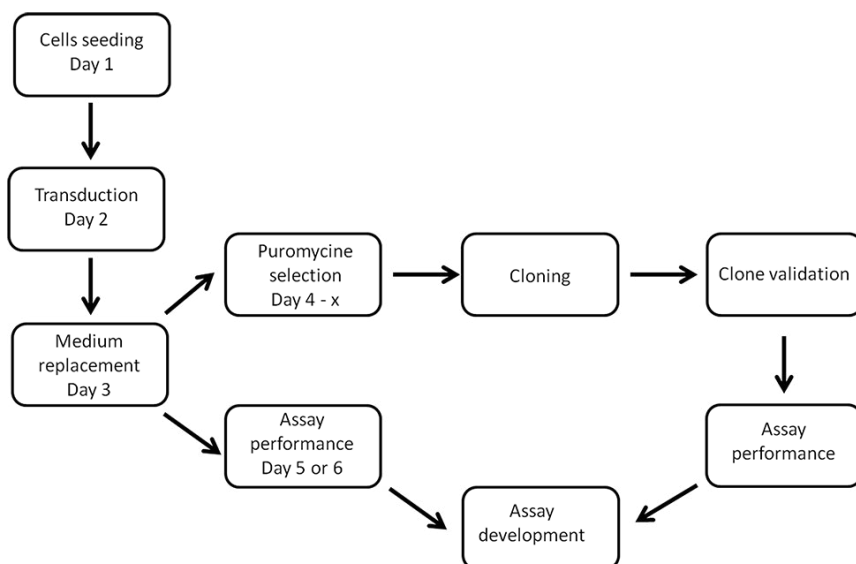
I. Introduction

Cell based reporter assays represent a valuable tool to investigate the biological effects of genes, as well as determining the mechanism of action of proteins, peptides, ligands, and small molecule compounds (natural products and synthetic ones) under physiological conditions.

The reporter systems are generated by fusion of the promoter region (part of the coding region of the gene of interest) with a reporter gene. The reporter itself, and its method of detection, vary between the different assays. Reporter activity is typically measured either by the fluorescence intensity of the reporter itself or by detection of a specific enzymatic activity of the reporter using luminescence measurement. Examples of convenient fluorescent reporters are variants of green fluorescent protein (GFP) from *Aequorea victoria* or variants of red fluorescent protein (DsRed) from *Discosoma* spp., both providing high-level fluorescence and minimal cytotoxicity (1). One of the most frequently used reporter based on luminescence measurement is luciferase from the firefly (*Photinus pyralis*), catalyzing oxidation of D-luciferin accompanied by light emission (2). There are fundamental application differences

between these reporters. Generally, fluorescent reporters are suitable for identification of cells or tissues expressing gene of interest. On the other hand, luciferase reporters are suitable for quantification of expression of such gene. In this respect, luciferase-based reporter assays using luminescence microplate readers have become increasingly popular for high-throughput analysis of chemical biology and drug discovery applications.

Here, I describe transduction of selected cell line by commercial lentivector containing Gli (key transcription factor of Hedgehog signaling pathway) responsive luciferase, to monitor small molecule compounds inhibitory effect on Gli dependent signaling pathway. Since this lenti reporter works well with virtually any cell type, including non-dividing or differentiated cells, the protocol can be used for any cell lines of interest. The protocol is applicable both for transient transduction studies and for stably transduced cell line generation. Instead of Gli containing lentivector, it is possible to use any other commercial luciferase expressing lentivectors for studying a specific cell signaling pathway of interest.



Scheme 20.1

Scheme of the procedure including both, transient as well as stable transduction.

II. Materials and instrumentation

Instruments

- Multi-channel pipettor (for example Eppendorf)
- Microplate shaker (Biosan)
- Haemocytometer (Hausser Scientific)
- Spectrophotometer plate reader equipped with luminescence detection mode (for example EnSpire, Perkin Elmer)
- Cell sorter (optional, for example FACSARIAII, BD)
- Multidrop reagent dispenser (optional, 5840300, ThermoFisher Scientific)
- Besides above mentioned, the procedure requires standard material and equipment for tissue culture (for details see Chapter 1)

Materials

White opaque flat bottom 96-well tissue culture plates (6005680, PerkinElmer), britelite plus Reporter Gene Assay System (6016766, PerkinElmer), cell culture plastics such as transparent 96-well plates, culture dishes, culture flasks (for example from TPP), SureEntry transduction reagent (336921, Qiagen), Lenti GLI reporter (CLS-3030L, Qiagen), Lenti positive controls (CLS-PCL or CLS-PCG - optional, Qiagen), puromycin (P8833, Sigma Aldrich),

minimum essential medium with Earle's salts and stable glutamine (MEM; D888, Diagnostics), MEM Non-essential amino acid solution 100x (M7145, Sigma Aldrich), 100 mM sodium pyruvate (SH30239.01, HyClone), GANT61 - an inhibitor of GLI-mediated gene transactivation (sc-202630, Santa Cruz Biotechnology), U-87 MG human glioblastoma cell line (ATCC HTB-14, ATCC), fetal bovine serum (FBS; FBS-12A, Capricorn Scientific), and Opti-MEM medium (11058021, Gibco).

III. Procedures

Solutions

Minimum essential medium (MEM)

To make a complete growth medium, add FBS to a final concentration of 10 %, sodium pyruvate to a final concentration of 1 mM and MEM Non-essential amino acid solution to a final concentration of 1x to MEM.

Britelite plus reagent (working solution)

Reconstitute one vial of britelite plus lyophilized substrate with 10 ml of britelite reconstitution buffer (both components should be equilibrated to room temperature (RT) prior to reconstitution). For a long-term storage, keep at -80 °C.

0.5 mg/ml SureEntry (working solution)

Dilute appropriate volume of SureEntry stock solution (10 mg/ml) in the cell culture medium to get a final concentration of 0.5 mg/ml.

5 mg/ml Puromycin (stock solution)

Dissolve 5 mg of Puromycin dihydrochloride in 1 ml of deionized water, aliquot and store at -20 °C.

Luciferase cell-based assay for monitoring Gli transcription factor activity

Cells seeding

1. Cultivate the required number of U-87 MG cells or any other cell line of your choice.
2. On the day of plating, harvest the cells and determine their density using a hemocytometer.
3. If necessary, add appropriate volume of MEM complete medium to get a final density of $0.5 - 1 \times 10^5$ cells/ml.
4. Plate 100 μ l of resuspended cells ($0.5 - 1 \times 10^4$) per well of the white opaque 96-well culture plate using the multi-channel pipettor or Multidrop dispenser (if available).
5. Incubate cells at 37 °C in CO₂ incubator overnight.

Transduction

1. Remove the culture medium and add appropriate volume of lenti positive control (GFP) to one well and Lenti Gli reporter to the remaining wells (typically corresponding to 10-50 multiplicity of infection; MOI).
2. Add SureEntry working solution to a final concentration of 4 μ g/ml in each well (needs to be optimized for the particular cell line, see IV. Comments).
3. Mix by pipetting or gently swirl the plate.
4. Incubate 16 - 20 hours at 37 °C in CO₂ incubator.
5. Replace the culture medium containing lentiviral particles with the fresh culture medium in all the wells.

Stable cell line generation (optional)

1. Replace the culture medium 48 hours post-infection with the fresh culture

medium containing puromycin at the final concentration of 3 μ g/ml. The minimum effective concentration for the selection of transduced cells needs to be determined from kill curve for the particular cell line.

2. Replace culture medium with the fresh, puromycin-containing culture medium. Repeat this step every 3 - 4 days until dead cells are no longer visible.
3. If possible, do the single cell sorting in 96-well tissue culture plate (1 cell per well). Otherwise perform cloning by limiting dilution to obtain monoclonal cell population (3).
4. Expand each clone and validate using GANT61 (inhibitor/activator of the signaling pathway studied). Expand the most responding clone for further treatment with small chemical compounds.

Treatment with small chemical compounds

1. Check the GFP expression in the well transduced by lenti positive control using the fluorescence microscope.
2. Treat the cells 72 or 96 hours after infection (depending on the cell type) with 2 - 4 different concentrations of the tested compounds or GANT61, for 2 - 3 periods (typically 6, 12, and 24 hours). Keep at least 3 wells untreated as controls. If necessary, the standard culture medium can be replaced with the serum-reduced Opti-MEM culture medium (containing 0.5 % of FBS) before the compound's treatment.

Assay development

1. Allow the plate to cool down to RT after removal from the incubator, for at least 30 minutes.
2. Add 100 μ l of britelite plus reagent to each well by multi-channel pipettor or Multidrop dispenser (if available).
3. Put the plate on microplate shaker for at least 1 minute to achieve complete cell lysis.
4. Measure luminescence by a spectrophotometer plate reader within 15 minutes after reagent addition for maximum sensitivity.
5. Analyze data to determine IC₅₀ for Gli inhibition of the compounds tested.

IV. Comments

- a) Generally, lentiviral particles represent highly effective vehicle for transfection and expression of reporter constructs. However, the optimization of the conditions is necessary for efficient transduction. It includes optimal SureEntry concentration, which can be determined by titration of SureEntry (starting at 2 µg/ml and increasing by 2-fold increments) to find the highest nontoxic concentration for the particular cell line.

b) It is also important to optimize the conditions of the treatment with the tested compounds. It includes time- and concentration-dependent studies to obtain maximum response.

c) To obtain reliable results, always include lenti positive control (lentiviral particles constitutively expressing firefly luciferase or GFP) as well as small chemical compound control (pathway specific inhibitor/activator) for assay execution.

d) For maximum reproducibility of experiments, make sure that you seed the same number of cells into each well. Mix the cell suspension thoroughly with a 5- or 10-ml pipette and while seeding cells, use the multichannel
- pipettor to mix 2 - 3 times the cell suspension between columns seeding.

e) Since the growth rates of various cell types vary greatly, it is important to take into consideration plating density to avoid cells overgrowing during long-lasting experiments.

f) FBS induces multiple signaling pathways, resulting in the cross-talk and high background in luciferase cell-based assays.

g) Because of difficulty in measuring the gene down regulation in terms of discrimination between proliferation inhibition (and/or cell death) and cellular down-regulation, the normalization is necessary. To differentiate between specific Gli (or any other transcription factor) inhibition and nonspecific cellular responses, it is recommended to perform concurrently MTS cell proliferation assay (for the detailed protocol see the Chapter 10) for normalization of Gli-dependent luminescence signal.

h) Commercial lentiviral vectors used within this chapter belong to the 3rd generation of replication-defective vectors and should be handled under BSL2 tissue culture control using protective equipment (see (4) for details).

V. Pitfalls

Problem	Cause and cure
Low transduction efficiency/low expression of reporter	• Target cell line may be difficult to transduce. Optimize the transduction protocol conditions (number of cells, MOI, SureEntry concentration). Use the GFP expressing lenti positive control for protocol optimization. • Inactivation of lentiviral particles during storage. Store lentiviral particles at -80 °C and avoid repeated freeze/thaw cycles. Do not use the particles beyond the expiration date. • The assay is done too early. Prolong the time of infection up to 96 hours before assay performance (depending on the cell line used).
Cellular toxicity	• SureEntry is toxic for target cells. Decrease the amount of SureEntry reagent or omit it entirely in case that the target cells are sensitive toward it.
High luminescence signal background	• FBS concentration in the culture medium interferes with signaling pathway studied. Replace the standard culture medium by the serum-reduced OptiMEM medium (containing only 0.5 % FBS).

VI. Acknowledgement

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Reporter based DNA demethylation detection system

Khushboo Agrawal

I. Introduction

CpG island hypermethylation causing gene silencing is well evident in cancers, and various other disease types. Despite major advances in the field of epigenetic research, the success of DNA demethylation-based epigenetic therapy remains elusive due to narrow therapeutic window. A plethora of naturally occurring epigenetic agents and synthetic molecules that can alter methylation patterns exist (1). However, the usefulness of many such agents for epigenetic therapy remains unknown. This underlines the need for a sensitive DNA demethylation detection system for large-scale screening of drug candidates with potent hypomethylation activity.

The classical approaches used to study DNA methylation status include bisulphite sequencing (2), methylated DNA immunoprecipitation combined with microarrays or next-generation sequencing (3), and immunohistochemistry (4). However, these techniques require fixation of cells, making it impossible to study DNA methylation ‘dynamics’ as a function of time. Besides, they are uneconomical and not

feasible for high throughput screening of DNA demethylating drugs.

The disadvantage of the strategies that do not permit the time-dependent analysis of DNA methylation dynamics has been circumvented by the establishment of a fluorescence-based DNA demethylation detection system (5), suitable for high content (HC) screening of epigenetic drug libraries on a high-throughput platform. The detection system takes advantage over conventional approaches as it facilitates the monitoring of DNA methylation dynamics as a function of time, and further allows the analysis of cytostatic/cytotoxic drug effects in conjunction with demethylation effects, in parallel, under the same experimental setting.

The detection system comprises a double-gene stably-transfected reporter cell line, HCT116-pFLJ-H2B, generated by utilizing enhanced green fluorescent protein (EGFP)-tagged FLJ32130 gene, discovered to be silenced in HCT116 human colorectal cancer (6), and red fluorescent protein (RFP)-tagged histone-2B gene (7). The

principle of the method is that in untreated HCT116 cells, EGFP fusion protein would not be expressed due to hypermethylated status of FLJ promoter. On the other hand, treatment of the reporter cells with hypomethylating drugs will result in demethylation of the FLJ promoter and subsequent expression of EGFP. The rationale behind the use of fluorescently tagged histone protein is to identify and quantify the cell number the cells during HC image analysis. Thus, using this reporter cell line, demethylation activity of the drugs can be assayed by quantifying the intensity of EGFP fluorescence following drug treatment. The intensity of EGFP fluorescence is directly proportional to demethylation potential of the drug. Likewise, cytostatic/cytotoxic drug effects can be monitored by counting the cell numbers in untreated and experimental wells, using RFP channel, utilizing continuous live cell fluorescence imaging.

II. Materials and instrumentation

All materials in contact with cells must be sterile and pyrogen free. Equivalent materials and instruments may be used.

Instruments

- Water bath (WNE 45, Memmert)
- MSC-Advantage™ Class II Biological Safety Cabinet (51025411, Thermo Fisher Scientific)
- Centrifuge (5810R, Eppendorf)
- Inverted Fluorescence and Phase Contrast Tissue Culture Microscope (CKX41, Olympus)
- Direct Heat CO₂ Incubator (51026406, Thermo Fisher Scientific)
- Vi-CELL™ Cell Viability Analyzer (383260, Beckman Coulter)
- Multidrop™ Combi Reagent Dispenser (5840300, Thermo Fisher Scientific)
- Cell Voyager High Throughput Cytological Discovery System (CV7000, Yokogawa Electric Corporation)
- Columbus Image Data Storage and Analysis System (PerkinElmer)

Materials

McCoy's 5A medium (M8403, Sigma-Aldrich), foetal bovine serum (P30-3302, PAN-Biotech), L-glutamine (G6392, Sigma-Aldrich), streptomycin sulphate (S9137, Sigma-Aldrich), penicillin G (73368-74-8, Biotika), phosphate buffered saline (PBS; P3813, Sigma-Aldrich), TrypLE Express (12604013, Thermo Fisher Scientific), dimethyl sulfoxide (D8418, Sigma-Aldrich), ethanol (100983, Merck), cell culture grade sterile water (W3500, Sigma-Aldrich), T75 tissue culture flasks (90076, Techno Plastic Products AG), 96-well tissue culture plates (92096, Techno Plastic Products AG), and CellCarrier 96-well clear bottom microplates (6005550, PerkinElmer).

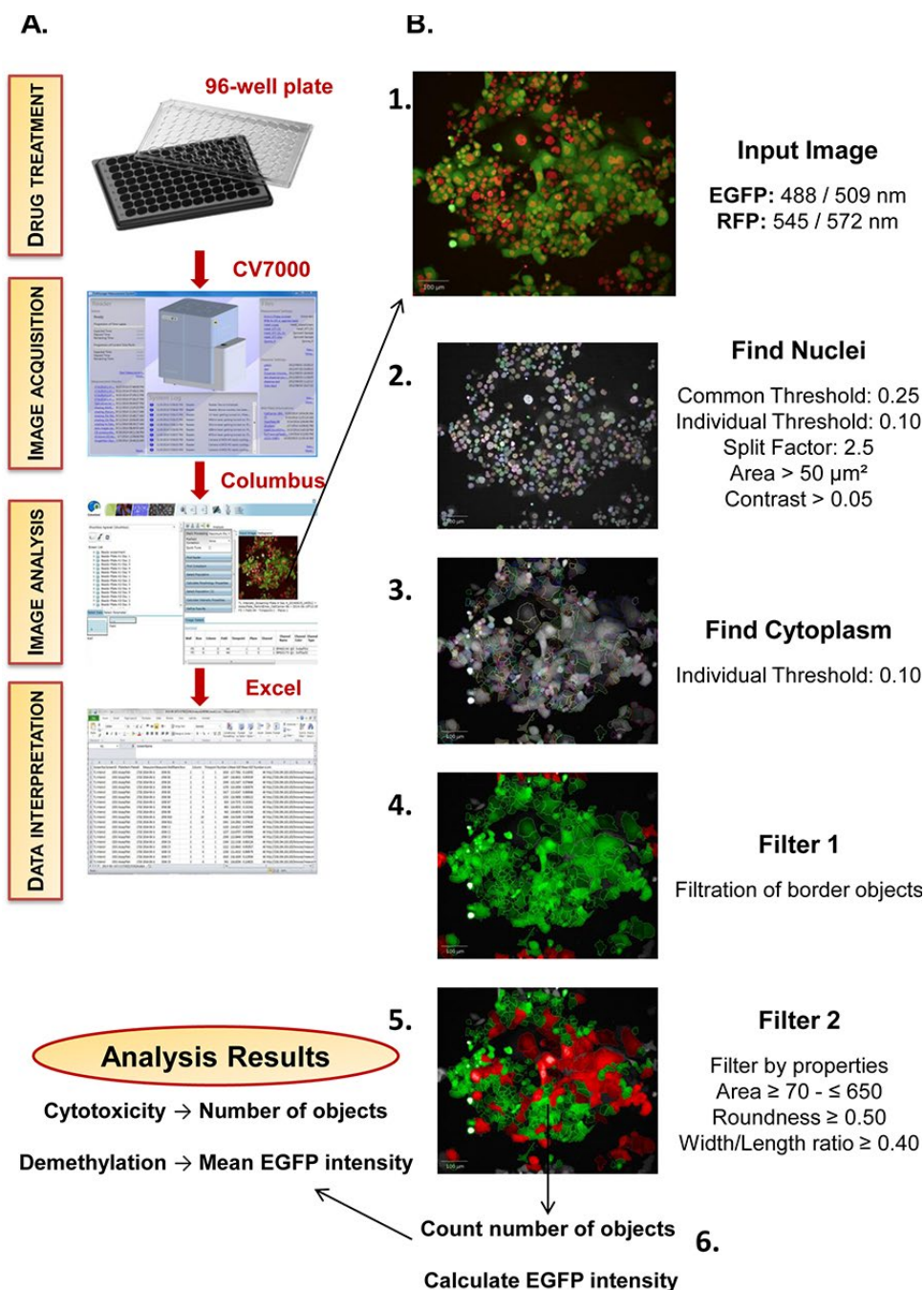
Other additionally used materials include single - (10 µl, 100 µl, 1000 µl) and multi-channel (300 µl) pipettes and pipettor with compatible tips, centrifuge tubes (1.5 ml, 15 ml, 50 ml) etc.

III. Procedures

Cell culture and drug treatment

The reporter cell line, HCT116-pFLJ-H2B can be obtained from the Laboratory of Experimental Medicine, at Institute of Molecular and Translational Medicine, Olomouc, Czech Republic.

1. Quickly thaw the frozen HCT116-pFLJ-H2B cells by gently swirling the cryovial in a 37 °C water bath. Dilute the thawed cells by transferring the content from the cryovial to a 15 ml centrifuge tube containing 10 ml McCoy's 5A growth medium, supplemented with 10 % foetal bovine serum, 3 mM L-glutamine, 50 µg/ml streptomycin, and 100 U/ml penicillin.
2. Centrifuge the cells at 1,500 rpm for 5 minutes and discard the supernatant. Resuspend the cell pellet in 15 ml of fresh growth medium, and plate the cells in a T75 tissue culture flask. Transfer the flask to a humidified incubator, maintained at 37 °C and 5 % CO₂. This additional centrifugation step ensures the removal of dimethyl sulfoxide (DMSO) from the culture medium.



Scheme 21.1. Overview of cellomics workflow in two-dimensional culture system.

3. On the next day, aspirate the culture medium and add 10 ml of PBS. Aspirate PBS from the flask. Then, add 20 ml of fresh growth medium, and transfer the flask to the incubator. This step is done to remove the floating dead cells and remaining traces of DMSO.
4. Check the cells under the microscope and confirm that confluence is 90-100 % on the day of the experiment. Aspirate the culture medium and wash 2-3 times with PBS, as described in the previous step. This washing step is required to completely remove the serum-containing medium in order to permit the activity of cell-dissociation reagent.
5. Add 3 ml TrypLE solution (cell dissociation reagent) to cells in T75 flask. Place the flask in the incubator for 5 minutes. Remove the flask from the incubator, and tap the sides of the flask to disperse the lumps of cells. Check under the microscope to confirm that cells have detached from the surface.
6. Add 7 ml culture medium to the flask, gently mix by pipetting and transfer the cell suspension to a 15 ml centrifuge tube. Centrifuge the cells at 1,500 rpm for 5 minutes. Addition of serum-containing culture medium stops the activity of protease enzymes in the cell-dissociation reagent.
7. Re-suspend the cell pellet in 10 ml of fresh growth medium. Count the cell density using Vi-CELL™ Cell Viability Analyzer. Consider the number of viable cells/ml for preparing the cell suspension in the next step.
8. Prepare the cell suspension at a density of 1.875×10^4 cells/ml. Dispense 80 μ l of the cell suspension (containing 1,500 cells) in desired number of 96-well clear bottom microplates, using Multidrop™ Combi Reagent Dispenser. Before dispensing the cell suspension, sterilize the dispensing cassette by spraying 70 % ethanol, and priming the cassette with ethanol followed by sterile water.
9. After 24 hours, add 20 μ l of culture medium with 5 different drug concentrations (diluted fivefold in a serial manner) to the wells containing 80 μ l of medium with cells. Treat the cells in the control wells with DMSO. Use DMSO concentration as in the medium

with the highest drug concentration (below 0.1 %). Use 96-well tissue culture plate as master plate to prepare the serial dilutions of the drugs using the multi-channel pipette.

10. Transfer the microplates to the CO₂ incubator.

Cellular imaging

For monitoring DNA methylation dynamics as a function of time, acquire HC cellular images at regular intervals from day 1-5, following 24 hours of drug treatment. Images can be acquired using Cell Voyager CV7000 High Throughput Cytological Discovery System, which features automated laser spinning disk imaging microscope with original confocal scanner unit, offering high resolution imaging, combined with a stage incubator, ideal for long term live cell imaging.

1. Approximately 1-2 hours before imaging, turn on the Cell Voyager Measurement System followed by the operating software, and allow the initialization of Reader Device and Camera Library. After initialization process is complete, open Reader Control screen to check the status of the Reader (press Get) and Lasers (press Get Status).
2. Prepare the live cell stage incubator. Set the temperature to 37 °C and CO₂ to 5 %, and press Get to allow the temperature and CO₂ to reach the set value. Meanwhile, switch on the water supply for Main Pump, Water Immersion Lens, and Stage Incubator. Turning on the water supply for each component takes 10 minutes.
3. When the live cell stage incubator is ready, create the Measurement Setting. To create a New Measurement Setting, select the PerkinElmer_#6005550_96 wells as the Plate Type. The selection of the plate type will lead to the Measurement Setting screen.
4. On the Measurement Setting screen, under the Well Plate Scan Setting tab, select the target wells to be scanned and acquisition points to be captured within each well, and click Apply to Channel Settings.
5. Go to the Action List tab and select Fluorescence Acquisition. Select channels (RFP: BP620/70 @1, EGFP: BP525/50 @2) for acquisition by clicking on Add Channels. Choose Method: Confocal Fluorescence

405/488/561 nm, and Objective: 20× PH Long W.D. for both channels.

6. After defining the acquisition settings, go back to the Action List tab, and select Software Focus. Choose RFP channel under the Software Focus tab, and roughly set the slice interval (e.g. -10 µm to 10 µm) and Exposure Time (e.g. 500 milliseconds) to start with. Load the plate and acquire images. For setting the focus, choose the well and/or field with the maximum number of cells. The parameters such as slice interval and exposure time, and target well and/or field can be varied during acquisition.
7. When the satisfactory images are obtained using the set focus and exposure time under RFP channel, select the EGFP channel, and acquire images using the same focus setting, but vary the exposure time during image acquisition. For setting the exposure time for EGFP, choose the well and/or field where maximum and minimum signals are expected following the drug treatment. The exposure time for EGFP channel is a crucial parameter. While choosing the exposure time, keep in mind that EGFP signals will increase with time on the following days. So carefully choose the exposure time to avoid saturation in case of increasing signals.
8. After setting the focus and exposure times for both channels, go back to Fluorescence Acquisition tab and acquire test images, simultaneously using both channels. Acquire test images for a number of different wells and/or fields to ensure that satisfactory images are obtained across the plate using the set parameters.
9. After creating the measurement setting, enter the field for Application Name (e.g. Experiment name_Well plate type_Username), and save the measurement setting.
10. Use the same measurement setting to acquire images from day 1-5 following the drug treatment, and for all the plates from the particular experiment.
11. To start the measurement on each day, go to Reader Control screen (i) Enter the field for Measurement Title (e.g. Experiment name_Plate1_Day1); (ii) under Operator tab, select your folder on the local drive or network drive where experiment will be saved; (iii) select your saved measurement setting under Measurement Setting tab,

and click Start Measurement. Once the measurement starts, Estimated Time, and Elapsed and Remaining Time for scanning of the experimental plate will be displayed on Reader screen.

12. When the plate measurement is finished, put the plate back in the CO₂ incubator for imaging on the next time intervals. Load another plate if any, and start the measurement again.
13. At the end of each plate measurement, the plate data are stored in a folder structure containing an index MRF file together with various single channel images as TIF.

High content image analysis

The HC cellular images acquired using Cell Voyager CV7000 can be analysed using Columbus Image Data Storage and Analysis System. The Columbus system is a universal, high volume image analysis and management platform available upon purchase from PerkinElmer. The software brings access to images from a wide range of sources including all major HC screening instruments, and can be remotely accessed from anywhere in the world, using an IP address or the name of your Columbus server created upon local installation.

1. To start using Columbus system, enter the IP address into your web browser (Internet Explorer version 9, 10, and 11/Firefox/Google Chrome/Safari 5.0 or later), and sign in using the Username and Password provided by your Columbus administrator.
2. Import the folder containing acquired TIF images into Columbus interface. To import the image files from the local drive or network drive, download Columbus Helper and install it on your computer. After installation, connect Columbus to the helper application. From the drop down menu, select CellVoyager MRF/TIF as the Import Type. Browse to select the Source Folder. Choose Import Mode as Normal, and enter the field for the Screen Name. Click Start Import. Once the import is finished, it will reflect as Completed under Job Status, and the data folder will appear on the Columbus home screen.
3. Click on Image Analysis button. To create the analysis, select a measurement in the Data Tree on home screen, after which cellular images for different wells and

number of captured fields will be displayed in the Image View section in a 96-well plate structure on the left bottom of the screen.

4. In the Image View section, select one single image (e.g. well, field, plane, or time point) as representative image, in order to test the sequence of image analysis building blocks. Then, start building the analysis by clicking plus sign. Choose the representative image from the wells and the fields with the maximum number of cells, and wells where maximum and minimum EGFP signal is expected. After building the analysis using the representative image, calibrate each building block of image analysis by going across several wells and several fields in each well to achieve the optimal setting.
5. Start building the analysis sequence by selecting Input Image block. Under this block select Stack Processing: Maximum Projection, Flatfield Correction: None, and leave Quick Tune as unchecked. Maximum Projection reduces all planes to one single image, and each pixel gets the maximum value across the stack planes. On selecting None option, uncorrected image is used as default. The use of Quick Tune might falsify the result.
6. Next, detect the cell nuclei under RFP channel. To detect regions on the image belonging to cell nuclei, select Method C under the Find Nuclei tab, and fine-tune various parameters such as Common Threshold, Individual Threshold, Split Factor, Area, and Contrast. Method C is most suitable for low background images with variations in size, intensity or contrast of nuclei, and bad splitting of stuck nuclei, especially in case of confluent wells.
7. After selection of the cell nuclei (Output Population 1), detect regions around nuclei belonging to cell cytoplasm under EGFP channel. To detect the cytoplasm, select Method A under the Find Cytoplasm tab, and set Individual Threshold for determining the intensity threshold for each object individually. Method A is most robust for finding cytoplasm, and is best applicable in cases where the intensity decreases with the distance from nucleus.
8. Following the detection of the cells (nuclei + cytoplasm) in the region of input image (Output Population 1), use the Select Population tab and tick Remove Border Objects using Method: Common Filters. The filtration of border objects is done to exclude the incompletely captured cell population.
9. After removal of the border objects using Common Filters (Output Population 2), calculate Morphology Properties (Area, Roundness, and Ratio Width to Length) using Standard Method, and then set the parameters to filter according to morphology properties under the Select Population tab. The filtration according to morphology properties is done to discard the cell clumps and false discovered objects.
10. In the final selected cell population (Output Population 3), calculate EGFP fluorescence intensity, using Standard Method under the Calculate Intensity Properties tab.
11. At last, define the final readout parameters of the analysis using the Define Results building block. Use Standard Output Method to define Mean: Mean EGFP intensity per well (Variable a) and Object Count: Number of objects in the current population (Variable b), and Formula Output Method: Formula a/b to define Mean EGFP intensity per cell. The number of objects represent the number of cells in each well, reflecting cytostatic/cytotoxic effects of the drugs, whereas, mean EGFP intensity per cell reflect demethylation efficacy of the drugs.
12. Once the good results are achieved, select a few other images to test and optimize the analysis with these images.
13. Save the analysis sequence (.aas file) to the database or the file system for later Batch Analysis, to analyse one or multiple measurements, using the same analysis script file.
14. To start the Batch Analysis, select one or multiple measurements in the Data Tree. The number of measurements selected for Batch Analysis is displayed in the Select Data section, and the selected measurements are listed in the Data Selection table.
15. Click to select the saved script file (.aas file), and then click Start Analysis. A background job will start, analysing all selected measurements using the selected script file. The progress of the analysis can be viewed using the Job Status screen.
16. When the analysis is finished for one or multiple measurements, export results in the

Excel file format. Go to Export Navigation Wizard, and select one or multiple analysed experiments from the Data Tree. Browse to select the Export Folder. Select Method: Export to Disk, Results: Excel (.csv), and click Start Export. Note: For exporting data, Columbus must be connected to the helper application, similar to data import, as described above.

17. The exported Excel file can be used for downstream processing and statistics, in order to biologically interpret the results.

IV. Comments

- This Chapter describes the detailed stepwise cellomics work-flow which can be easily adopted for (i) culturing of HCT116-pFLJ-H2B reporter cells, and drug-treatment; (ii) HC imaging of reporter cells following the drug treatment; and (iii) processing of HC imaging data for analysing induced demethylation and cytostatic/cytotoxic drug

effects in two-dimensional culture system. However, apart from two-dimensional culture system, the reporter cells can also be used to study DNA demethylation dynamics in three-dimensional (3D) cell culture models. Additionally, the detection system also supports the *in vivo* monitoring of demethylation efficacy of potential lead compounds from *in vitro* screens in tumour xenografts. For studying DNA demethylation dynamics in 3D systems, spheroids of HCT116-pFLJ-H2B cells can be generated, and for *in vivo* screening of lead compounds, HCT116-pFLJ-H2B reporter cells can be xenografted into SCID mice, following the procedure described elsewhere (5).

V. Pitfalls

Problem	Cause and cure
No signal or weak EGFP signal	• Exposure time was too small and/or the demethylation activity of the tested drug was low. • Increase the exposure time while capturing images under EGFP channel.
Saturated EGFP signal	• Exposure time was too long. • Decrease the exposure time while capturing images under EGFP channel.
Difficult to split stuck nuclei	• High confluence of cells in wells. • Decrease the number of cells at the time of plating.
High image background in all wells	• Presence of phenol-red in the culture medium. • Use the culture medium without phenol red.

VI. Acknowledgement

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Identifying senescent cells using microscopy or flow cytometry

Matthew Lacey

I. Introduction

At the most fundamental level, senescence is a stable and irreversible form of cell cycle arrest (1), which can be caused by many different factors, as is illustrated in Figure 22.1. Senescence is generally categorised into two distinct groups: replicative senescence, which is driven by telomere shortening, and stress-induced premature senescence (SIPS), which is driven by a larger number of factors (2,3).

Despite a multitude of differing causative factors, senescence induction is controlled by two major molecular pathways, the p53-p21 and p16^{INK4a}-Rb networks (1). Both telomere shortening and other cellular stressors that cause senescence result in DNA damage and thereby activate DNA Damage Repair pathway (1,3,5). This in turn leads to the stabilisation of p53 and an increase in p53 levels (1,5), which then results in an increase in production of the CDK4 and its inhibitor p21 (1,5). The p16^{INK4a}-Rb pathway functions in a similar, although independent, manner. Senescence inducing stress causes an increased in p16 (another CDK4/6 inhibitor) production (1). Cell cycle arrest is ultimately

achieved by the inhibition of CDK4 and CDK6; preventing the phosphorylation of Rb (1). This is not a comprehensive account of either the p53-p21 or p16^{INK4a}-Rb pathways, more detailed accounts can be found in the works of Childs et al. (1) and McHugh & Gil (5).

While increased levels of p53, p21 and p16^{INK4a} are indicative of senescence, and their relative levels are often increased in senescent cells (6), they are not reliable markers of senescence. This is because in some senescent cells there is no discernible change in their relative levels; in particular in the case of p53 (6) and cells with senescent phenotypes but not expressing p16 (7).

Characteristics of Senescent Cells

There are a number of phenotypic differences between replicating and senescent cells, and these phenotypic differences are significant and can be used to distinguish senescent cells from replicating cells. Senescent cells are morphologically distinct from replicating cells (Figure 22.2) with senescent cells being larger and flatter than their replicating counterparts (8).

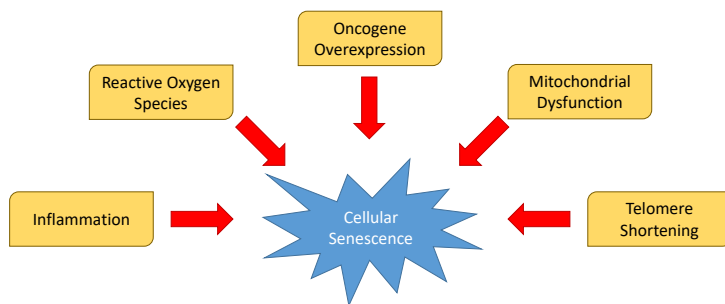


Figure 22.1. Illustrated causes of cellular senescence. Figure derived from Di Micco *et al.*, 2006 and McHugh & Gil, 2018 (1,4).

Senescent cells also gain additional characteristics such as: increased β -galactosidase activity, known as senescence-associated β -galactosidase (SA- β -gal); senescence associated heterochromatic foci (SAHF), increased presence of γ -H2AX, and the aforementioned changes in the expression of p16^{INK4a}, p53, and p21 (8,9). Furthermore, senescent cells gain a biologically-active and pro-inflammatory secretory phenotype, known as the Senescent Associated Secretory Phenotype (9,10).

Although these characteristics of senescent cells that have been mentioned can be used to identify them and differentiate them from other cell types, it is important to note that there is, at time of writing, no single universal marker for cellular senescence (11). The fact that senescent cells can have variable phenotypes, such as: differences in SASP factors between senescent cells (12), the total absence of SASP when the senescence is caused by the overexpression of cell cycle inhibitors (13), and a variable expression level of p53 and p16 between different senescent cells (6,7) complicates their use as markers of senescence. While many studies do use these traits as markers of senescence (11), the most commonly used marker is SA- β -gal activity (1,14). It is for this reason that two protocols for the detection of SA- β -gal are outlined below.

Kit SA- β -Gal Detection

II. Materials and instrumentation

Materials

Cell Signalling Technology Senescence β -Galactosidase Staining Kit #9860 (or another similar kit), 10 $\times$ PBS: Distilled water (1l), NaCl (80 g), KCl (2 g), Na₂HPO₄·12H₂O (32.1 g),

KH₂PO₄ (2 g), distilled Water, DMF or DMSO, 70% Glycerol, HCl and NaOH for pH adjustments, pipettes, microcentrifuge tubes, suitable cell culturing container (flask, Petri dish, or well plate), 15 ml or 50 ml centrifuge tubes, Parafilm (or equivalent), and Tin foil.

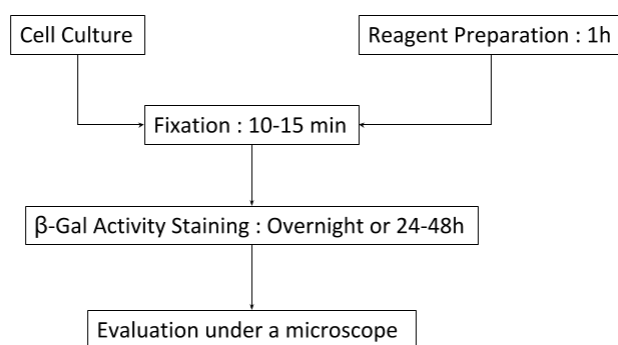
Instruments

Water bath, pH meter, incubator, light microscope, and laminar flow cabinet.

III. Procedures

Procedure

1. All reagents from the kit should be thawed and heated to 37 °C in a waterbath.
2. Calculate the volume of solutions required. One ml of Fixative Solution and 1 ml of β -Galactosidase Staining Solution are required for an area of 9 cm², the approximate surface area of a 35 mm Petri dish, or a single well in a 6 well plate. All measurements given hereafter are intended for this volume, but the measurements can be scaled up or down as required.
3. Prepare the reagents from the kit
 - a) Fixative solution: 10 $\times$ stock solution is to be diluted with distilled water to a 1 $\times$ concentration.
 - b) Staining solution: 10 $\times$ stock solution is to be diluted with distilled water to a 1 $\times$ concentration.
 - c) X-gal(5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)solution: Create a 20 mg/ml stock solution by dissolving 20 mg of X-gal



Scheme 22.1

in 1 ml of DMF or DMSO.

d) β -Galactosidase Staining Solution: Mix the following for a 1 ml solution: 1x Staining solution (930 μ l), 100 $\times$ Solution A (10 μ l), 100 $\times$ Solution B (10 μ l), 20 mg/ml X-gal solution (50 μ l). *Ensure the final pH of this solution is 6.0, (adjust the pH with NaOH or HCl), for the most accurate results.*

4. Aspirate the growth medium from the cell culture.
5. Wash the cells with 1 $\times$ PBS (the volume of 1 $\times$ PBS used should equal the volume of growth medium).
6. Add the appropriate volume of 1 $\times$ Fixative solution to the cell culture. Incubate at room temperature for 10-15 minutes.
7. Aspirate the 1 $\times$ Fixative Solution
8. Wash the fixed cells twice with 1 $\times$ PBS
9. Add the appropriate volume of 1x β -Galactosidase Staining Solution into the

fixed cells. Seal the vessel (Petri dish or plate) with Parafilm to prevent evaporation and crystal formation and then incubate for up to 48 hours (at least overnight) at 37 °C in a dry, CO₂ free incubator.

10. Check for the development of blue colour under a microscope (200x magnification). Once sufficiently stained, aspirate the β -Galactosidase Staining Solution and replace with 70% glycerol.
11. Stained samples overlaid with 70% glycerol can be stored at 4 °C for a longer period of time (up to 6 months or more).

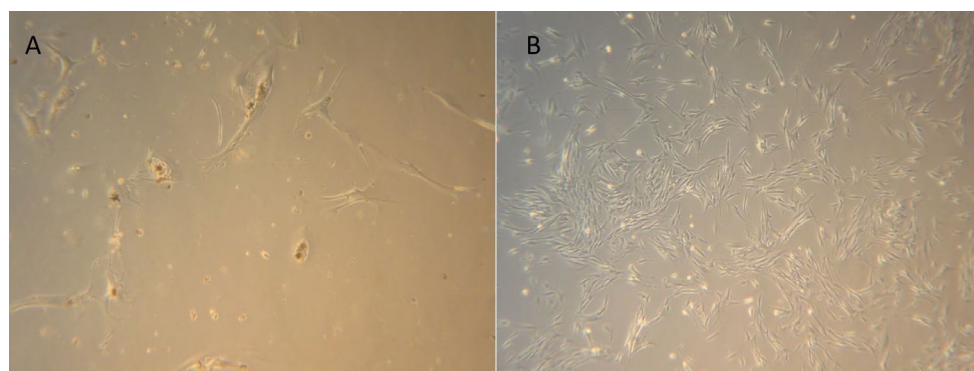


Figure 22.2. Images of senescent MCR5 cell culture (A) and proliferating MRC5 cell culture (B). The senescent cells are distinctly larger than their proliferating counterparts.

IV. Comments

- Note solutions A and B can take up to an hour to thaw.
- The protocol given by Cell Signalling recommends the use of DMF for the preparation of the X-gal solution, however, DMF can be substituted with DMSO.
- Any excess X-gal solution can be stored at -20 °C, but it must be kept in the dark and shielded from light. If it is not used within

4-6 weeks then a new X-gal solution should be prepared.

- If there is no dry, CO₂ free incubator available for the staining reaction (Step 9), the plate should be sealed with tin foil (note: parafilm is not air-tight). This is because the pH of the reaction can be affected by CO₂ which can in turn affect the staining results.

V. Pitfalls

Problem	Cause and cure
False Positive Results	• β-Galactosidase Staining Solution pH too low, adjust pH to 6.0 using NaOH. • CO₂ may have decreased pH, carry out the experiment in a CO₂ free incubator or an airtight container. • Cells may have become over confluent and/or have become quiescent, which results in SA-β-gal expression (14,15). • Some cells (e.g. Macrophages) have naturally higher levels of β-gal expression (1).
False Negative Result	• β-Galactosidase Staining Solution pH too low, adjust pH to 6.0 using NaOH

Flow Cytometry S-A β-Gal Detection

II. Materials and instrumentation

Materials

C₁₂FDG, a fluorogenic substrate for β – galactosidase (Cat.no.: 25583; Cayman Chemicals, Dissolved in DMSO to a final concentration of 20mM), Baf A1 [(100nM), Dissolved in DMSO to a final concentration of 0.1 mM], DAPI (300 nM) Solution, 10× PBS: Distilled water (1l), NaCl (80 g), KCl (2 g), Na₂HPO₄·12H₂O (32.1 g), KH₂PO₄ (2 g), Trypsin or suitable alternative (e.g. Gibco TrypLE Express)

Instruments

Flow cytometer, micro-centrifuge, incubator, and laminar flow cabinet.

Procedure

1. Add 100 nM Baf A1 to culture medium for lysosomal alkalisation and incubate for 1 hour at 37 °C in an incubator with humidified air and 5% CO₂.
2. Add C₁₂FDG to the Baf A1-containing medium (33 μM final concentration of 33 μM) and incubate for a further 2 hours at 37 °C in an incubator with humidified air and 5% CO₂.
3. Wash cells with ice cold 1× PBS.
4. Fix cells with 4% formaldehyde at room temperature
 - a) 5-15 minutes
 - b) Remove medium, add the formaldehyde solution.
5. Stain nuclei with DAPI Nuclear Stain Solution
 - a) Wash cells 1-3 times in 1× PBS

- b) Add 300 nM DAPI solution (enough to cover the cells)
- c) Incubate for 1-5 minutes in a dark area
- d) Remove the DAPI solution.
6. Wash stained cell culture with 1× PBS (2-3x).
7. Trypsinize cell culture and collect cell suspension in a centrifuge tube
 - a) Examine cells under a microscope to ensure that they have detached before collecting the cell suspension.
8. Centrifuge cells at 500×g for 5 minutes at 4 °C to collect them.
9. Re-suspend the cell pellet in ice cold 1× PBS.
10. Analyse using a suitable flow cytometer (e.g. FACSCanto from BD Biosciences).

Non-Adherent Cell Cultures

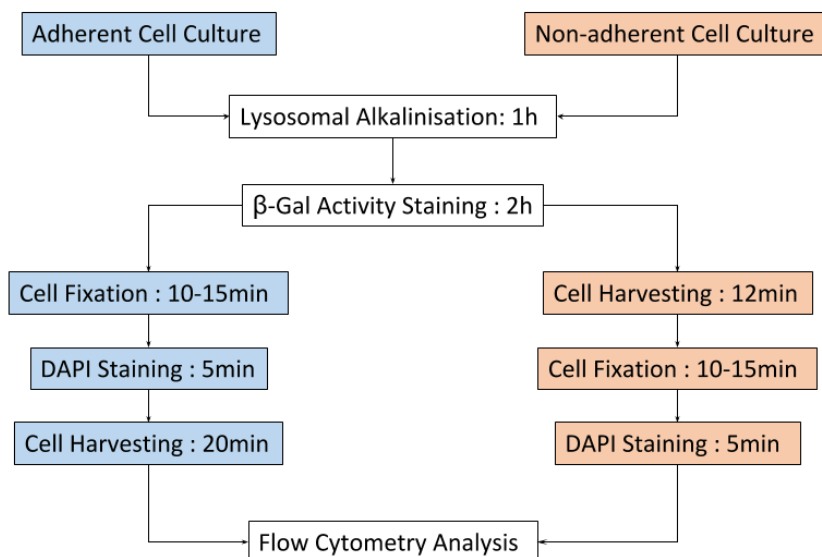
For non-adherent cell cultures, follow steps 1 and 2 as above, and then proceed as per the following steps:

1. Centrifuge cell culture at 500×g for 5 minutes at 4 °C.

2. Wash the pellet with 1× PBS, and centrifuge again if necessary.
3. Suspend the cell pellet in 4% formaldehyde for fixation (5-10 minutes).
4. Centrifuge at 500×g for 5 minutes at 4 °C and re-suspend in 300 nM DAPI solution for 1-5 minutes (keep in dark place).
5. Analyse via FACS.

IV. Comments

- When using non-adherent cell cultures do not wash cells with 1× PBS after the DAPI staining in order to prevent the stain from leaving the permeable cells via diffusion.
- If live cells are to be used, skip Step 4 (Fixation) and Step 5 (DAPI staining) of the standard protocol or skip Step 5 (Fixation) and Step 6 (DAPI staining) of the alternative protocol for non-adherent cells.
- This method is primarily derived from the works of Kovacicova et al. (16) and Cahu & Sola (17).



Scheme 22.2

V. Pitfalls

Problem	Cause and cure
Damaged Cells (No Result)	Senescent cells can be fragile, use pipette tips with larger diameter openings, pipette the cells more gently and adjust centrifuge speeds.
False Positive Results	- Adherent cell culture may have become over confluent and/or have become quiescent, which results in SA-β-gal expression (14,15) - Some cells (e.g. Macrophages) have naturally higher levels of β-gal expression (1)

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Methods for detection and induction of proteotoxic stress response in mammalian cells

Zdeněk Škrott

I. Introduction

Considering the huge amount of proteome, millions of aberrant proteins are produced. The proper folding and maintaining correct structure of proteins is extremely challenging, as proteins are constantly exposed to many environmental stressors (1). Since damaged or misfolded proteins may seriously interfere with many cellular processes, the cells evolved sophisticated multi-layered system for protection called protein quality control, which includes Unfolded protein response (UPR) (2) and Heat shock response (HSR) (3).

As malfunction of protein quality system and proteotoxic stress is associated with many health conditions, including several neurodegenerative diseases or certain types of cancer (4), it is thus important to have diagnostic and experimental methods for detection of proteotoxic stress. Both UPR and HSR stress responses consist of orchestrated cascade of proteins, functioning as sensors, transducers, or effectors.

UPR can be monitored by the activation of three main sensor pathways - IRE1 α (endoribonuclease inositol-requiring enzyme 1- α), PERK

(protein kinase RNA-like endoplasmic reticulum kinase), and ATF6 α (activating transcription factor 6 α) (5), which are regulated by the important chaperone, protein BIP (also known as GRP78 – glucose-regulated protein 78). Activated PERK phosphorylates translation incitation factor eIF2 α (eukaryotic translation initiation factor 2 α), which in turn decreases total protein synthesis to reduce the load of new proteins, and concomitantly, to allow preferential translation of ATF4 (activating transcription factor 4). Additionally, activated IRE1 α induces alternative splicing of transcription factor Xbp1 (X-box binding protein 1) (6). UPR activation can be easily monitored by the increased protein levels of these important effectors, including BIP, phospho-eIF2 α , ATF4, spliced form of Xbp1 or pro-apoptotic protein CHOP (CCAAT-enhancer-binding protein homologous protein) (7) (Fig. 23.1).

Similarly, HSR is also regulated mainly by transcription factors, among them HSF1 (Heat shock factor 1) is assumed as the critical one, and could serve as direct and specific marker of HSR activation (8). Under normal conditions, it is sequestered in the cytoplasm in inhibitory complex

with HSP70 and HSP90 chaperones. Upon accumulation of aberrant proteins, HSP proteins dissociate and liberated HSF1 is activated, leading to its oligomerization, phosphorylation and translocation to the nucleus, where it binds heat shock elements on DNA (forming so called nuclear stress bodies -NSB) (9). Phosphorylated form of HSF1 can be detected by Western blot and NSB can be easily visualised by immunofluorescence, providing suitable markers of HSR activation (Fig. 23.2a). Additionally, protein level of the main HSR chaperone HSP70 (10) could serve as a complementary marker of fully activated HSR (Fig. 23.2b).

While both UPR and HSR can be monitored by several ways, including RT-PCR of spliced variant of Xbp1, qPCR, reporter assays or transgenic models (11).

This protocol will be limited to easy, robust and highly applicable and widely used methods based on Western blot and Immunofluorescence.

Proteotoxic stress can be induced by several ways, but the most versatile are chemicals directly disrupting protein conformation or interfering with processes required for proper protein control including protein folding, modifications, degradation, and/or transport. UPR can be easily elicited by two commonly used toxins – thapsigargin, a specific endoplasmic reticulum Ca^{2+} ATPase inhibitor causing protein unfolding, or tunicamycin, an inhibitor of glycoprotein synthesis. Protein stress could be also triggered by inhibitors of protein degradation (inhibitors of the proteasome – MG132, bortezomib, or p97 segregase – NMS-873, CB-5083) (11, 12), or general protein disruptors such as dithiothreitol. HSR is typically induced by elevated temperature or exposure to heavy metals or heavy metal containing compounds (13).

II. Materials and instrumentation ¹

Materials

Required solutions:

10x TBS

Dissolve in 1 L of distilled water (adjust pH to 7.5):

- 24 g Tris base (Sigma Aldrich, 77-86-1)

- 88 g NaCl (Sigma Aldrich, 31434)

10x Running buffer

Dissolve in 1 L of distilled water (no pH adjustment required):

- 30 g Tris base (Sigma Aldrich, 77-86-1)
- 144 g glycine (Sigma Aldrich, G8898)
- 10 g Sodium dodecyl sulphate (Sigma Aldrich, L3771)

10x Transfer buffer

Dissolve in 1 L of distilled water (no pH adjustment required):

- 30 g Tris base (Sigma Aldrich, 77-86-1)
- 144 g glycine (Sigma Aldrich, G8898)

1x Laemmli buffer

- 62.5 mM Tris-HCl pH 6.8 (Sigma Aldrich, 77-86-1)
- 25% glycerol (Sigma Aldrich, G5516)
- 2% Sodium dodecyl sulphate (Sigma Aldrich, L3771)
- 0.01% bromophenol blue (Sigma Aldrich, B0126)
- 200 mM dithiothreitol (Sigma Aldrich, D0632)

Antibody dilution solution for

Immunofluorescence

- DMEM culture media (Biosera, LM-D1110/500)
- 20% fetal bovine serum (Gibco, 10437036)
- 0.1% sodium azide (Sigma Aldrich, S2002)

10x PBS buffer (phosphate buffered saline; stock solution)

- Dissolve in 1 L of distilled water (adjust pH to 7.4):
- 1.37 M NaCl (Sigma Aldrich, 31434)
- 27 mM KCl (Sigma Aldrich, 60130)
- 100 mM Na_2HPO_4 (Sigma Aldrich, S5136)
- 18 mM KH_2PO_4 (Sigma Aldrich, P5655).

Ponceau S staining solution

- 0.1% Ponceau S (Sigma Aldrich, P3504)
- 5% acetic acid (Sigma Aldrich, A6283)
- distilled water

1× PBS (working solution)

- Dilute ten times the stock solution of 10× PBS in deionized water.

1× Transfer buffer

- To 100 ml of 10× Transfer buffer add 100 ml of methanol and fill to 1L with deionised water.

1× Running buffer

- Dilute ten times the stock solution of 10× Running buffer in deionized water.

1× TBS Tween

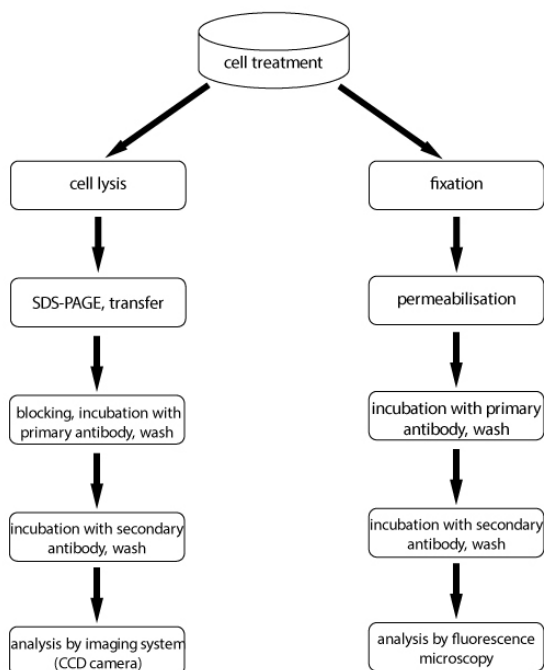
- Dilute ten times the stock solution of 10× TBS in deionized water, add Tween 20 to 0.1% final concentration.

Other material: nitrocellulose membrane (Bio-rad, 1620115), Mini-Protean TGX gels (Bio-Rad,) ELC detection reagent (Thermo Scientific, 34080), 4% formaldehyde solution (Sigma Aldrich, HT501128), mounting medium (Vectashield, H-1000), tunicamycin (Sigma, T7765), TritonX-100 (Sigma Aldrich, T9284), thapsigargin (Sigma, T9033), DAPI (Sigma, D9542), and following antibodies, for example: anti-Xbp1 (1:500; Santa Cruz Biotechnology, cat. n.: sc-7160), p-eIF2 α (Cell Signaling, cat. n.: 3597), ATF4 (Merck Millipore, cat. n.: ABE387), HSP70 (Enzo, cat. n.: ADI-SPA-830), HSF1 (Cell Signaling, cat. n.: 4356), CHOP (Cell Signaling, cat. n.: 2895), BIP (Abcam, cat.n.: a21685) appropriate secondary antibodies, for example: goat-anti mouse IgG-HRP (GE Healthcare, NA9310V), goat-anti rabbit (GE Healthcare, NA9340V), and Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, A11001).

Instruments

- Mini Rocker-Shaker (Biosan)
- Heating magnetic stirrer MSH-300i (Biosan, BS-010309-AAA)
- Thermo Shaker TS-100C (Biosan)
- Mini protein tetra system (Bio-Rad)
- Trans Blot SD (Bio-Rad)
- Power Pack HC (Bio-Rad)
- Water bath (Mettler)
- Imaging system equipped with CCD camera (such as ChemiDoc, Bio-Rad)
- Fluorescent microscope (such as Zeiss Axioimager Z.1)

III. Procedures



Scheme 23.1

A. Detection of UPR activation by Western blot

1. Treat the cells as needed. Include positive control (tunicamycin 2 μ g/ml and/or thapsigargin 1 μ M and/or NMS-873 5 μ M for 6 hours).²
2. Aspirate the media and wash briefly the cells with 1x PBS, remove PBS.
3. Lyse the cells directly with 1× Laemmli buffer (approximately 500 μ l of buffer to 6 cm Petri dish).
4. Boil the lysate for 5 minutes at 95 °C on shaker.³
5. Load equal amounts of protein lysate (approximately 30 μ l of lysate per well) and run the gel for 1÷2 hour at 10 V/cm.
6. Wash the gel and membrane in 1× Transfer buffer and perform protein transfer for 1 hour at 12 V.
7. Check transfer efficiency by staining with

- Ponceau S (5 minutes) and then wash the membrane 3 times in 1x TBS-Tween for 5 minutes.
8. Block the membrane in 5% milk in 1x TBS-Tween for 1 hour at room temperature. Wash with 1x TBS-Tween.
 9. Incubate with selected primary antibodies against selected UPR markers⁴ such as Xbp1s, ATF4, phospho-eIF2 α , BIP, CHOP (for example: anti-Xbp1¹⁵ (Santa Cruz Biotechnology, n.: sc-7160, dilution: 1:500), p-eIF2 α 6 (Cell Signaling, cat. n.: 3597, dilution: 1:500), ATF4 (Merck Millipore, cat. n.: ABE387, dilution: 1:500), CHOP (Cell Signaling, cat.: 2895, dilution: 1:500), BIP (Abcam, cat.n.: a21685, dilution 1:1,000) for 1 hour at room temperature. Dilute the antibodies in 1x TBS-Tween containing 5% milk.
 10. Wash the membrane with 1x TBS-Tween 3 times for 5 minutes.
 11. Incubate the membrane with appropriate secondary antibodies for 1 hour at room temperature.
 12. Wash the membrane with 1x TBS-Tween 3 times for 5 minutes.
 13. Incubate the membrane with ELC reagent according to manufacturer instructions and acquire the image.

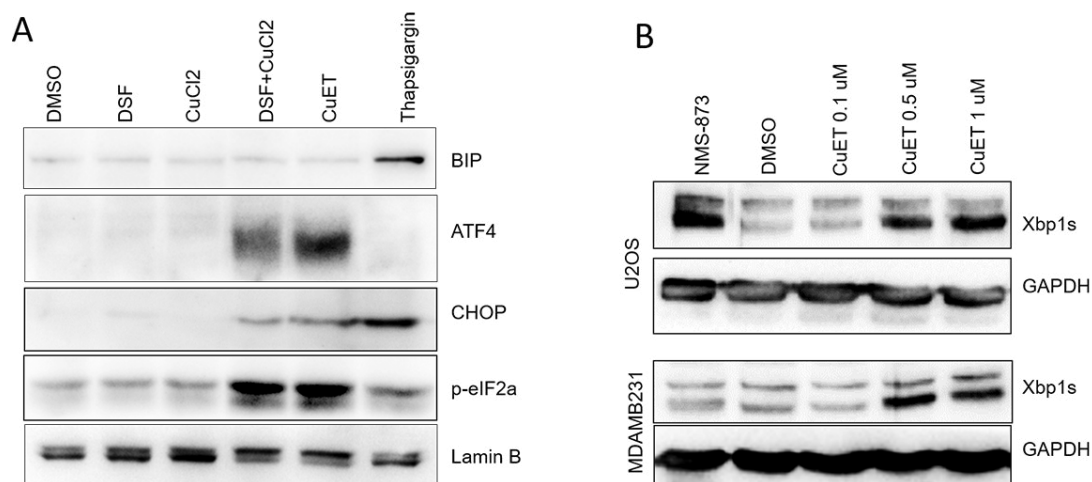


Figure 23.1. WB analysis of selected UPR markers.

(A) PC3 prostate cancer cells were treated with tested compounds and inducers of UPR (Thapsigargin 1 μ M or copper-diethyldithiocarbamate complex (CuET) 0.5 μ M, an inhibitor of protein degradation) for 6 hours. Elevated levels of BIP, ATF4, p-eIF2 α , and CHOP indicates activation of UPR. Lamin B used as loading control (Note, diverse arms of UPR are induced differently by certain compound). (B) Xbp1s induction in MDA-MB-231 and U2OS cancer cell lines by p97 inhibitor NMS-873 (5 μ M) and CuET.

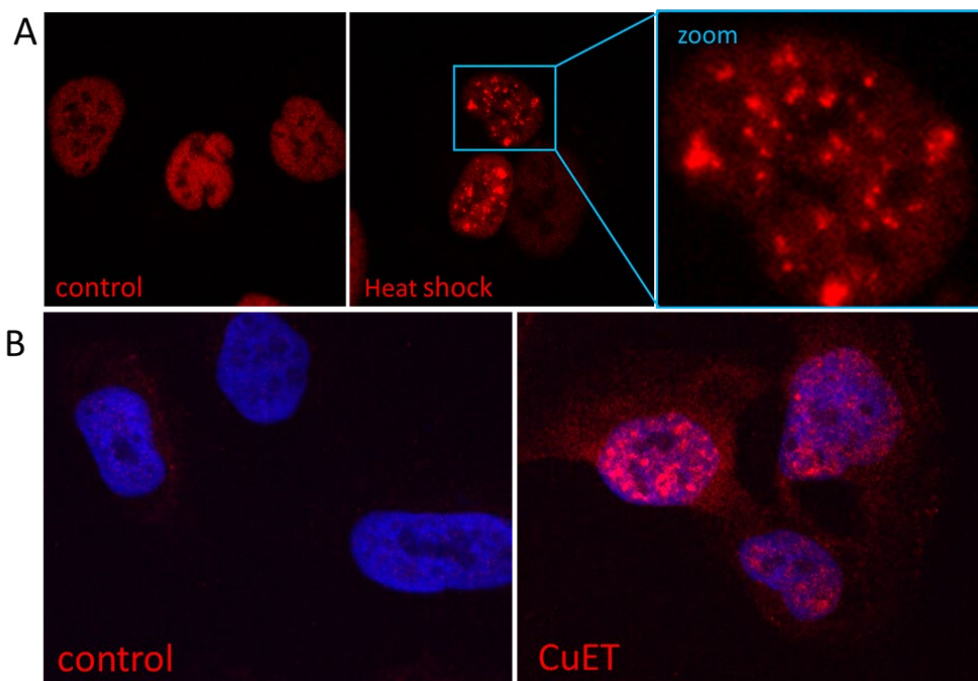


Figure 23.2. IF analysis of selected HSR markers.

(**A**) HSF1 staining in U2OS cancer cell line. Cells were mock treated or exposed to mild heat shock in water bath (42 °C for 1 hour). Typical HSF1 stress foci documents activation of HSR. (**B**) HSP70 staining (red) in U2OS cancer cell line. Cells treated by heavy metal containing compound (CuET 1 μ M, 3 hours) exhibit higher levels of HSP70 confirming activation of HSR. DAPI counterstain in blue.

B. Detection of HSR activation by Immunofluorescence

1. Treat the cells on coverslips as needed. Include positive control (cells incubated in water bath for 2 hours at 42 °C).
2. Aspirate the media and wash briefly the cells with 1× PBS, remove PBS.⁷
3. Fix the cells in 4% formaldehyde solution (Sigma Aldrich, HT501128) for 15 minutes.
4. Aspirate formalin, wash briefly with 1× PBS, remove PBS.
5. Permeabilise the cells with 0.5% TritonX-100 (Sigma Aldrich, T9284) in distilled water for 10 minutes. Wash 2 times with 1× PBS.
6. Incubate the coverslips with selected primary antibodies against certain HSR markers, such as HSF1 or HSP70 (for example: HSP70 (Enzo, cat. n.: ADI-SPA-830, dilution: 1:500), HSF1 (Cell Signaling, cat. n.: 4356, dilution: 1:500) for 1 hour at room temperature. Dilute antibodies in antibody dilution solution.
7. Wash the coverslips 3 times with 1× PBS for 5 minutes, remove PBS.
8. Incubate the coverslips for 1 hour at room temperature with appropriate secondary antibodies, for example: (Alexa Fluor 488 goat anti-mouse antibody (Invitrogen, A11001, dilution 1:1000). Dilute antibodies in antibody dilution solution.⁸
9. Wash the coverslips 3 times with 1x PBS for 5 minutes, remove PBS.
10. Stain the cell nuclei with DAPI solution (1 μ g/ml in distilled water) for 2 minutes. Wash briefly DAPI with distilled water.
11. Air dry the coverslips, mount coverslips with mounting medium and seal it with nail polish.⁹
12. Acquire the image.

IV. Comments

- 1 The mentioned manufacturers of the listed instruments were used during the protocol testing or development.
- 2 The concentration used and duration of treatment vary across cell types and optimization may be necessary.
- 3 The lysate could be very viscous and shaking reduces sample viscosity. Extensive vortexing or sonication can be also used.
- 4 Distinct arms of UPR may be activated activated differently by various treatments. For reliable confirmation of UPR activation, several proteins should be monitored.
- 5 Xbp1 antibody works better when incubated overnight at 4°C, dilution 1:1,000.
- 6 Phospho-eIF2α antibody should be diluted in 5% bovine serum albumin in 1× TBS-Tween. Also blocking should be done in albumin solution. Using of milk may increase background.
- 7 Heat shock response can be also monitored

by Western blot. In that case, the general protocol described for UPR can be followed. The same HSF1 and HSP70 antibodies can be used. Activated HSR is manifested by elevated levels of HSP70 and phosphorylation of HSF1, which is detectable as a shift of HSF1 band on the gel.

- 8 To prevent potential bleaching of fluorescent signal, this (and following) step should be done in dark.
- 9 The samples are stable for several weeks when stored at 4 °C or -20 °C.

VI. Acknowledgement

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V. Pitfalls

Problem	Cause and cure
<i>UPR detection by WB</i>	
Low or no signal	• Change the treatment conditions of positive controls (used concentration, incubation time) as effective induction of UPR may vary greatly among different cell types. • Alternatively, when different antibodies are used, consult manufacturer instructions for proper dilution of antibody.
<i>HSR detection by IF</i>	
Low or no signal	• Change the treatment conditions of positive control (use the temperature in range 42 °C ÷ 45 °C and incubate for 1 ÷ 4 hours). Do not overcharge the cells, as it may result in quick cell death. Check the cells under microscope. • Alternatively, when different antibody against HSP70 is used, check if it cross-react with close relative protein HSC70, which is expressed constitutively and may hide HSP70 induction.

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Multiparametric analysis of spheroid integrity by brightfield high-content microscopy

Viswanath Das

I. Introduction

Multicellular spheroids of cancer cells closely model the key characteristics of *in vivo* tissues, such as multicellular arrangement, stress, hypoxia, cell-cell interactions, and extracellular matrix deposition (1). These physiologically-relevant conditions, which are absent in two-dimensional cultures, better reproduce cellular response to external stimuli, including anti-cancer drugs. Spheroids are thus becoming a fundamental research tool for cancer drug discovery and validation of potential hits from high throughput drug screenings (1).

Liquid-overlay technique is a frequently used method of spheroid generation, and a number of groups have successfully used this method for drug target validation studies (2–8). We have recently modified this technique for drug screening under a high throughput setting (5, 9). These modifications address the frequently encountered issues, such as well-to-well variability in spheroid morphology and growth due to evaporation-induced uneven loss of medium in 384-well plates. Data from our and other published work

clearly show that the formation of non-uniform spheroids compromises the significance and relevance of data from pharmacological assays (5, 10, 11).

The spheroids generated using our method are imaged using a bright-field CV7000 high-throughput microscope, and the acquired images are quantified using an in-house, semi-automatic programming routine in MATLAB to measure spheroid area, major and minor axes, perimeter, and solidity. Note that the semi-automated routine is compatible only with the images acquired using the CV7000 high throughput microscope.

In this chapter, the step-by-step use of the semi-automated routine is described (Scheme). These steps are particularly important when analyzing a partially disintegrated, drug-treated spheroid that does not have a clearly defined boundary for the measurement of cross-sectional area.

II. Materials and instrumentation

Instruments

- MATLAB software (MathWorks, version R2017a or above)
- ImageJ software (NIH ImageJ, recent version)
- Desktop computer/laptop compatible with MATLAB and ImageJ

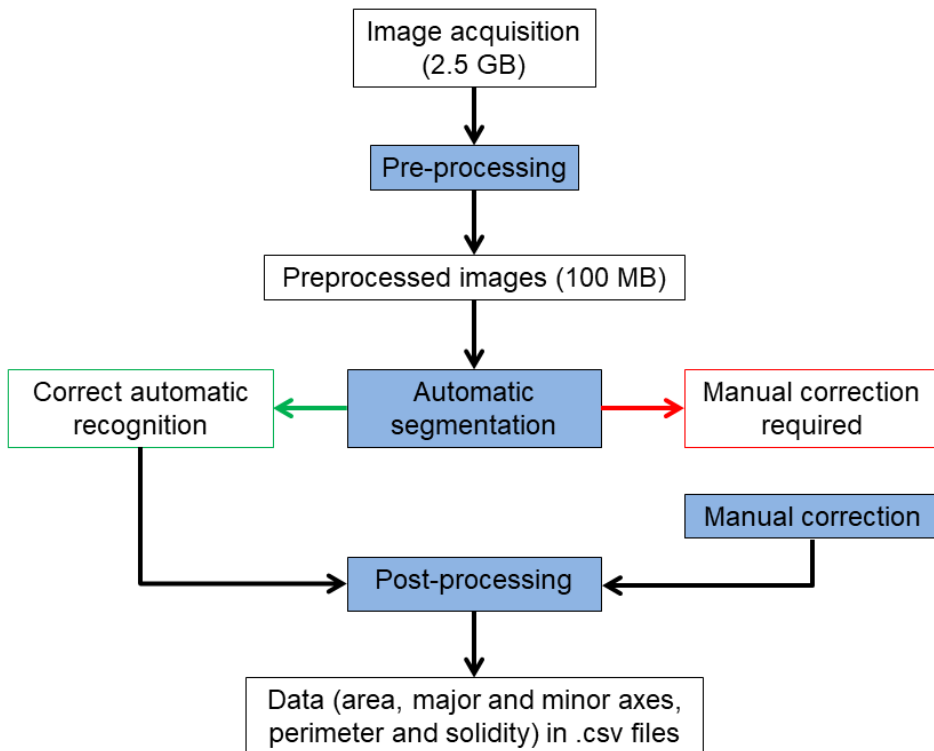
III. Procedures

A. Preparation for semi-automated image analysis

1. Acquire images of the spheroids using a high-content automated imaging system and a 4x air objective as described in Chapter 2.
2. Create a folder and name it ,analysis'. Create a subfolder called ,source" within the analysis folder and copy all images to be analyzed

into this folder. The images are expected to be grayscale TIFF in 16-bit depth. The ,source' folder will contain several images of a single spheroid per well due to z-stack imaging. The filename of images will be in a form that the well ID is between the ultimate and penultimate ,_' symbols (For example, in AssayPlate_PerkinElmer_CellCarrier-384_A10_T0001F001L01A01Z01C01.tif, A10 denotes the well location, Z01 denotes the z-plane height number, and C01 denotes the channel number).

3. Copy the routine files A1PreProcessJinak.m, A2MeasureJinak.m, A3MeasureCorrectionJinak.m and A4PostProcessJinak.m into the ,analysis' folder (not the source folder). Contact author to obtain the routine files.



Scheme 24.1

A workflow showing the steps involved in the semi-automated image analysis routine.

B. Pre-processing of images

1. Double click A1PreprocessJinak.m to open MATLAB.
2. Type A1 in the operation window and press the TAB key on the keyboard to select A1PreProcessJinak.m. Press ENTER to start the routine. This routine goes through the 'source' folder to find the most focused image from the series of z-stack images of a spheroid. After finding the image, the routine crops the borders to ca 80 % and writes the image in Portable Network Graphics (PNG) format into a 'pre-processed' folder. This folder will appear in the 'analysis' folder.
3. The cropped images are identified by the well-ID. For example, if AssayPlate_PerkinElmer_CellCarrier-384_A10_T0001F001L01A01Z01C01.tif is the most focused image of well A10, the cropped pre-processed image file will be written as A10.png. This step compresses the images by almost 2 orders of magnitude without affecting the pixel quality.

C. Identification of spheroid in the pre-processed image

1. After the completion of the pre-processing step, type A2 in the operation window followed by the TAB key to select the A2MeasureJinak.m. Press ENTER to start the routine. The routine will pick the images from 'pre-processed' folder and try to identify the spheroid. If the automatic identification is ascertained, spheroid characteristics (area, solidity, perimeter, the major and minor axes of the fitted ellipse) are measured. The preview of the identified spheroid is saved into a 'preview_ok' folder. If the routine is uncertain on the automatic identification of a spheroid, the routine still measures all spheroid characteristics; however, the preview of the uncertain spheroid is saved into a 'preview_manual' folder.
2. Check the images in the 'preview_ok' folder for the proper image segmentation and move all non-segmented images to the 'preview_manual' folder for visual correction.

D. Correction of uncertain images

1. Type A3 in the operation window followed by the TAB key to select the A3ManualCorrectionJinak.m. Press ENTER to start the routine. The routine walks through the 'preview_manual' folder to correct any errors in the segmentation of images.
2. To make a manual correction, follow the instructions in the figure title. By pressing 'n', all the characteristics will become NaN, i.e., not a number, undefined. By pressing '0', all the characteristics will become zero. To remove the automatic selection, press 'p' and use the computer mouse to draw the correct border around the spheroid.
3. If the routine crashes in the middle of the process, delete all images that were already corrected from the 'preview_manual' folder and re-run the routine again. The 'measure_results.mat' file contains the data and is updated after each image is analyzed.

E. Post-processing and data extraction

1. Type A4 in the operation window followed by the TAB key to select the A4PostProcessJinak.m. Press ENTER to start the routine. The routine will now produce 1x .CSV file for each of the tumor characteristics and saved them in the analysis folder.

IV. Comments

Although there are many semi-automated/automated software available for spheroid image analysis, measurement of the sizes of complete-to-partially disintegrated spheroids, after drug treatment, is prone to errors. In the first step of the presented routine, the best-focused image is automatically selected from the set of z-stack images by computing the norm of the gradient of the image and selecting the one with the largest 0.999 quantiles. This is a robust and noise-insensitive measure of the maximum.

Next, the darker borders are cropped, and the processed images stored at the original resolution in PNG format. This process reduces the total file size produced by the entire 384-well plate from

2.5 gigabytes to ca 100 megabytes. Subsequently, the center of the spheroid is found using a convolution with a circular filter of user-defined radius (in this case, it is 20 pixels; Figure 24A). A clear local minimum of the filtered image provides the position of the center (Figure 24A). Going from this center in concentric annuli, the median grayscale value, M , in each shell is computed as a function of the shell radius R . Thereafter, the first maximum of the numerical derivative of the function $M(R)$ is found (Figure 24B). The point MOPT of this maximum is taken as the optimal threshold and the image is segmented by threshold at this level.

The spheroid is identified as the largest central segment of the image. This procedure usually works better than standard threshold technique because it is able to separate the spheroid core from the disintegrating parts that often result from drug treatment (Figure 24C, D). Moreover, the approach adopted here makes implicit

use of the sphericity of the spheroid. Simple threshold routines, or algorithms based on active contours, do not exploit the a-priori knowledge that a spherical object is to be found. When the spheroid is clearly detected and delineated, the maximum MOPT is clear and single. The routine then measures the characteristics of the spheroid, such as area, major and minor axes, perimeter, and solidity of the fitted ellipse, and saves a preview of the segmented image. If the maximum is not clear and/or multiple local maxima are present, a preview of the suggested segmentation is saved into a correction folder. In the penultimate step, the routine walks the user through the correction folder to manually adjust the suggested segmentation by moving the threshold up or down and selecting a polygonal region of interest, to cut-off possible redundant parts of the mask. After this manual correction, the routine measures and saves the spheroid characteristics as described above.

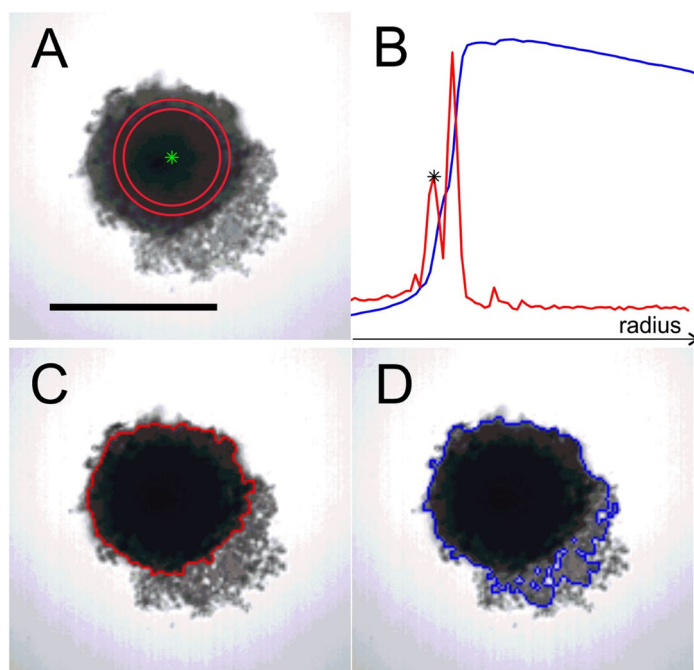


Figure 24. Illustration of the image segmentation procedure.

(A) The center of the spheroid is marked with a green asterisk. An example of the annulus is drawn in red. (B) The median grayscale value in each shell is plotted as a function of the shell radius (blue line). The numerical derivative of this curve is computed (red line). The first maximum of the numerical derivative is found (black asterisk). (C) The resulting correct segmentation of the image into the spheroid core and the background with the disintegrating spheroid parts. (D) Incorrect segmentation obtained by Otsu's method. 4x objective; Scale bar = 500 μm .

V. Pitfalls

Problem	Cause and cure
Routine crashes while running	• Script error. Contact the authors to check the script. • Check if the images are in the ,source' folder, and in the correct format as indicated in Step A.3.
Routine cannot identify spheroids	• Check that the exposure time is set to 11 ms and the lamp intensity is around 10÷12 % in CV7000 high throughput microscope.
Calculated measurement values (area, and minor and major axes) are extremely high.	• This can arise when analyzing partially disintegrated spheroids. Check if the routine identified the disintegrated parts of spheroids as an object. Use the A3ManualCorrectionJinak.m to manually select the correct spheroid boundary.

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Raman imaging of adherent cells

Vlastimil Mašek and Martina Medvedíková

I. Introduction

The aim of this chapter is to describe the general workflow of the model task – study testing the compound intracellular distribution on an adherent cell line model using label-free Raman microscopy.

Raman spectroscopy, and also infrared spectroscopy (IR), are often considered "vibrational" spectroscopies. Term vibrational comes from the fact that Raman as well as infrared spectra contain information about vibrations of atoms or groups of atoms within a molecule. Vibrational frequencies of a given molecule are independent on the method used, Raman or IR, but intensities of individual vibrational bands differ dramatically in Raman and IR spectrum because of different origin of both physical phenomena. Raman and IR spectra are often complementary – bands intensive in Raman spectrum are weak in IR and vice versa. Both Raman as well as IR spectrum serves as the "fingerprint" of the chemical composition of the sample. The character of the sample usually determines which method will suit better for the analysis. The advantage of Raman

spectroscopy is roughly one order of magnitude better spatial resolution (approximately 250 nm in lateral direction for 532 nm excitation) and relatively small and easily correctable signal from water which opens possibilities for many *in vivo* applications. On the other hand, there is no risk of sample damage by excitation laser radiation and there is no fluorescence in IR spectra which is often an intrinsic attribute of a biological sample. More detailed overview of Raman and IR spectroscopy/imaging can be found for example here (1).

One key feature is common for both methods – vibrational spectroscopy reflects inherent chemical composition of the sample and it doesn't require any sample staining.

Typical Raman spectrum of a cell can be divided into the main spectral regions:

- fingerprint region ($600 \div 1,500 \text{ cm}^{-1}$);
- amide I and amide II region ($1,500 \div 1,800 \text{ cm}^{-1}$);
- "silent" region ($1,800 \div 2,500 \text{ cm}^{-1}$);
- higher-wavenumber region ($2,500 \div 3,800 \text{ cm}^{-1}$)

First two spectral regions typically correspond to bending and carbon skeleton fingerprint vibrations, whereas the last one is associated with stretching vibrations such as S-H, C-H, N-H and O-H (2). The spectral region in between is often called “silent” because only limited types of vibrations can be seen here. Based on the vibrational spectra of a pure molecule the structure of a given molecule can be determined or at least estimated (2, 3). But this is possible only for small molecules. In case of cells or tissues, this is not possible because there are thousands of various molecules within the focal volume. It is necessary to look at each Raman spectrum of a Raman scan/map as a sum of contributions from all molecules located in the same point (4).

II. Materials and instrumentation

Materials

Ultrapure water for UHPLC & LC-MS (Merck Milli Q system), dimethyl sulfoxide (DMSO; 1.01952.1011, Merck Millipore), formalin solution, neutral buffered, 10% (HT501128, Sigma-Aldrich); sodium azide (S2002, Sigma-Aldrich), syringe filters 0.22 μm (99722, TPP), Petri dishes with the glass bottom (for example: Cell Imaging Dishes, 0030740009, Eppendorf; Confocal dishes for microscopy, 734-2905, VWR).

Instrumentation:

Confocal Raman microscope Alpha300R+ (WITec, Germany) with following accessories:

- Excitation: 532 nm, 45 mW at the sample.
- Grating: 600 lines/mm.
- Microscope objective: water dipping objective Nikon CFI Apo NIR 60x, numerical aperture (NA)=1.0, free working distance 2.8 mm.
- Scanning: piezo-driven x-y-z stage, range 200 μm in x-, y- direction and 20 μm in z-direction, precision 2 nm in x- and y-direction and 0.2 nm in z-direction.
- Software: dedicated WITec Suite 5.1 package including WITec Control software for measurement control and WITec Project Plus software for data evaluation.

III. Procedures

Solutions

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na_2HPO_4
- 18 mM KH_2PO_4

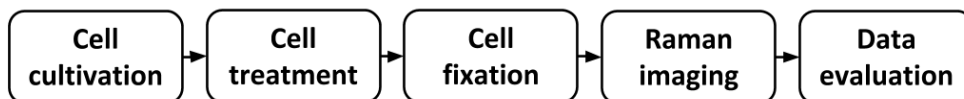
Dissolve appropriate amounts of the above-mentioned chemicals in deionized water (eight tenth of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Sterilize in an autoclave. Store at room temperature; after opening, store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of 10× PBS in deionized water. Store at 4 °C.

Cell cultivation, treatment and fixation

1. Cultivate cells in Petri dishes with the cover glass integrated into the bottom of the dish. For details concerning cell cultivation see Chapter 1.
2. If cells are at 50 – 75 % confluence add the tested compound solution.
3. Fix the treated cells with formalin. For small Petri dishes (approximately 35 mm diameter) use the following protocol. It should be done inside a flow-box to minimize microbial as well as dust particle contamination.
 - a. Remove the cultivation media.
 - b. Rinse the cells in Petri dish thoroughly with 1× PBS buffer to remove potential floating particles, remove the buffer.
 - c. Add 1 ml of 10 % buffered formalin solution and incubate 10 minutes at room temperature (RT).
 - d. Remove the formalin solution and rinse thoroughly with 1× PBS buffer to wash away formaldehyde residuals and potential floating particles.
 - e. Add 1 ml of 1× PBS buffer containing 0.01 % sodium azide to prevent bacterial contamination. Exchange the buffer after 30 minutes. Repeat the buffer exchange after another 30 minutes. Proceed with Raman analysis or store the sample at 4 °C.
 - f. If the sample has been stored, always exchange the 1× PBS buffer containing 0.01 % sodium azide prior Raman analysis.



Scheme 25.1

Protocol for Raman imaging of adherent cells:

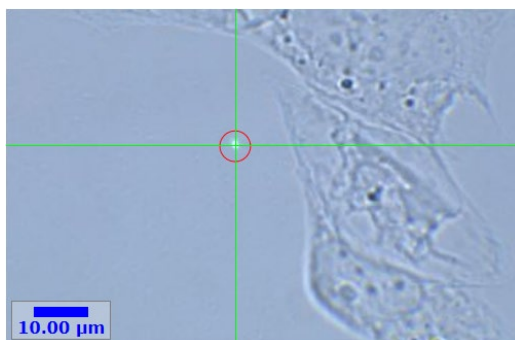


Figure 25.1. *Laser spot calibration.*

Raman imaging

The following protocol describes the workflow using WITec Alpha300R+ confocal Raman microscope which has some unique features (for example z-stabilization procedure); however, general principles described here can be adopted also for other Raman imaging systems.

1. Turn on the Raman system. To reach the temperature stability of all its components turn on the complete Raman system at least 1-hour prior imaging of cells.
2. Calibrate the positioning stage. For perfect sample adjustment, it is recommended to perform the sample positioning stage calibration procedure after each time the system was turned off.
3. Calibrate the spectrograph. Within spectrograph the Raman signal is projected on the CCD detector which means that the horizontal axis of the raw output is in pixel units. The transformation into cm^{-1} (wavenumber) scale is done using automated procedure which employs built-in calibration source. Due to the temperature fluctuations calibration may shift so it is recommended to perform spectrograph calibration prior measurement.
4. Determine the laser spot. The user determines manually the trace of excitation laser on the free substrate (Fig. 25.1). This step is important because it links together bright-field image and Raman image coordinates.
5. Check the system performance using silicon intensity test. Measurement of the silicon wafer standard using the same parameters as it is stated in the installation protocol (maximum laser power, same objective, same integration time) serves as a quick and easy performance test. Silicon peak intensity at 520 cm^{-1} must be comparable to the installation value (Fig. 25.2).
6. Set the spectral center. Typically, spectral center is set at $2,100 \text{ cm}^{-1}$, because this value allows user to see Rayleigh line at one end of the spectrum together with O-H stretching vibrations of water on the other side. To make data treatment easier and uniform it is recommended to keep this value for a given set of experiments.
7. Focus on the cells of interest. In BF mode, use the x-, y- positioning stage to find cells of interest. Using z-coordinate of the microscope, adjust the focus, set z-coordinate of the microscope to $Z=0$.

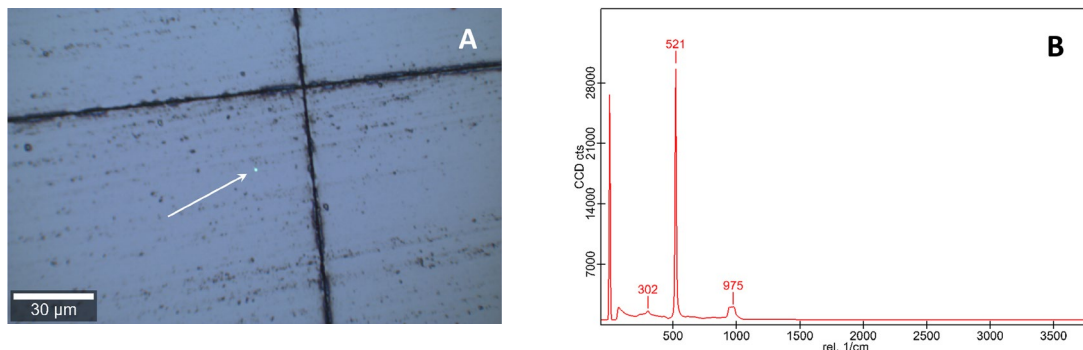


Figure 25.2. A) Silicon wafer standard BF image; B) corresponding Raman spectrum.

8. Determine the substrate surface using line scan. Substrate surface z-coordinate determination is the crucial step because Raman scanning parameters as well as z-stabilization parameters are related to this value.

- In Raman mode, perform the line scan perpendicularly to the free substrate, 50 equidistant steps within the range $<+10; -10>$ μm around microscope $Z=0$ position, this should be broad enough to include buffer-substrate boundary.
- Use the line scan data to calculate the graph of water intensity as a function of z-coordinate (it forms a negative edge course (Fig. 25.3A) - the water signal decreases as the laser beam is focused deeper into the substrate material). The surface corresponds to the inflection point of the course which can be easily determined as the z-coordinate of the 1st derivative minimum Z_{DM} (Fig. 25.3B).
- Go to the microscope z-coordinate

$Z = Z_{DM}$ and set it zero. Now the substrate surface is at zero z-coordinate.

9. Select reference point on free substrate, use Raman water peak spectral region (3,250-3,700 cm^{-1}) and negative edge course (Fig. 25.3A) as parameters for z-stabilization algorithm. Given Raman system has a built-in feature which compensates z-coordinate drifts. Basically, it is the substrate surface determination procedure (see step 8), at given point algorithm fits the negative edge course of the water signal from only 5 z-coordinates (+5; +2.5; 0; -2.5; -5 μm), calculates the position of the surface and compensates potential changes. This procedure is launched after one line of the Raman map has been scanned. Though such correction prolongs measurement time, it makes the analysis more efficient because it can compensate gradual drift of several μm which would normally move the cell out of focus.
10. Make BF image stack of selected cells. In

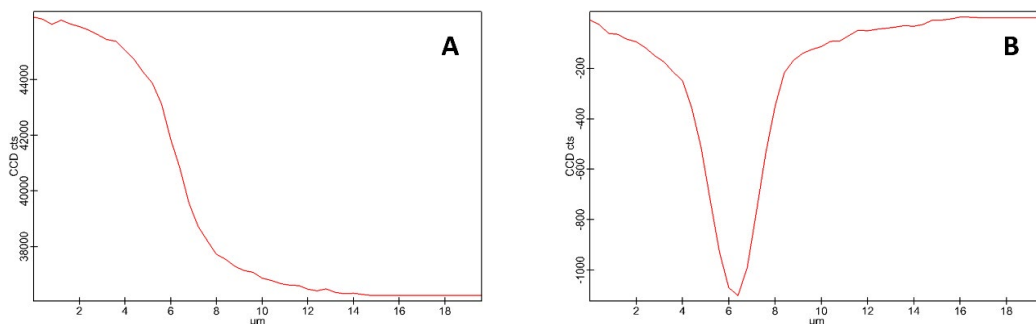


Figure 25.3. Graph of water intensity as a function of z-coordinate (A) and its 1st derivative (B), horizontal scale in μm, vertical scale corresponds to Raman intensity (counts on CCD detector).

BF mode, acquire a stack of several images around substrate focus (typically at $Z=+4; +2; 0; -2; -4 \mu\text{m}$), it will give you better idea of the selected cell(s) and its (their) structure.

11. Determine z-coordinate of the Raman scan. Typically, the Raman map "cuts" the cell in the middle parallel to the substrate surface. To estimate cell thickness, perform quick "depth scan" – Raman scan perpendicularly to the substrate within the range 15 - 20 μm , using coarse raster (1 - 2 pixel/ μm) and 100-250 ms integration time (Fig. 25.4).
12. Set the raster step size of the scan = 3 pixel/ μm . Although the piezo stage allows extremely small steps (less than 10 nm), the real Raman mapping will be done using much coarser raster which takes into account the optical resolution of the Raman microscope as well as sampling frequency criteria. Using Sparrow criterion (5, 6), the lateral spatial resolution is equal to $0.47 \cdot \lambda / \text{NA}$, where λ is the excitation wavelength. For 532 nm excitation and $\text{NA}=1.0$, the theoretical resolution is 250 nm. According to Nyquist theorem (5, 6), sampling frequency (raster) must be equal or better than **2.3 pixel/resolution**. In this case, it is 2.3 pixel/250 nm which is equal to 1 pixel/109 nm. It means that for given Raman system makes sense to measure Raman map containing 9 pixel/ μm . Beyond that limit, the data acquired cannot improve the reconstruction of the object of interest. In real situation the raster is a trade-off between the desired information and the time spent. For illustration, Raman map of just one cell at above-mentioned limit resolution takes several hours. In general, Raman maps at 2-3 pixel/ μm provide reasonable insight into intracellular structure and chemical composition (Fig. 25.5).
13. Set the integration time to 250 ms. Integration time is period necessary for acquisition of the spectrum at single point of the Raman map. It is also a trade-off and it depends on the range of spectral features we are looking for. In the case that the tested compound exhibits intense spectral markers which can be easily identified within Raman spectrum of a cell, the shortest integration time (39 ms for the given Raman system) can be used. In the case of evidence of subtle spectral

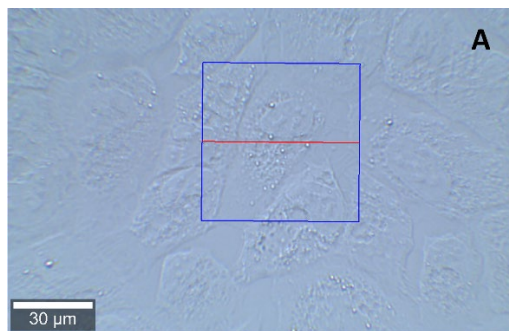


Figure 25.4. (A) Field of view in BF mode including area of interest (blue) and depth scan trace (ground plan) (red), (scale bar - 30 μm); (B) Raman image of the corresponding depth scan reveals that the cell thickness is about 6 μm thus the z-coordinate of the Raman scan = +3 μm (scale bar - 10 μm).

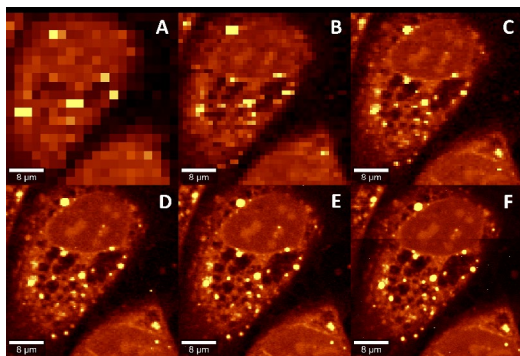
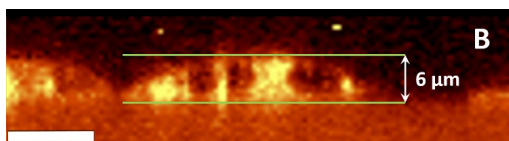


Figure 25.5. The effect of sampling frequency on the Raman image quality. (A) 0.5 pixel/ μm ; (B) 1 pixel/ μm ; (C) 2 pixel/ μm ; (D) 3 pixel/ μm ; (E) 4.5 pixel/ μm ; (F) 9 pixel/ μm . Acquisition times: (A) 1.5 minute; (B) 4.5 minute; (C) 36.5 minute; (D) 67 minutes; (E) 128 minutes; (F) 423 minutes. Due to the short acquisition times, z-stabilization was not used for (A) and (B).

changes, 10× longer integration time may be used at the expense of image quality. In general, integration time within the range of 100 ÷ 250 ms provides reasonable spectra quality (signal to noise ratio).

14. Launch Raman scan.

Data evaluation

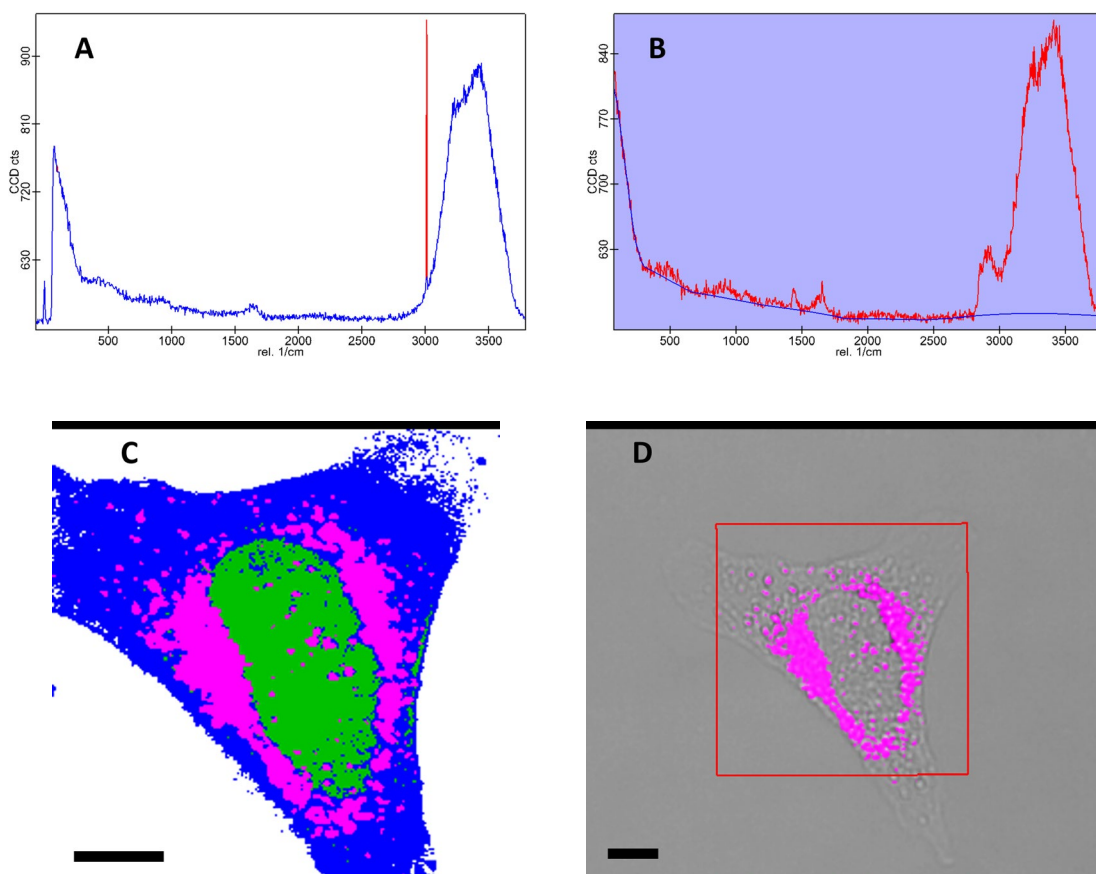
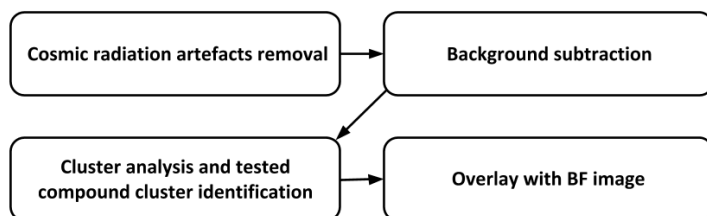


Figure 25.6. (A) The typical appearance of a spike (red) - spectral artefact caused by impact of cosmic radiation on the CDD detector. Spikes are usually very narrow (typically 1 - 3 pixels) and can be detected and corrected by dedicated algorithms. (B) Background (blue line) subtraction from the model spectrum (red) from the Raman map. (C) The result of the cluster analysis, pink color represents cluster corresponding to the tested compound. (D) BF image of the cell overlaid by tested compound cluster. Red square correlate with Raman scan area. Scale bar 10 μm .



Scheme 25.2.

Basic treatment steps of spectral data obtained during Raman scan of a cell. Corresponding examples are shown at Fig. 25.6.

IV. Comments

CaF₂ substrates for Raman imaging of cells.

Due to inherent strong Raman signal, plastic materials are not being used as substrates. For Raman imaging, cells are grown typically on CaF₂ or glass substrates. The advantage of CaF₂ is its flat spectral background containing only one sharp band at 324cm⁻¹ (Fig. 25.7) which can be easily identified within acquired spectra of a cell. On the other hand, cells don't adhere to CaF₂ so willingly as to glass and moreover, CaF₂ is relatively expensive material. Glass is inexpensive but its spectral background affects the data to a larger extent and the data often require further treatment. As there are many types of glass and various manufacturers, the spectral fingerprint may also vary. It is highly recommended to check the chosen substrate for the potential fluorescence background. An alternative approach is to use glass substrate only for initial tests with the model cell line and compound studied.

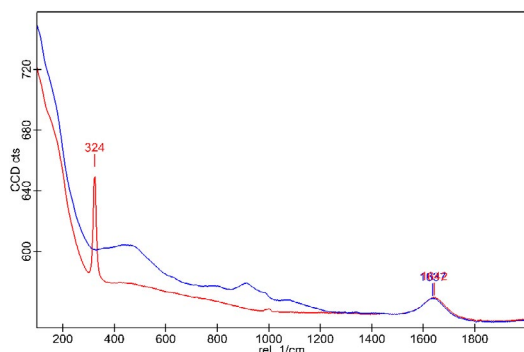


Figure 25.7. Spectral features of CaF₂ (red) and glass (blue).

Excitation wavelength

For Raman imaging of cells, 532 nm is the excitation of the first choice. In case of samples with the fluorescent background, higher excitation wavelengths (with lower energy) can be used - typically 633 nm or 785 nm. Disadvantage of these excitations is lower Raman intensity and lower spatial resolution.

Although lasers used for this Raman system are designed to work continuously, to prolong their lifetime, it is worth turning them OFF when not used. If you expect pause in the measurement longer than 1 hour, turn OFF the laser. If no measurements are planned during the weekend, SHUT DOWN the whole system.

Cell cultivation, treatment and fixation

When using cover glass alone as a substrate there is a risk that it may slide during "zig-zag" Raman scanning causing noticeable shift between bright-field (BF) and Raman image. Fixing the cover glass to the bottom of the scanning vessel using an adhesive may cause contamination of the buffer by adhesive particles which can be trapped by laser beam and their spectral features appear as an artefact in the data. So the procedure is much easier using Petri dishes with the integrated cover glass. As the cover glass serves here just as a substrate and the focus is located above the material, thickness of the integrated cover glass is of no importance.

Extreme care must be taken when using lab-markers as they contain highly fluorescent pigments. Such contamination can render the sample useless for Raman analysis.

Since the tested compounds are often water-insoluble, solvents are frequently used in the cell-based assays, typically DMSO, dimethylformamide (DMF), methanol etc. If possible, maximum concentration of the solvent must be $\leq 0.1\%$ (7).

Bad solubility of the tested compound(s) often results in the precipitation during cells treatment procedure. It is important to wash away potential precipitated particles prior cells fixation to reduce the risk of spectral artefacts in the data.

Formalin fixation procedure (8) is fully compatible with Raman imaging of cells. Other fixatives like methanol, ethanol or acetone can also be used but they are rather invasive as it will wash away all the lipids. Moreover, they could also wash away the tested compound.

V. Pitfalls

Problem	Cause and cure
Horizontal “lines” in Raman map	Contaminating particles (e.g. bacteria, impurities or precipitates from the buffer). Rinse the sample with the filtered and sterile immersion liquid (buffer/water) containing bacteriostatic agent (sodium azide).
Z-stabilization failed	Focus is too far from the surface and the algorithm doesn’t converge. Do the vertical line scan within broader range (for example $z = [+10; -10] \mu\text{m}$) to find the substrate surface and repeat the z-stabilization procedure.
Slight shift between Raman and bright-field image	Change in the optical path. Perform the laser spot calibration procedure.
Silicon intensity test failed	Change the optical path or laser failure. Re-align the optical path and/or check the laser output power using power meter.
PBS buffer interfere with the sample (tested compounds)	Other common water-based buffers or just distilled/deionised water can be used instead.

VI. Acknowledgement

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Cell cycle analysis by flow cytometry

Ivo Frydrych

I. Introduction

Flow cytometry represents a laboratory technique that is used for measuring the physical and chemical properties of live cells or other biological particles. We can simply measure cell size, shape, and internal complexity. By using a fluorescent chemical compound (fluorochrome) to detect a specific cellular component, the component can be further investigated. In addition, several metabolic processes can be monitored. The core and one of the first application to be developed in the field of flow cytometry is the measurement of DNA content. Staining of the cells by stoichiometric DNA-binding dyes, allow estimating proportions of cells in G1, S, and G2/M phases. Moreover, by measuring the DNA content of individual cells, we obtain information about their ploidy (1). Finally, DNA content quantification may reveal the presence of apoptotic cells, characterized by fractional DNA content. A vast array of DNA fluorochromes can be used for cell staining. The most common represent propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI). Also, flow cytometry allows more sophisticated methods, such as simultaneous measuring of DNA content and another parameter, e.g. BrU/BrdU incorporation or cell cycle related proteins

(2).

In this chapter, I present methods allowing an analysis of cells distribution within the major cell cycle phases (Unit A.), DNA replication analysis (B.), analysis of RNA synthesis (C.), and analysis of cell population undergoing mitosis (D.). The method for DNA content based apoptotic cell detection by flow cytometry is described elsewhere (see Chapter 27). All protocols are given for analysis of fixed cells.

II. Materials and instrumentation

Instruments

Regarding instrumentation, manufacturers of the most common flow cytometers include Becton Dickinson (BD) and Beckman Coulter. We routinely use a FACSCalibur (Becton Dickinson). The software for estimation of the percentage of cells in different cell cycle phases is available from different companies, e.g. Verity Software House (ModFit) or Phoenix Flow Systems (MultiCycle). Common software for acquiring and analyzing

data from flow cytometers represents CellQuest from BD. Other equipments needed comprise rotary shaker (Biosan), water bath (Biosan), micropipettes (Eppendorf), pH meter (Mettler Toledo), centrifuge (Eppendorf), scale (Scaltec), and vortex mixer (Genie).

Materials

A. Cell cycle analysis

Propidium iodide (PI, Cat. No. P4170), Triton X-100 (Cat. No. 93443), Ribonuclease A from bovine pancreas (RNase, Cat. No. R5503) and Sodium citrate tribasic dihydrate (Cat. No. C8532) are obtained from Sigma-Aldrich. FACS tubes 12x75 mm (Cat. No. 352054) are from BD.

B. Analysis of DNA replication

Propidium iodide (PI, Cat. No. P4170), RNase (Cat. No. R5503), Triton X-100 (Cat. No. 93443), bovine serum albumin (BSA, Cat. No. A3059), Tween20 (Cat.No. P2287), sodium tetraborate decahydrate (Cat. No. B9876) and 5-Bromo-2'-deoxyuridine (BrdU, Cat. No. B5002) are obtained from Sigma-Aldrich. Monoclonal anti-BrdU antibody is from Exbio (Cat. No. 11-286-C025) and secondary Anti-mouse IgG F(ab')₂ fragment-FITC antibody is from Sigma-Aldrich (F2883). FACS tubes 12x75mm (Cat. No. 352054) are from BD.

C. Analysis of RNA synthesis

Propidium iodide (PI, Cat. No. P4170), RNase (Cat. No. R5503), NP-40 (Cat. No. 74385),

bovine serum albumin (BSA, Cat. No. A3059), 5-Bromouridine (BrU, Cat. No. 850187) and formaldehyde solution (Cat. No. F8775) are obtained from Sigma-Aldrich. Monoclonal anti-BrdU antibody is from Exbio (Cat. No. 11-286-C025), secondary Anti-mouse IgG F(ab')₂ fragment-FITC antibody is from Sigma-Aldrich (F2883) and Glycine is from Amsbio (Cat. No. 23390.03). FACS tubes 12x75 mm (Cat. No. 352054) are from BD.

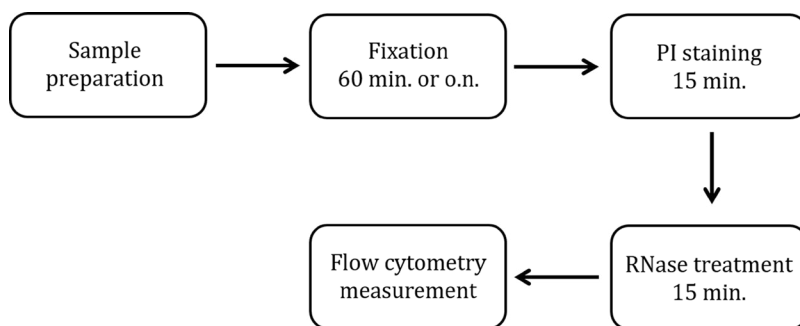
D. Analysis of mitotic cells

Propidium iodide (PI, Cat. No. P4170), RNase (Cat. No. R5503) and Triton X-100 (Cat. No. 93443) are obtained from Sigma-Aldrich. Fetal bovine serum (FBS, Cat. No. FBS-12A) is from Capricorn Scientific. Primary Anti-phospho-Histone H3 antibody is from Millipore (Cat. No. 06-570). Secondary Alexa Fluor 488 goat IgG is from Invitrogen (A11070). FACS tubes 12x75 mm (Cat. No. 352054) are from BD.

III. Procedures

A. Cell cycle analysis

The following procedure describes the analysis of cells distribution in different cell cycle phases. For DNA content quantitation, propidium iodide staining is used. The binding of the dye to DNA is stoichiometric and thus the fluorescence intensity of the stained cells is proportional to the cellular DNA content.



Scheme 26.1

Solutions

Citrate buffer stock solution (10× concentrated)

Dissolve 5.68 g Sodium citrate tribasic dihydrate in 500 ml of deionized water. Store at 4 °C for up to 3 months.

Citrate buffer working solution (1× concentrated)

Dilute appropriate volume of citrate buffer stock solution tenfold with deionized water. Store at 4 °C for up to 3 months.

2% Triton X-100/citrate buffer stock solution

Mix 2 ml of Triton X-100 with 100 ml of citrate buffer stock solution. Store at 4 °C for up to 3 months.

Propidium iodide solution (PI, 50 µg/ml)

Dissolve 2.5 mg PI in 5 ml of 2% Triton X-100/citrate buffer stock solution and fill up to a total volume of 50 ml with deionized water. Every time prepare fresh.

RNase solution

2 mg/ml citrate buffer working solution. Store at -20 °C for up to 1 year.

Cells fixation

1. Collect the cells by centrifugation at 800 g for 5 min at RT.
2. Wash the cells by adding an appropriate volume of PBS.
3. Spin down, aspirate supernatant and add ice-cold 70% ethanol (2 ml/1×10⁶ cells) dropwise.
4. Keep at least 1 hour or overnight at -20 °C.

Cells staining

1. Set up a water bath incubator temperature to 37 °C.
2. Spin down at least 1×10⁶ of fixed cells at 800 g for 5 min at RT.
3. Aspirate supernatant, add 1 ml citrate buffer working solution, mix thoroughly by pipette and add an additional 4 ml of citrate buffer working solution.
4. Spin down at 800 g for 7 min at RT.
5. Aspirate supernatant, add 600 µl PI solution and vortex maximally.

6. Incubate for 15 minutes in a water bath (37 °C) in the dark.
7. If the aggregates are visible, run the suspension through 50 µm pore size filter.
8. Add 500 µl RNase solution and continue water bath incubation for an additional 15 minutes in the dark.
9. Keep the sample in the fridge for at least 1 hour to stabilize the sample.
10. Analyze the samples by flow cytometer.

B. Analysis of DNA replication

This method is based on analysis of DNA replication measured simultaneously with DNA content. It relies on S phase marker 5'-bromo-2'-deoxyuridine (BrdU) incorporation by actively dividing cells, detected by an antibody specific against BrdU. Antibody binding requires denaturation of DNA induced by exposing the cells to acid (3).

Solutions

• 1mM BrdU solution

Dissolve 3.071 mg BrdU in 10 ml of cell culture medium. Filter-sterilize, aliquot and store at -20 °C.

• 2 N HCl/0.5% Triton X-100 solution

• 0.1 M borax solution (pH 8.5)

Dissolve 3.82 g sodium tetraborate decahydrate in 100 ml of distilled water and adjust to pH 8.5.

• Washing solution

0.5% Tween20/0.1% BSA in PBS (PBS-T)

• Propidium iodide solution (50 µg/ml PBS)

• RNase solution

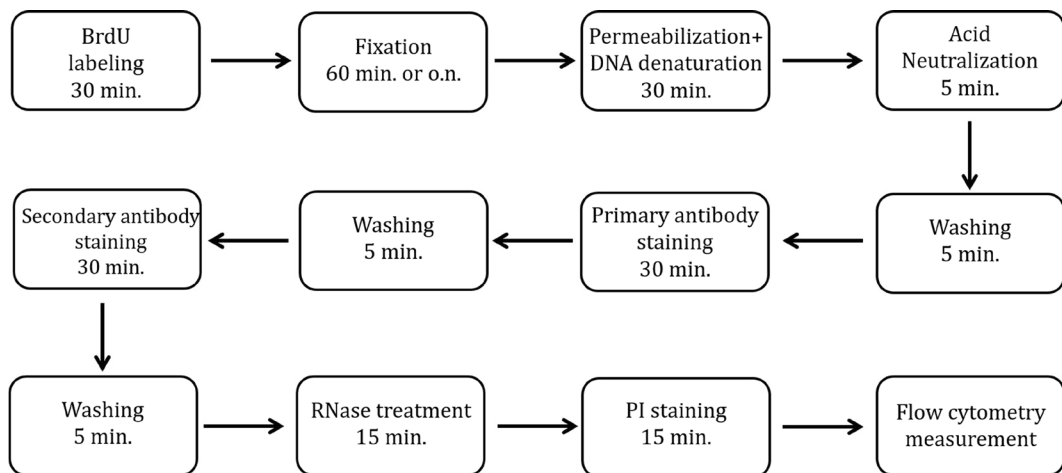
10 mg/ml of PBS.

• Anti BrdU antibody solution

Dilute 1:250 in PBS.

• Secondary antibody solution

Dilute 1:250 in PBS.



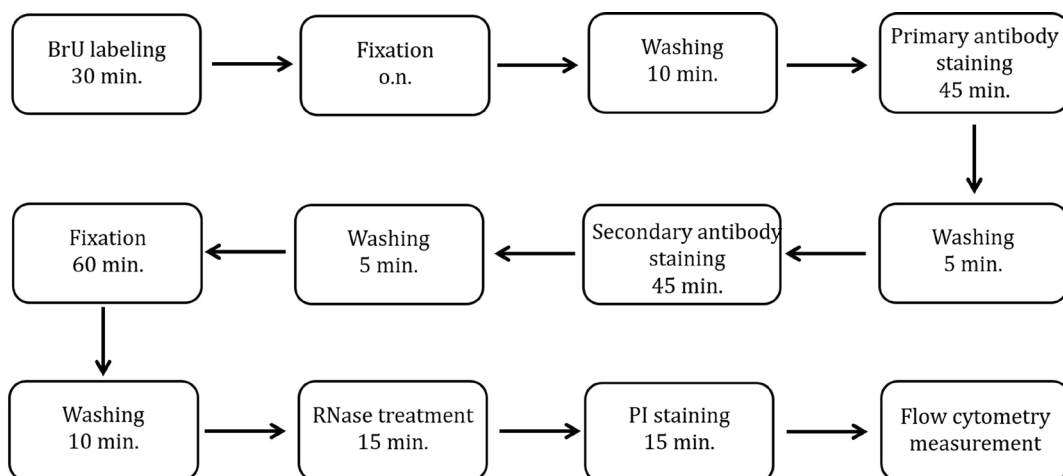
Scheme 26.2

Steps

1. Add the corresponding volume of 1mM BrdU to the culture media to get a final concentration of 10 μ M and incubate for 30 minutes at 37 °C in a CO₂ incubator.
2. Transfer cell suspension into the tube and spin down at 500 g for 5 minutes at 4 °C.
3. Wash the pellet once with 3 ml of PBS, centrifuge (500 g/5 min/4 °C) and aspirate supernatant.
4. Add 2 ml ice-cold 70% ethanol dropwise with constant vortexing and store the sample at -20 °C overnight.
5. Transfer 1 ml or at least 1x10⁶ of the fixed cell suspension to clean tube and centrifuge at 500 g for 5 minutes at RT.
6. Aspirate supernatant and add 2 ml 2N HCl/Triton X-100 solution dropwise with constant gentle vortexing.
7. Incubate for 30 minutes in the dark with occasional gentle vortexing.
8. Centrifuge at 500 g for 5 minutes at RT.
9. Aspirate supernatant and add 2 ml borax solution.
10. Centrifuge at 500 g for 5 minutes at RT.
11. Aspirate supernatant and add 2 ml PBS-T solution while vortexing.
12. Centrifuge at 500 g for 5 minutes at RT.
13. Aspirate supernatant, add 200 μ l anti-BrdU antibody solution and mix gently. Incubate for 30 minutes at RT.
14. Wash with 2 ml PBS-T solution and centrifuge at 500 g for 5 minutes at RT.
15. Aspirate supernatant and add 200 μ l secondary antibody solution. Incubate at RT in the dark for 30 minutes.
16. Wash with 2 ml PBS-T solution and centrifuge at 500 g for 5 minutes at RT.
17. Aspirate supernatant and add 100 μ l RNase solution. Incubate at RT in the dark for 15 minutes with occasional mixing.
18. Finally, stain cells by adding 600 μ l propidium iodide solution and leave the sample in the dark for 15 minutes with occasional mixing.
19. Keep the sample in the fridge for at least 30 minutes before flow cytometry analysis.

C. Analysis of RNA synthesis

Analysis of RNA synthesis is based on the incorporation of brominated RNA precursor 5-Bromouridine (BrU) into RNA of target cells. The RNA substituted by BrU is detected upon cells permeabilization by anti-BrdU antibody cross-reacting specifically with BrU.



Scheme 26.3

Solutions

- **100 mM BrU**

Dissolve 32.3 mg BrU in 1 ml of cell culture medium. Filter-sterilize, aliquot and store at -20 °C.

- **Fixing solution**

1% formaldehyde/0.05% NP-40 in PBS

- **1% glycine/PBS solution**

- **Washing solution**

0.1% BSA/0.1% NP-40 in PBS

- **Propidium iodide solution**

50 µg/ml of washing solution

- **RNase solution**

10 mg/ml of washing solution

- **Anti BrdU antibody solution**

Dilute 1:250 in washing solution.

- **Secondary antibody solution**

Dilute 1:250 in the washing solution.

Steps

1. Add the corresponding volume of 100mM BrU solution to the culture media to get a

final concentration of 1mM and incubate for 30 minutes at 37 °C in a CO₂ incubator.

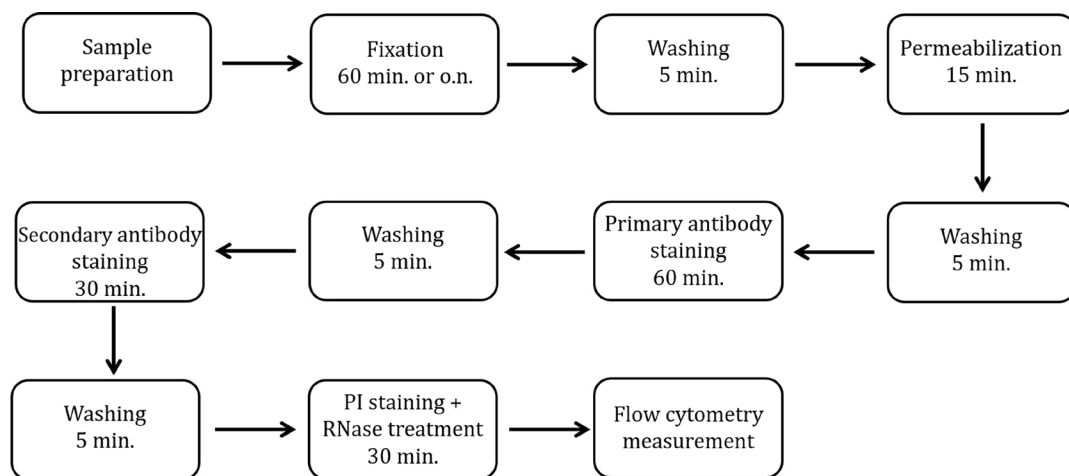
2. Transfer cell suspension into tube and centrifuge at 500 g for 5 minutes at 4 °C.
3. Wash the pellet with 3 ml of PBS, centrifuge (500 g/5 min/4 °C) and aspirate supernatant.
4. Add 2 ml fixing solution, vortex gently and place the sample on a rotator (gentle rotation) for 15 minutes at RT.
5. Store the sample at 4 °C until analysis, but at least overnight.
6. Transfer a volume of at least 2x10⁶ of the fixed cell suspension to clean tube and centrifuge at 500 g for 5 minutes at 4 °C.
7. Aspirate supernatant and add 3 ml glycine/PBS solution, vortex and centrifuge at 500 g for 5 minutes at 4 °C.
8. Aspirate supernatant and wash with 3 ml of PBS solution. Centrifuge at 500 g for 5 minutes at 4 °C.
9. Aspirate supernatant, add 100 µl anti-BrdU antibody solution and mix gently. Incubate for 45 minutes at RT.
10. Add 3 ml washing solution, vortex and centrifuge at 500 g for 5 minutes at 4 °C.
11. Discard supernatant, add 100 µl secondary antibody solution and mix gently. Incubate for 45 minutes at RT in the dark.
12. Add 3 ml washing solution, vortex and

centrifuge at 500 g for 5 minutes at 4 °C.

13. Aspirate supernatant, add 1 ml of fixing solution and vortex at maximum speed.
14. Incubate for 1 hour at 4 °C and then centrifuge at 500 g for 5 minutes at 4 °C.
15. Aspirate supernatant, wash with 3 ml glycine/PBS solution and centrifuge at 500 g for 5 minutes at 4 °C.
16. Discard the supernatant and add 100 µl RNase solution. Incubate at RT in the dark for 15 minutes.
17. Finally, stain cells by adding 600 µl propidium iodide solution and incubate at RT in the dark for 15 minutes with occasional mixing.
18. The sample can be analyzed immediately after propidium iodide staining.

D. Analysis of mitotic cells

By using single parameter DNA analysis, it is not possible to distinguish between G2 and mitotic phase cells. The following procedure describes a method combining DNA analysis simultaneously with a mitotic phase specific marker (phospho-histone H3). Antibody used in this protocol specifically recognizes Histone H3 phosphorylation on Ser10, which is considered to be a crucial event for the onset of mitosis (4).



Scheme 26.4

Solutions

- **0.25% Triton-X100/PBS solution**

- **Washing solution**

1% FBS/PBS

- **Propidium iodide (PI)/RNase solution**

Dissolve 50 µg PI and 0.5 mg of RNase per 1 ml of washing solution (or PBS).

- **Anti-phospho-Histone H3 antibody solution**

Dilute 1:500 in the washing solution.

- **Secondary antibody solution**

Dilute 1:500 in the washing solution.

Steps

1. Spin down at least 1×10^6 of cells at 800 g for 5 minutes at RT.
2. Aspirate supernatant and add 1 ml ice-cold 70% ethanol dropwise. Keep at least 1 hour or overnight at -20 °C.
3. Add 1 ml washing solution, vortex and centrifuge at 800 g for 5 minutes at RT.
4. Discard supernatant, add 1 ml cold Triton-X100/PBS solution while vortexing.
5. Incubate for 15 minutes on ice.
6. Add 10 ml washing solution, vortex and centrifuge at 800 g for 5 minutes at RT.
7. Aspirate supernatant, add 100 µl anti-phospho-Histone H3 antibody solution and incubate for at least 1 hour at RT. Vortex gently after 30 minutes.
8. Add 10 ml washing solution, vortex and centrifuge at 800 g for 5 minutes at RT.
9. Discard supernatant, add 100 µl secondary antibody solution and incubate for 30 minutes at RT in the dark.
10. Add 10 ml washing solution, vortex and centrifuge at 800 g for 5 minutes at RT.
11. Aspirate supernatant and add 700 µl PI/RNase solution. Incubate for 30 minutes at 37 °C in the dark.
12. The sample can be analyzed immediately or you can keep it for several hours at 4 °C in the dark.

IV. Comments

- a. In general, above-described protocols suppose a basic knowledge of users on the field of flow cytometry principles and practices. Despite the fact, that all of the protocols are connected with a FACSCalibur instrument, the methods apply to other FACS machines.
- b. The first step common to all protocols comprises cell suspension fixing. For that purpose, alcohol or aldehyde based fixative are commonly used. Ethanol fixation should be done carefully with ice-cold ethanol added dropwise while vortexing. This ensures all the cells to be fixed with minimum risk of clumping. Ethanol-fixed cells can be stored for several months at -20 °C, thus the cells can be prepared and collected independently of the timing of their analysis. On the other hand, formaldehyde-fixed cells (analysis of RNA synthesis) are stable only for up to 2 weeks when stored at 4 °C.
- c. All presented protocols include RNase solution treatment before PI addition, to ensure specific PI incorporation into DNA of analyzed cells. The RNase should be free of DNase activity.
- d. PI is excited by blue light (490 nm) from the 488-nm line argon laser, which is available on most flow cytometers. Since PI is an intercalator, it is important to be careful in its disposing and handling.
- e. BrU and BrdU are light sensitive and should be added in the dark. The time needed for BrU/BrdU labeling strongly depends on cell division rate. While primary cells may need up to 24 hours, for rapidly proliferating cells, 30 minutes is enough. The exact time required to get the optimal signal to noise ratio should be optimized for particular cell lines.

V. Pitfalls

Problem	Cause and cure
No signal	• Bad pH of washing solutions or RNase solution. Check the pH of washing solutions or RNase solution. Check the osmolarity of the solutions. • PI staining is absent. Check the color of the sample solution (should be slightly pink). • Cell loss during washing steps and/or aspiration. Carefully aspirate supernatant, leaving at least 50 µl of the sample in the tube.
Poor-quality signal	• Degraded primary or secondary antibody. Use fresh antibodies. • Insufficient RNase treatment. Check for pH of RNase solution or prepare fresh one.

VI. Acknowledgement

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Detection of apoptosis by flow cytometry

Ján Gurský

I. Introduction

Cell death plays a pivotal role in embryonal development, tissue homeostasis, tumorigenesis, the immune system, and at the same time constitutes a major therapeutic target for the management of multiple human disorders. Over the past decade, the knowledge in the field continues to progress and novel signaling pathways, with diversity in initiation and execution of cell death are the basis for updated classification. According to the recommendations of Nomenclature Committee on Cell Death in 2018 (1), at least 12 types of cell death are proposed including intrinsic apoptosis, extrinsic apoptosis, mitochondrial permeability transition (MPT)-driven necrosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, autophagy-dependent cell death, anoikis, and immunogenic cell death.

Regarding the cell death type discrimination, one of the early questions to be addressed is the diagnosis of apoptotic features presence. Some methods are based on typical morphological changes during apoptotic cell death such as

blebbing of the plasma membrane, chromatin condensation, nuclear fragmentation or the formation of apoptotic bodies (2). Other approaches are focused on changes to mitochondrial membrane permeability during apoptosis (3) that result in the release of specific proteins from the intermembrane mitochondrial space, such as APAF and cytochrome c. Biochemical features of apoptotic cells offer another opportunity and include externalization of phosphatidylserine on the outer leaflet of the plasma membrane (4), activation of caspases or presence of caspase-substrates cleavage (for example cleaved PARP1).

In this chapter, a protocol for analysis of externalized phosphatidylserine (PS) using flow cytometry and protocol for FACS quantification of caspase activation are described (5,6). Both methods are sensitive and enabling quantitative analysis of thousands of cells. Briefly, externalized PS is detectable by Annexin V, which has a high affinity for PS. Annexin V conjugated to fluorochromes serves as a probe for flow cytometric detection of cells that are undergoing apoptosis. The crucial point of the assay is discrimination of live intact cells from

already permeabilized cells. Therefore, Annexin V staining is performed in combination with vital cell-impermeant DNA dye (such as 7-AAD, PI), allowing for identification of permeabilized cell, positive for DNA dye. Only intact, non-permeabilized cells (negative for DNA dye) with externalized PS, thus positive for AnnexinV, are considered as apoptotic (Fig. 27.1). The second method is based on the use of the fluorogenic substrate, that contains a cleavage site for caspase-3/7 in the form of four-amino acid peptide (DEVD), conjugated to nucleic acid-binding dye. The reagent is intrinsically non-fluorescent as the DEVD peptide inhibits the ability of the dye to bind to DNA. After cleavage of DEVD peptide by activated caspase-3/7, dye binds to DNA and produces a fluorogenic response, that can be detected using flow cytometry. Similarly to Annexin V assay, identification of permeabilized cells is essential, so vital cell-impermeant DNA dye is included in the staining procedure. Following flow cytometric analysis, non-permeabilised cells with fluorogenic substrate-generated signal represent the apoptotic population with activated caspases 3/7 (Fig. 27.2). Because no single parameter defines apoptosis reliably, a combination of different measurements is strongly recommended.

II. Materials and Instrumentation ¹

Materials

- **100 mM Hepes pH 7.4 (stock solution)**

Dissolve Hepes (Sigma Aldrich, H4034) in deionized water (eight-tenths of the final volume), adjust the pH with 1 M NaOH to 7.4) and add deionized water to the final volume. Sterilize the solution by passage through a 0.22- μ m filter. Store at 4 °C.

- **2 M NaCl (stock solution)**

Dissolve NaCl (Sigma Aldrich, 31434) in deionized water (eight-tenths of the final volume). Add deionized water to the final volume. Sterilize by filtration and store at room temperature.

- **1 M KCl (stock solution)**

Dissolve KCl (Sigma Aldrich, 60130) in deionized water (eight-tenths of the final volume). Add

deionized water to the final volume. Sterilize by filtration and store at room temperature.

- **1 M MgCl₂ (stock solution)**

Dissolve MgCl₂.6H₂O (Sigma Aldrich, M2393) in deionized water (eight-tenths of the final volume). Add deionized water to the final volume. Sterilize by filtration and store at room temperature.

- **Annexin reaction buffer (working solution) (7)**

- 10 mM Hepes/NaOH (pH 7.4)
- 140 mM NaCl
- 4 mM KCl
- 0.75 mM MgCl₂

Mix 100 mM Hepes/NaOH pH 7.4 (10% of final volume), 2 M NaCl (7% of final volume), 1 M KCl (0.4% of final volume), 1 M MgCl₂ (0.075% of final volume) and add deionized water to the final volume. Sterilize by filtration and store at 4 °C.

- **100 mM CaCl₂ (stock solution)**

Dissolve CaCl₂ (Sigma Aldrich, C5670) in deionized water (eight-tenths of the final volume). Add deionized water to the final volume. Sterilize by filtration and store at room temperature.

- **Fetal Bovine Serum**

Fetal bovine serum for standard mammalian cell culture application, (for example Gibco, Thermo Fisher, 10270106)

Solutions used for the FACS analysis of Annexin V binding

- Annexin V conjugated to a fluorochrome (stock solution)

For example, APC-Annexin V, recommended dilution 1:20 (BD Biosciences, 550474) (use the dilution of the annexin-conjugate recommended by the manufacturer).

- Cell impermeant DNA dye (stock solution)
- For example 7-AAD, recommended dilution 1:20 (BD Biosciences, 559925) (use the dilution of the dye recommended by the manufacturer).

Solutions used for the FACS quantification of caspase-3/7 activation

- **Fluorogenic caspase substrate (stock solution)**

For example CellEvent® Caspase-3/7 Green Detection Reagent, stock solution 2 mM, recommended final concentration 500 nM (Thermo Molecular Probes Cat.no. C10423) (use the dilution of the dye recommended by the manufacturer). Before use, prepare working solution of Cell Event Reagent by dilution 1:320 in DMSO. Use 8 µl of working solution per sample (total sample volume 100 µl) to reach 500 nM final concentration. Prepare working solution just before use.

- **Cell impermeant DNA dye (stock solution)**

For example DAPI, (Sigma Aldrich, D9542). Dissolve DAPI in deionized water. Aliquot and store at -20 °C.

Prepare working solution 10 µg/mL. Aliquot and store at -20 °C.

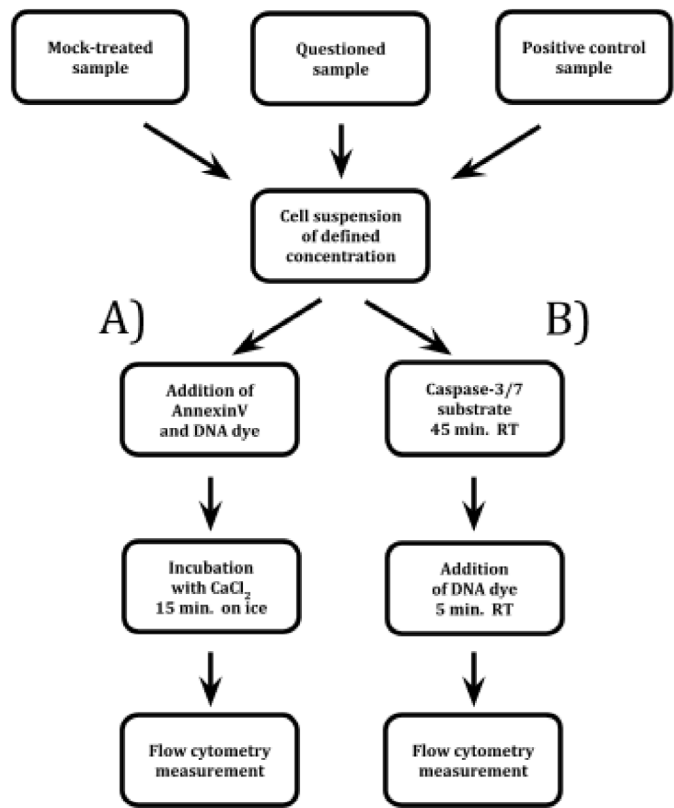
Instruments

- BD FACSVerse (BD Biosciences)
- Heating magnetic stirrer MSH-300i (Biosan, BS-010309-AAA)
- Vortex Genie 2 (Scientific Industries, SI-0236)

III. Procedures

Scheme (for details follow the procedure protocol)

- FACS analysis of Annexin V binding
- FACS quantification of caspase-3/7 activation using fluorogenic substrate



Scheme 27.1.

A. FACS analysis of Annexin V binding

1. Harvest cells, to include detached cells also collect the cell culture media.²
2. Centrifuge cells (5 min, 250 g, 4 °C).
3. Resuspend cells in precooled Annexin reaction buffer and from this step keep cells on ice.
4. Count cells.
5. Centrifuge cells (5 min, 250 g, 4 °C).
6. Resuspend cell pellet in cold Annexin reaction buffer to obtain a concentration of 1.1×10^6 cells/mL.³
7. Mix 90 μ l of cell suspension with 10 μ l of AnnexinV/impermeant DNA dye mixture to obtain final volume 100 μ l.^{3,4}
8. Optional: For compensation determination and/or quadrants set up following single-stained controls should be prepared: Annexin V-only stained sample and DNA dye-only stained sample.
9. Add 2.5 μ l of CaCl_2 to each sample 15 minute before the acquisition and mix gently.⁵
10. Incubate the samples for 15 minutes on ice in the dark.
11. Acquire the stained cells by flow cytometry.
12. Use the mock-treated sample to define the gating of cells and to set up the analysis quadrants (Fig. 27.1).¹⁰

B. FACS quantification of caspase-3/7 activation using fluorogenic substrate

1. Use cell suspension as prepared in step 6 of procedure 'A. FACS analysis of annexin V binding procedure in this chapter.^{3,6}
2. Mix 90 μ l of cells suspension with 2 μ l of Fetal bovine serum.⁷
3. Prepare the working solution of the fluorogenic caspase substrate.
4. Add 8 μ l of pre-diluted fluorogenic caspase substrate to obtain the desired final concentration in 100 μ l volume. Mix gently.⁸
5. Incubate the samples for 45-60 minutes at room temperature in the dark.
6. Add 5 μ l of 10 μ g/mL DAPI solution to each sample and mix gently.⁹
7. Incubate for 5 min at room temperature in the dark.
8. Acquire the samples by flow cytometry.
9. Use the mock-treated sample to define the gating of cells and to set up the analysis quadrants (Fig. 27.2).¹⁰

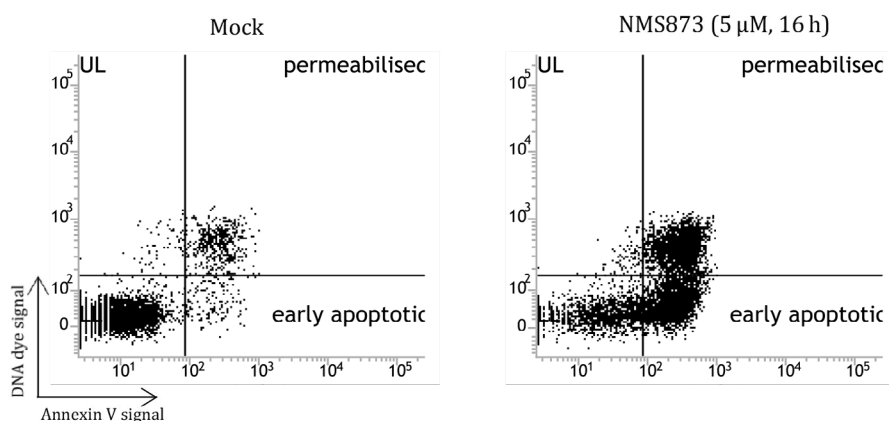


Figure 27.1. Annexin V binding analysis

Analysis of annexin V signal in AMO-1 cells: left mock-treated, right NMS873 (p97 inhibitor, 5 μ M, 16 h) treated sample. Permeabilized and dead cells are positive for cell-impermeant DNA dye (upper quadrants), whereas live cells with intact membrane are negative for DNA dye (both bottom quadrants). Cells, that are both intact (negative for DNA dye) and positive for Annexin V (right bottom quadrant) are considered to be early apoptotic. Adapted from Skrott, Z., et al., 2017, Alcohol-abuse drug disulfiram targets cancer via p97 segregase adaptor NPL4. *Nature* 552(7684): p. 194-199 (5).

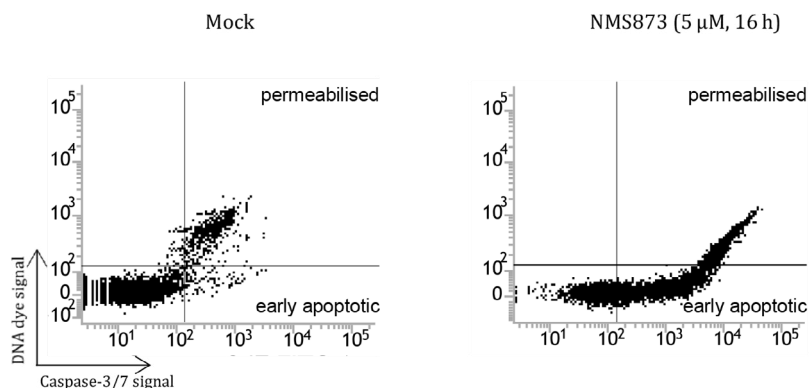


Figure 27.2. Analysis of caspase 3/7 activity

Analysis of caspase 3/7 activity signal in AMO-1 cells: left mock-treated, right NMS873 (p97 inhibitor, 5 μ M, 16 h) treated sample. Permeabilized and dead cells are positive for cell-impermeant DNA dye (upper quadrants), whereas live cells with intact membrane are negative for DNA dye (both bottom quadrants). Cells, that are both intact (negative for DNA dye) and positive for caspase 3/7 activity (right bottom quadrant) are considered as early apoptotic. Adapted from Skrott, Z., et al., 2017, Alcohol-abuse drug disulfiram targets cancer via p97 segregase adaptor NPL4. *Nature*, 552(7684): p. 194-199 (5).

IV. Comments

- 1 The mentioned manufacturers of the listed instruments were used during the protocol testing or development.
- 2 Induction of cell death using various agents may lead to detachment of adherent cells. To analyze the whole cell culture population, collecting the cell culture media containing floating cells is necessary.
- 3 The goal is to stain an identical amount of cells in the same final volume for each sample. In this case, 1×10^5 cells in 90 μ L volume are used for the next step of the procedure.
- 4 The Annexin V concentration optimal for cell model being used should be determined empirically, vendor-recommended working dilution could serve as a starting point.
- 5 Annexin V binding to phosphatidylserine is strongly calcium-dependent. However, calcium at high concentration is harmful to cells. The abundance of CaCl_2 needed for the specific cell model should be titrated, to use the minimum required concentration.
- 6 We highly recommend applying both FACS Analysis of Annexin V binding and FACS quantification of caspase-3/7 activation using fluorogenic substrate procedures on aliquots of the same sample. In this case, cell

suspension prepared in steps 1-6 of FACS Analysis of Annexin V binding procedure is utilized by the FACS quantification of caspase activation procedure. Otherwise, cell suspension at a concentration of 1.1×10^6 cells/ml for quantification of caspase activation could be prepared in other appropriate suspension buffer (e.g., phosphate buffered saline, complete media, etc.). For more information, see the instruction of the fluorogenic substrate manufacturer.

- 7 If cell suspension is prepared in the inorganic buffer, supplementation of the reaction mixture with fetal bovine serum is recommended, to minimize the starvation-induced stress during subsequent incubation.
- 8 The final concentration of fluorogenic caspase substrate optimal for used cell model should be determined empirically, vendor-recommended working dilution could serve as a starting point. Following single-stained controls should be prepared for compensation determination: Annexin V-only stained sample and DNA dye-only stained sample.
- 9 Make sure to include proper apoptosis-inducing agent as a positive control (e.g., NMS873, staurosporine, camptothecin).

V. Pitfalls

Problem	Cause and cure
<i>Annexin binding</i>	
No signal	• The Annexin V final concentration should be optimized, vendor-recommended working dilution could serve as a starting point. • Make sure to include proper apoptosis-inducing agent as a positive control (e.g.: NMS873, staurosporine, or camptothecin).
<i>Caspase-3/7 analysis</i>	
Poor-quality signal	• Prolong the incubation time, optimize the final concentration of the fluorogenic caspase substrate. • Determine the appropriate time-point for initiation of apoptosis after induction with an apoptosis-inducing agent.

VI. Acknowledgment

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Detection and quantification of cellular antigens using flow cytometry

Petr Džubák

I. Introduction

The molecular composition of the cell is the information which is essential for the understanding of the molecular processes and could be used for cellular characterization and cells classification. As one of the most important examples of cellular markers is well-established CD (clusters of differentiation) nomenclature of cellular antigens, useful for leucocytes characterization and markers of differentiation, activation or lineage. CD nomenclature was established in 1982 and since that time is continuously developed by HLDA (Human Leukocyte Differentiation Antigens) workshops. New CD markers are regularly introduced and the last set counting 371CD molecules was announced at HLDA10 meeting held in 2014 in Wollongong, Australia. More additional information is on the HLDA web pages www.hcdm.org.

Combinations of CD antigens (CD3, CD4, CD8, CD14, CD16/56, CD19, and CD45) for peripheral blood lymphocyte characterization are well known for years and described with all details in many flow cytometry handbooks and

protocols (1). However, as the list of the CD markers is growing, well known antigens such as P-glycoprotein, MDR1, and ABCB1 are being renamed (e.g. to CD243). P-glycoprotein functions as an efflux pump and is expressed in normal tissues such as gut mucosa, endometrium or bile canaliculi. In the pathological tissue, the P-glycoprotein has a major impact on multidrug resistance, particularly of tumors with a broad substrate spectrum (2). P-glycoprotein expression in tumor tissue and prognostic or predictive roles for cancer treatment are intensively studied. In the past, we have demonstrated its important role in the development of tumor resistance in cancer cell lines (3). If the cellular population is positive to the P-glycoprotein expression, the functional characterization of the P-glycoprotein is strongly recommended. Method for P-glycoprotein functional evaluation is shown in the dedicated chapter. In this chapter, we will demonstrate a generally applicable method of cellular antigens labeling and quantification by flow cytometry on the example of CD243 (P-glycoprotein) (3).

Expression of the proteins can be measured by a plethora of proteomic methods like Western blot, immunofluorescent microscopy, ELISA,

and mass spectrometry, but flow-cytometry remains as one of the most frequently used cellular biology techniques. The big advantage of the flow cytometry is the single cell analysis of the population of thousands of cells in a relatively short time and possibility to combine different antibodies and markers to select and study population of our interest. The disadvantage of the method is the inability to see the microscopic image of the cells and to distinguish cellular localization of the signal. To eliminate the bias introduced to our measurement by non-specific antibody binding, the parallel use of isotype antibody control is strongly recommended.

II. Materials and Instrumentation Materials

• Rinsing buffer (RB)

Dissolve 0.1 g of BSA, fraction V (A5611, Sigma-Aldrich) and 20 µl of Nonidet P-40 (21-3277, Sigma-Aldrich) in 20 ml of Cell Wash (349524, Becton-Dickinson). Store at room temperature for 24 hours.

• Solution for dilution of blocking buffer

Dissolve 0.5 g of BSA, fraction V (A5611, Sigma-Aldrich) in 100 ml of Cell Wash (349524, Becton-Dickinson). Store 2ml aliquots at -20 °C. Before using the aliquots must be tempered to room temperature.

• Permeabilizing solution

Dilute 10x FACS Permeabilizing solution 2 (340973, Becton-Dickinson) by deionized water (ninth-tenth of the final volume). Store at 4 °C and discard after 24 hours.

• Fc Receptor saturation solution

Mix carefully 10 µl of Fc Receptor saturation reagent (5200-01, Southern Biotech) in 30 µl of Solution for dilution of blocking buffer. The amount is sufficient for blocking of one sample. Store at 4 °C.

• Working solution of P-glycoprotein primary antibody

Mix 4 µl of monoclonal Anti-P-glycoprotein antibody (Sigma-Aldrich, P7965) with 1ml of RB. Store at 4 °C.

• Working solution of isotype control

Mix 7.1 µl of isotype control (IgG1 Isotype Control, unconjugated, Sigma-Aldrich, M5284) with 1ml of RB. Store at 4 °C.

• Working solution of secondary antibody

Mix 4 µl of secondary antibody (Anti-mouse IgG Fab2 fragment – FITC antibody Sigma-Aldrich, F2883) with 1ml of RB. Store at 4 °C.

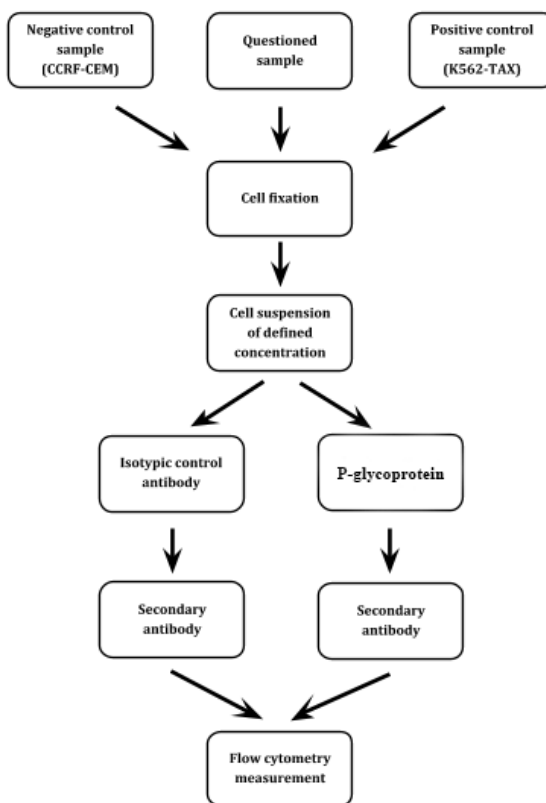
• General chemicals and material

Methanol (100837, Supelco), Falcon tubes 12x75 mm (Becton-Dickinson).

Instruments¹

- BD FACS Calibur (BD Biosciences)
- Centrifuge (5810R, Eppendorf)
- Vortex Genie 2 (Scientific Industries, SI-0236)

III. Procedures



Scheme 28.1.

FACS analysis of P-glycoprotein expression

FACS analysis of P-glycoprotein expression

1. Harvest cells, to include detached cells also collect the cell culture media.
2. Centrifuge cells (5 min, 250 g, 4 °C) and remove supernatant.
3. Resuspend cells in Cell Wash solution.
4. Centrifuge cells (5 min, 250 g, 4 °C) and remove supernatant.
5. Gently mix the cells by pipette or vortex to disintegrate clumps and add ice cold (-20 °C) methanol dropwise. At that point you can store the cells in the freezer for weeks.
6. Centrifuge cells (5 min, 500 g, 4 °C) and remove supernatant.
7. Add 2 ml of Cell wash solution.
8. Count cells and take
 - a) $5 \cdot 10^5$ cells per one sample for P-glycoprotein antibody labeling .
 - b) $5 \cdot 10^5$ cells per one sample for isotype control. Label tubes accordingly.
9. Centrifuge cells (5 min, 500 g, 4 °C) and remove supernatant.
10. Add 0.5 ml of Permeabilizing solution, centrifuge cells (5 min, 500 g, 4 °C) and remove supernatant.
11. Add 2 ml of RB, centrifuge cells (5 min, 500 g, 4 °C) and remove supernatant.
12. Add 40 µl of Fc Receptor saturation solution, incubate for 10 minutes.
13. Wash cells twice by 2 ml of RB at the same centrifugation settings.
14. Add 40 µl of the Working solution of P-glycoprotein primary antibody and incubate in the dark for 30 minutes at room temperature. (In the case of isotype control use 40 µl of the Working solution of isotype control.)
15. Wash cells twice by 2 ml of RB at the same centrifugation settings.
16. Add 40 µl of the Working solution of the secondary antibody and incubate in the dark for 30 minutes at room temperature.
17. Wash cells twice by 2 ml of RB at the same centrifugation settings.
18. Remove supernatant and resuspend pellet in 500 µl of RB and store sample in the dark at room temperature.
19. Acquire the stained cells by flow cytometry on the same day.
20. Use the Isotype control sample to define the measurement settings, particularly the settings of sample intensity. The recommended intensity value for the Isotype control is to set at median 10 RFU (Figure 28.1) as is expected for a negative sample.
21. Analyze in flow cytometry and compare the intensity (relative fluorescence units - RFU) of the sample fluorescence labeled by isotype control (Isotype antibody) and P-glycoprotein antibody.

IV. Comments

- The mentioned manufacturers of the listed instruments were used during the protocol testing or development.
- Induction of cell death using various agents may lead to detachment of adherent cells. To analyze the whole cell culture population, collecting the cell culture media containing floating cells is necessary.
- The goal is to stain an identical amount of cells in the same final volume for each sample.
- To set isotype control on the same level for all samples is the important prerequisite for a successful comparison. The isotype control can set the same level of the background as it can differ in between the different cell lines or after the treatment by different conditions.
- Make sure to include proper positive and negative controls (e.g. K562-TAX or CCRF-CEM)

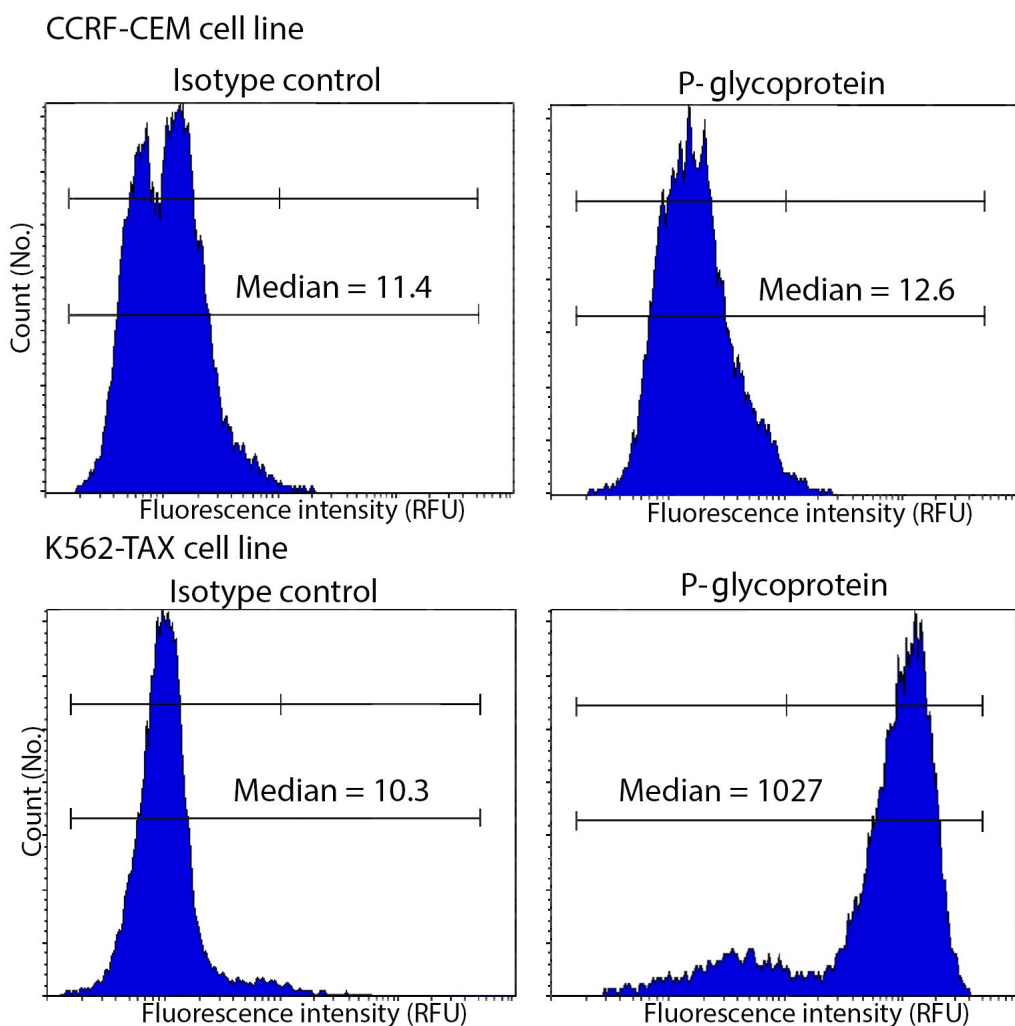


Figure 28.1. P-glycoprotein analysis

Example of P-glycoprotein analysis in cancer cell lines: CCRF-CEM acute lymphoblastic leukemia cell line without expression of the P-glycoprotein; K562-TAX myeloid paclitaxel-resistant cell line with high P-glycoprotein expression. Isotype control is used for the uniform settings of the instrument for comparative measurements and is set as close as possible to agreed values. In our case the median of fluorescence = 10 RFU.

V. Pitfalls

Problem	Cause and cure
Antibody	
Low signal of positive population	• A decomposed antibody, test on a reference sample. A decomposed fluorochrome, ie PE last maximum 6 months, FITC is a little more stable, ALEXA is far more stable. • Protect antibody to light and store in proper conditions. • Make sure to include positive and negative controls (e.g., K562-TAX, CCRF-CEM).
Processing	
False negativity of the signal	• Part of the cells were not in contact with the antibody. Avoid drying of the cells on the wall of the tubes.
Low amount of the cells on the end of the protocol	• Cells were aspirated during the preparation. Check the cells under the fluorescence microscopy and count and dilute them again. • Aspirate supernatant with caution when washing the cells, leaving at least fifty microliters of the sample at the bottom.

VI. Acknowledgment

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Analysis of mitochondria function by flow cytometry

Juan B. De Sanctis

Introduction

Mitochondria function has been widely used to screen metabolic activity, differentiation, senescence, and different types of cell death (1-6). Since mitochondrial function is represented by ATP production, two key elements have been defined. One is related to oxidative phosphorylation and proton pump and the other mitochondrial role in cell senescence and death. The proton pump efficiency is defined as $\Delta\psi$ and is a combination of both the mitochondrial membrane potential ($\Delta\psi$, a charge or electrical gradient) and the mitochondrial pH gradient (ΔpH , an H^+ chemical or concentration gradient). In order to avoid the effect of other organelles on mitochondria function, several researchers prefer to use purified mitochondria, which can also be screened by flow cytometry. There are several methods to purify mitochondria and also several commercial kits (7). However, yield and quality of mitochondria may differ among the methods used (7).

Several fluorochromes have been designed to monitor the membrane potential, radical formation, pH gradients, and mitochondria

death. However, not all probes that have been available in the market can be used for measuring mitochondrial function and cell death (8-10). The most useful probes are: 1) TMRM (tetramethylrhodamine methyl ester), or TMRE (tetramethylrhodamine ethyl ester). They are often used in quenching mode ($2\div 10\ \mu\text{M}$), to monitor acute changes in $\Delta\psi$ after dye loading and washout. 2) Rhodamine 123 ($5\div 10\ \mu\text{M}$) is used for assessment of electrical gradient and radical production with similar fashion as the dye above; it is not very useful in cell death. 3) JC-1 (tetraethylbenzimidazolylcarbocyanine iodide), usually $20\ \mu\text{M}$, and JC-9 (3,3'-dimethyl- α -naphthoxacarbocyanine iodide) $5\text{-}10\ \mu\text{g}$, have been used effectively for monitoring burst and cell death but as membrane potential monitors have not been beneficial due to aggregate formation unless vancomycin is used. 4) DiOC6 (3,3'-dihexyloxacarbocyanine iodide) has been widely used for flow cytometry, and it is similar to JC-1 and JC-9, with higher concentration $200\ \mu\text{M}$, yet depending on the type of experiments significant leak can be observed along with precipitation. Most of the fluorochromes can also be used in confocal or fluorescence microscope.

Several companies provide kits for assessing mitochondrial function by flow cytometry or fluorescence microscopy. The kits are based upon different fluorochromes, and consequently, the choice of probe depends on what do you want to assess.

If you are working with polymorphonuclear cells, or any cell that may produce a large number of radicals, you have to take into account peroxide production generated by the holoenzyme NADH oxidase. Moreover, if you are working in the mouse or rat model with these cells, the production of nitric oxide radical and eventually peroxynitrate formation has to be taken into account (11-13). All those radicals may interfere with the assay and give high background. Rhodamine 123 is highly reactive to both radicals as well as the TMR probes (8). One way to avoid the generation of those radicals is to use calcium chelating agents (11-12). It is important to note that Calcium uptake by mitochondria can be significant for mitochondrial function and death (14).

Thus, depending on the aim of the study, the right protocol and fluorochrome should be used in order to avoid problems in the interpretation of the results.

II. Materials and instrumentation

Instrumentation any flow cytometer with 3 PMT or three colours if you are using JC-1, JC-9 or DiOC6 and 2 if you are only recording one colour like TMRM or 123 Rhodamine.

Cell types

Adherent or not adherent cells or tissue can be used. The critical issue is to have the cells on the solution at 2 million cells/ml for labelling. Depending on the flow cytometer, the sample can be in higher or lower volume.

Chemicals

- TMRM can be purchased as a kit MitoProbe™ TMRM Kit for Flow Cytometry from Thermo Fisher, M20036, or only the fluorochrome from the same

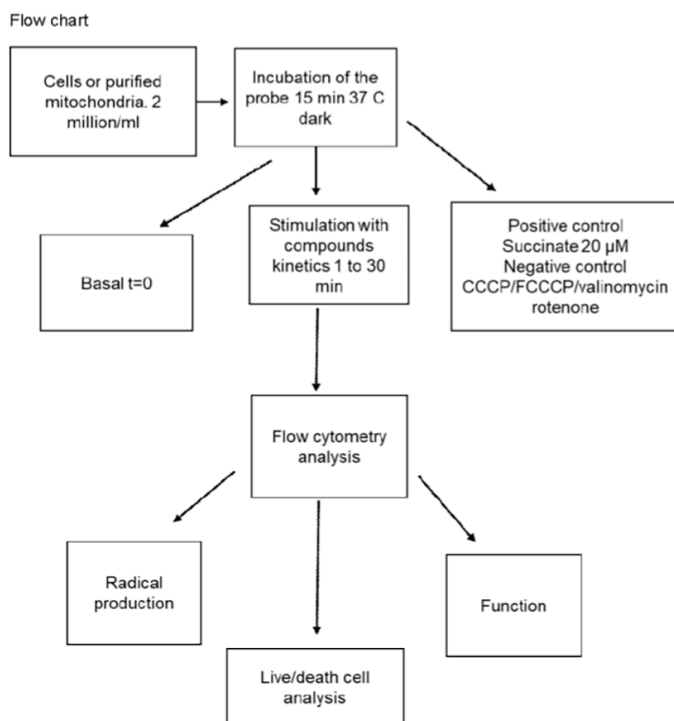
company T668. The kit contains the probe in solution as well as the inhibitor for mitochondria activity CCCP (Carbonyl cyanide 3-chlorophenylhydrazone). A stock solution of the fluorochrome 20 μ M in DMSO is usually used for 30 days. The amount of fluorochrome usually used is 1 μ l of the stock solution per 2 million cells or the equivalent number of purified mitochondria. For the inhibition of mitochondrial oxidative phosphorylation 1 μ l of the stock solution, 50 mM CCCP can be used per 2 million cells.

- Rhodamine 123 (Sigma-Aldrich, R8004; Thermo Fisher, R302). The fluorochrome can be dissolved in ethanol or DMSO at a concentration of 50 μ M for the stock solution. Use 1 μ l of the stock solution per 2 million cells or the equivalent number of purified mitochondria.
- JC-1 (Thermo MitoProbe™ JC-1 Assay Kit, M34152 or T3168 for the dye alone; Sigma-Aldrich, T4069; Cayman Chemical, 701560 JC-1 Mitochondrial Membrane Potential Flow Cytometry Assay Kit). A stock solution of 200 μ M in DMSO is prepared, and 2 million cells can be labelled with 1 μ l of the stock solution.
- JC-9 (3,3'-dimethyl- α -naphthoxacarbocyanine iodide), (Thermo Fisher, D22421). Usually, a 5 mM stock solution in DMSO is used, depending on the cell type (amount of mitochondria per cell) concentrations range between 1 to 10 μ g/ml of fluorochrome for 2 million cells.
- DiIOC6 (Thermo Fisher, D273; Enzo biosciences, ENZ-52303). A 0.1 mM stock solution in DMSO is prepared, and usually, 1 μ l can be used to label 2 million cells. Since this fluorochrome may leak from mitochondria, after the kinetics is performed, it is suggested to fix the cells with paraformaldehyde 2 mM for 10 min at 4 degrees and then washed before flow cytometry analysis.
- Succinate (Sigma-Aldrich, 224731). Prepare a fresh solution of succinate 20 mM in PBS before the experiment and keep it at 4 °C. Use 10 μ l of the solution as a positive induction of mitochondrial respiration (complex III).

- Oligomycin (Calbiochem, 495455) (inhibit mitochondrial ATP formation). The oligomycin can be used alone or as a mixture to inhibit completely oxidative phosphorylation. Usually, a 50 μM oligomycin is dissolved in DMSO, and 1 μl is used to inhibit oxidative phosphorylation of 2 million cells. Alternatively, for complete inhibition, cells can be treated with a final concentration of 5 μM oligomycin 1.5 μM FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), Sigma Aldrich cat # C2920 and 1 μM rotenone + 100 nM antimycin A as final concentration (Sigma Aldrich catalogue number 83-79-4 and 1397-94-0, respectively). The total inhibition of oxidative phosphorylation is often referred to as a mitochondrial stress test.
- Sodium azide (Sigma Aldrich, S2002), inhibit partially mitochondrial ATP formation. Sodium azide 20 mM can be prepared in PBS and kept in the cold 4 $^{\circ}\text{C}$. Usually 2 mM is enough to inhibit partially membrane potential. Sodium azide can be used along with FCCP 2 μM or valinomycin 1 μM final concentrations.
- CCCP Carbonyl cyanide 3-chlorophenylhydrazone (Sigma-Aldrich, C2759), mitochondrial membrane potential disrupter but also interferes mitophagy. Usually, a 100 μM solution in DMSO is prepared and 10 μM final concentration is usually used to inhibit the mitochondrial function of 2 million cells.
- Valinomycin (Sigma-Aldrich, V0627), used to depolarise mitochondria at concentrations ranging 1 μM . Moreover, valinomycin avoids JC-1, JC-9 and DiOC6 aggregation. At higher concentrations, valinomycin can be used to induce cell apoptosis and membrane depolarization.

Solutions

10 $\times$ PBS buffer (phosphate buffered saline; stock solution): Prepare the following solution in autoclaved distilled water: 1.37 M NaCl (Sigma Aldrich, 31434), 27 mM KCl (Sigma Aldrich, 60130), 100 mM Na_2HPO_4 (Sigma Aldrich, S5136), 18 mM KH_2PO_4 (Sigma Aldrich, P5655). The, adjust the pH to 7.4, filter it, autoclave it and



Scheme 29.1.

store at 4 °C. Use 1× PBS as a working solution.

PBS gel (15). If you are working with B lymphocytes, macrophages, neutrophils or eosinophils for adherent cells so it will not obstruct the tubing of the flow cytometer. PBS with 0.1 % gelatine, Sigma-Aldrich, 48723), 2 mM EDTA (Sigma-Aldrich, E6758), 5 mM glucose (Sigma-Aldrich, G8270).

Purification of mitochondria. Purification can be performed using commercial kits. However, the efficiency of mitochondria purification depends on the cell or tissue type.

III. Procedures

1. Cell lines

The cell lines should be analysed before confluence; the cells should not be overgrown. If the cell lines are adherent, remove non-adherent cells by washing the flask with warm PBS. Then remove the cells either by standard trypsin or else by cold PBS gel using a rubber policeman. Cells are then washed in PBS and resuspended at a concentration of 2 million cells/ml. Remember that the cells always have to be in suspension and do not aggregate since it may obstruct the lines or the flow cell of the flow cytometer.

2. Mitochondria isolation protocol

Usually, mitochondria procedures involve several centrifugation steps and specialized buffers in order to maintain not only mitochondrial viability but function. Several commercial kits are available that facilitates mitochondrial separation and usually are straightforward and fast protocols. One of the most recommended kits in the literature is Quiagen Qproteome mitochondria isolation kit Cat No./ID: 37612. However, others like mitochondria Isolation Kit for Cultured Cells (Thermo Fisher, 89874) and the MITOISO2-1KT from Sigma-Aldrich are also recommended. For specific tissues, you may want to use sucrose gradients as described by Clayton and Shadel (16). Mitochondria need to be fresh to be analysed by flow cytometry.

3. Labelling and kinetics

Labelling of the mitochondria using cells in suspension or isolated mitochondria is as follows. Use 2 million cells/ml in PBS and incubate at 37°C for 15 minutes, in the dark, to incorporate more than 95 % of the fluorochrome. There are several probes depending on your needs: 1) radical production: TMRM 1 µl of stock solution (20 µM), Rhodamine 123 1 µl of stock solution (50 µM), 2) mitochondrial function and viability JC-1 1 µl of stock solution (200 µM), JC-9 5 µl of stock solution (5 mM), DiOC61 µl of stock solution (0.1 mM). After the incubation, the kinetics is started with the different compounds to be tested the incubation is in a water bath at 37 °C and the reaction is stopped at 4 °C. A time 0 tube, after the 15 min incubation, is used as a basal assessment. Then the rest of the treatments at different time are assessed. It is recommended to analyse the samples within 30 minutes after the assay to avoid probe leakage.

Negative controls: cells incubated with final concentration of oligomycin 5 µM, FCCP 2 µM, CCCP 10 µM, valinomycin 1 µM, sodium azide 2 mM or a combination of oligomycin, FCCP, 1 µM rotenone + 100 nM antimycin A or valinomycin plus CCP or FCCP should completely depolarise the mitochondrial membrane.

Positive controls: cell are incubated with succinate 2 mM final concentration. Succinate repolarises mitochondrial membrane since it is an inductor of complex III of the oxidative phosphorylation and consequently more ATP is formed.

In order to assess either apoptosis or autophagy, the kinetics can be prolonged depending on the inductor. Otherwise, the cells can be treated, and afterwards labelled to record the possible effect on cell death. Mitophagy can be recorded as early as 1 hr and could be inhibited by CCCP or FCCP. As stated before, for apoptotic or autophagy assessment, the choice of fluorochrome is crucial, JC-1 and JC-9 are recommended.

4. Flow cytometry analysis

Adjust the cytometer depending on the sample, adjust for cell size and granularity you can purchase beads to verify the size of your sample.

Thermo Fisher, Sigma Aldrich, Biolegend have several particle sizes and colour depending on the cell type and fluorochrome used. Moreover, the same companies sell particles which can be used to quantify fluorescence if required. Once the setting for the cells is performed, it is useful to verify cell viability using propidium iodide 5 μ l of a solution of 1 mg/ml (Thermo Fisher, P3566) and incubate for at least 5 minutes in the dark. Dead cells should be discarded from the analysis, and an electronic map with viable cells should be used to record fluorescence intensity.

The staining protocol is simple and straightforward but dependent upon the fluorochrome used. Most of the fluorochromes label mitochondria after a maximum of 15-minute incubation at 37 °C. One key issue is the use of the inhibitors for membrane depolarisation as negative controls and the use of succinate for induction of ATP production, positive control. Recall that if you are testing compounds that induce apoptosis, membrane depolarisation may be very fast depending on the lipophilicity of the compound tested. In some cases, tested compounds can be either quench or compete for the fluorochrome. In order to assess this possibility, it is recommended to use another probe and redo the experiments.

For JC-1, JC-9 and DiI OC6 recall that you have to use two PMT and should compensate both colours with around 10 % (please see figure for recommended analysis).

5. Flow cytometry protocol for isolated mitochondria

Essentially, the protocol of Daniele *et al.* (17) is useful for several approaches. However, it is highly recommended, as they describe in their

latest publication (18) to purchase, beads 3000 Series Nanosphere™ Size Standards from Thermo Fisher, to do the singlet analysis and to identify different mitochondria sizes and avoid noise in the analysis. The isolated mitochondria would be similar in size to most bacteria, and consequently, the setting should be set to quantify particles size 200 nm. For this purpose, the forward and side scatter should be set at 10 (log 1). Adjust the voltage and gain with nanobeads, Thermo Fisher Nanosphere™ Size 200 nm cat 3200A, to have a concentric electronic map, with low variation coefficient (< 2%). Eventually, you can purchase, upon request to the manufacturer, FITC labelled nanobeads. Run a sample of unlabelled mitochondria to assess background fluorescence, and verify possible subpopulations. Elaborate a maximum of 3 maps from 200 nm 400 nm, i.e. a maximum of twice the size of the first map. Mitochondria size is dependent upon the sample used. New biogenic mitochondria are smaller than old mitochondria (17). Label mitochondria as described before with any of the fluorochromes and do the analysis. Adjust fluorescence intensity at log 10, use gain set to avoid background noise. Then, start the kinetics of the analysis.

The positive control Adjust the intensity of the FITC fluorescence to log 1 and measure the variance of the peak (heterogeneity of the beads).

It is recommended to use several protocols for assessing mitochondria function due to the fact several mitochondrial functions can be targeted by treatment: oxidative phosphorylation with $\Delta\psi_m$ changes, inhibition of biochemical pathways, Krebs cycle, beta-oxidation, transaminase activity and radical production or inhibition of radical production (Superoxide dismutase).

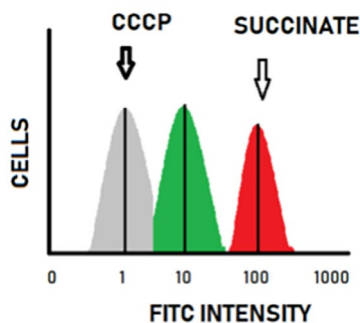


Figure 29.1. Analysis of single colours, probe Rhodamine 123 or TMRM

IV. Comments

Mitochondrial metabolism is essential to assess cell function, senescence and death. Using the right probe, you can be able to identify modulation of mitochondria function. Mitochondria from any species can be analysed either as part of a cell or else purified. Flow cytometry allows the possibility to increase the analysis of mitochondria function, and it is a useful tool in pharmacology, toxicology, immunology and biochemistry. In a short period, you can be able to do several kinetic experiments and analyse the effect on size, morphology and death directly.

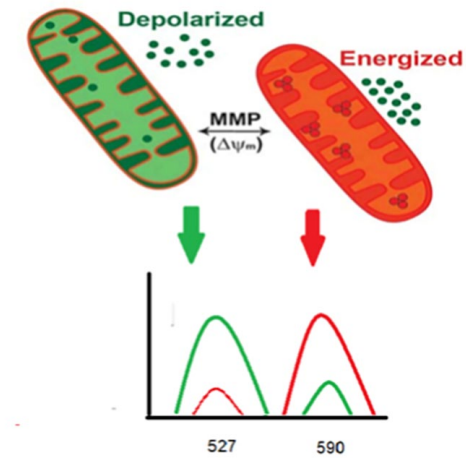


Figure 29.2. The mean channel of fluorescence intensity is modified by the treatment. Positive and negative controls.

V. Pitfalls

Problem	Cause and cure
Lack of fluorescence	• Cell death or mitochondrial death. • Fluorochrome did not penetrate the organelle. Either degraded or not optimal concentration.
Too much fluorescence	• Adjust the PMT voltage. There may be too much fluorochrome. • Check the pH of the solution running the sample.
Too low ratio for JC-1/JC-9	• Check for dye precipitation on a fluorescence microscope. • Check the colour of the stock solution; if it is red, it is degraded.
Cell morphology changes in the electronic map	• Induction of apoptosis or necrosis. Check osmolarity of the solutions. Label with propidium iodide.
A negative control is not working	• The compound may be degraded. Check PMT and background fluorescence.
A positive control is not working	• The compound may be degraded. Check PMT and background fluorescence.
Cell or mitochondria leaking	• Fix cells or mitochondria with 2 % paraformaldehyde.

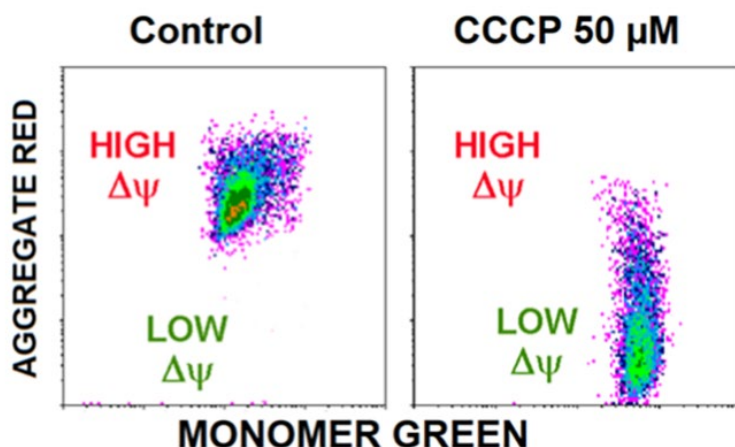


Figure 29.3. Analysis of JC-1 on mitochondrial function.

VI. Acknowledgment

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Functional test of MDR transporters

Lenka Hrubá

I. Introduction

Cancer is a globally widespread group of diseases that, despite comprehensive treatment options, ends in the death of a patient in many cases. Statistics show that worldwide there were 18.1 million reported new cases of cancer in 2018 (1) and resulted in 9.6 million deaths in the same year (2).

One of the reasons for treatment failure is the ability of cancer cells ability to resist the action of cytostatic agents (3). This resistance in cells can occur at the first administration of the drug (primary resistance) (4, 5) or during treatment (secondary resistance) (4). Moreover, resistance can arise not only due to the currently administered substance but also for the other drug with a similar chemical structure (cross-resistance) or a completely different structure and function (multidrug resistance, MDR) (5).

Fundamental problem in the cancer cell resistance is the intra-tumour heterogeneity (cellular differences within a single neoplasm), which can be caused by a large number of proliferation cycles and by increasing mutation rates. The result is the emergence of tumour

subpopulations that may be variously sensitive to treatment (6).

Multidrug resistance proteins

Mechanisms of MDR due to altered expression of MDR proteins have been extensively described (8- 10). The best known MDR protein is P-glycoprotein (Pgp), which is an ATP-dependent efflux pump. Its physiological function is to protect the organism against toxic agents, but its overexpression makes cancer cells more resistant to drug therapy (11- 13). Another protein connected with MDR is a member of the multidrug resistant protein (MRP) protein family, which is also a group of ATP-dependent membrane transporters (7). Since 1992 eight members of MRP family have been described (15- 19). Two of the MRP family members, MRP1 and MRP2, are studied particularly in relation to anti-cancer drugs (7).

Another protein connected with MDR phenotype is lung resistance-related protein (LRP), which is the major vault protein (MVP). MVP is one of the three components (MVP,

TEP1, VPARP) of vaults that are connected with many physiological functions of cells, especially nucleus-cytoplasm transport (8). LRP is expressed in tissues such as peripheral lung cells and bronchial epithelial cells (9), and overexpressed in many types of multidrug-resistant cancer cell lines (22, 23).

The breast cancer resistance protein (BCRP) is another MDR-associated protein. It was first described in MCF-7/AdrVp human breast carcinoma cells in 1998, and interestingly these cells didn't have altered expression of MDR1 or Pgp (10). BCRP is not only overexpressed in human breast carcinoma cells but also in colon carcinoma, myeloma, gastric carcinoma and fibrosarcoma cells (11). Stem cells frequently BCRP is highly expressed in stem cells (12). BCRP is also expressed in normal tissues, especially in tissues of organs associated with distribution and elimination of drugs or toxic xenobiotics. High expression of BCRP is usually detected in liver, kidney, small intestine and blood-brain and placental barriers, where it works as an efflux pump (24, 26, 27).

Resistant cancer cell lines as a research model

One of the research targets of Institute of Molecular and Translational Medicine is selection and characterisation cell lines that are resistant to various drugs. In 2002, Noskova et al. published results of *in vitro* analyses of chemoresistance profile of resistance cell lines derived from CCRF-CEM, K562, A549 and MDA MB 231. Expression of MDR proteins was also tested for all lines (resistant and parental).

Combine with the previous paragraph was found that cell line K562-TAX (cell line derived from K562 with resistance to taxol) has high expression levels of Pgp and low expression of MRP1 and LRP. On the other hand, cell line A549-VCR (cell line derived from A549 with resistance to vincristine) expresses high levels of MRP1 and low levels of Pgp and LRP. Therefore, K562-TAX and A549-VCR cell lines were ideal models for testing the activity of MRP1 and Pgp.

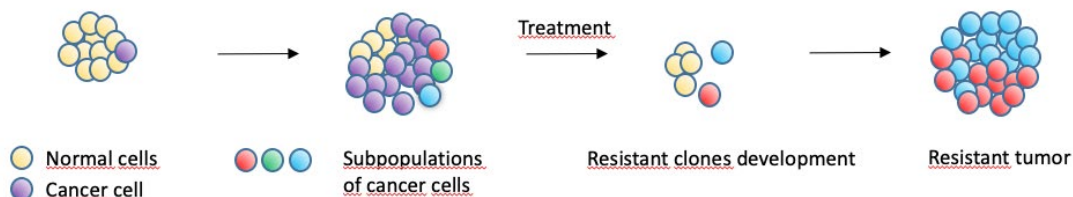


Figure 30.1 Intra-tumour heterogeneity

II. Materials and instrumentation

Instrumentation

Multidrop combi (Thermo Scientific), Echo 550 liquid handler (Labcyte), Centrifuge 5810 R (Eppendorf), HERAcell 150i CO₂ incubator (Trigon-Plus), EnVision Plate Reader (Perkin-Elmer), FACSCalibur (Becton Dickinson), Scalted analytical scale, Typhoon FLA 9000, Titramax 100 (Heidolph).

Reagents

Daunorubicine (Cat. No. 23541-50-6), cyclosporine A (Cat. No. 30024-25MG), probenecid (Cat. No. P8761-25G), Triton X-100 (Cat. No. 93443), Nonidet P-40 (Cat. No. 74385-L), sodium deoxycholate (Cat. No. 30970-1006), KCl (Cat. No. P9541-500g), antibiotics (penicillin-streptomycin), fetal bovine serum (Cat. No. FBS-12A), DMEM (Cat. No. D6429, Sigma Aldrich), NaOH (Cat. No. 1310-73-2) from Microchem,

TrisCl (Cat. No. 3718005) from Serva, RPMI cell culture medium (BE12-115F) from Lonza, phenol red-free cell culture medium [Cat. No. 11835063 (RPMI); Cat. No.21041025 (DMEM)] from ThermoFisher Scientific, 1× PBS.

Material

CellCarrier 384 TC (black, clear bottom) from Perkin Elmer, six-well tissue culture plate from TPP.

Cell cultures

Cell line K562-TAX cultivated in RPMI medium (supplemented with 10% FCS, 1× penicillin-streptomycin), MDCK1-MDR1 grown in DMEM medium (supplemented with 10 % FCS, and 1× penicillin-streptomycin). A549-VCR cells were cultivated in same medium as MDCK1-MDR1. All cell lines were maintained at 37 °C and 5 % CO₂.

A549-VCR cell line was selected by growing cells in medium supplement with 10× IC₅₀ concentration of vincristine. Only the selected cells were used for the experiment.

Lysis buffers

- Lysis buffer A: 0.2 % Triton X-100/0.01 M NaOH
- Lysis buffer B: 1 % sodium deoxycholate, 40 mM KCl, 20 mM TrisCl
- Lysis buffer C: 1 % Nonidet P-40, 1 % sodium deoxycholate, 40 mM KCl, 20 mM Tris

III. Procedures

Detection of MDR transporter activity by flow cytometry

Steps:

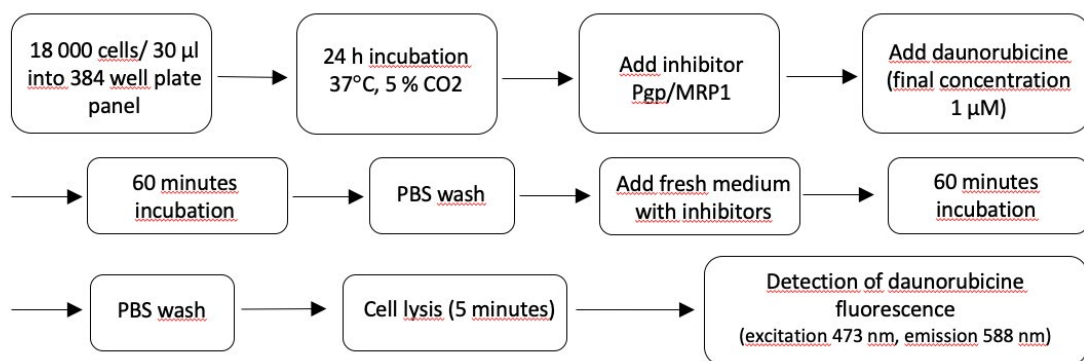
1. Seed 500 000 cells in 2 ml of medium/per well into 6 well plate
2. Incubate overnight at 37 °C, 5 % CO₂
3. Add inhibitors of MDR transporter at concentrations provided below:
 - Cyclosporine A: 0.39 µM, 1.56 µM, 6.25 µM, 25 µM, 100 µM
 - Probenecid: 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM

- One well is used as control (no inhibitor)
4. Add daunorubicine at a concentration of 1 µM (20 µl from the stock 100 µM solution).
 5. Incubate for 60 minutes in the dark at 37 °C, 5 % CO₂
 6. Remove media, wash cells twice with 1 ml fresh media
 7. Add 2 ml of fresh media containing cyclosporine or probenecid.
 8. Incubate for 60 minutes in the dark, 37 °C, 5 % CO₂
 9. Analyze cells using a flow cytometer

An optimised method for screening of potential inhibitors of MDR transporters

Steps

1. Seed 18 000 cells into 30 µl of medium per well in a 384 well plate (black, clear bottom) by multidrop, and centrifuge for 1 minute (1 100 rpm, 21 °C)
2. Incubate cells overnight at 37 °C, 5 % CO₂
3. Add inhibitors of MDR transporters using ECHO at following concentrations:
 - Cyclosporine A: 0.83 µM, 1.66 µM, 6.25 µM, 12.5 µM, 25 µM, 50 µM, 100 µM, 166 µM
 - Probenecid: 0.125 mM, 0.25 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM, 3 mM
4. Add 1 µM daunorubicine using ECHO, and centrifuge the plate (1 100 rpm, 21 °C)
5. Incubate for 60 minutes in dark, 37 °C, 5 % CO₂
6. Remove the drug-containing culture media by turning the plate upside down, and wash cells twice with 1× PBS (use a Multidrop to add the PBS)
7. Add 30 µl of fresh medium containing MDR inhibitors and centrifuge for 1 minute (1 100 rpm, 21 °C)
8. Incubate 60 minutes incubation in dark at 37 °C, 5 % CO₂
9. Remove the culture media, and wash cell as described in Step 6
10. Add 10 µl lysis buffer (1% Nonidet P-40, 1% sodium deoxycholate, 40 mM KCl, 20 mM TRIS), centrifuge for 1 minute (1 100 rpm, 21 °C)
11. Incubate for 5 minutes in dark on Titramax 100
12. Measure daunorubicine fluorescence using a Typhoon FLA 9000



Scheme 30.1. Functional test MDR transporters

IV. Comments

There are two ways to test the activity of MDR transporters – the first using a 6-well plate and the other in a miniature format using a 384-well plate.

Flow cytometry method (6 well plates)

The advantage of this method is the ease of sample preparation and an excellent signal-to-background ratio. The fluorescence of daunorubicin is measured in living cells.

The disadvantage of this method includes the need for a significant volume of the reactant (20 µl of inhibitors/ 20 µl of daunorubicine). This can be an issue when compounds are scarce. Moreover, the longer analysis time (analysis of 1 compound at 5 concentrations takes 3 hours) can be cumbersome. It is possible to analyze only 10 compounds per day by this method, which may not be practical when dealing with a large compound set.

Screening method (384-well plates)

For drug screening purposes, the miniaturization of the flow cytometry of this method was needed. We optimised reaction volume and changed manual steps to automated steps. We

miniaturized the assay from 6-well plates to 384-well plates, with the possibility of analysis of more than 10 compounds at different concentrations at a given time. We needed to optimize the method for quantification of daunorubicine fluorescence – new reaction volume was too small for flow cytometry. The easier solution was to scan the fluorescence in the entire panel. In this way, we can detect fluorescence of all 384 wells in a few seconds.

To measure the fluorescence evenly throughout the well, it was necessary to lyse the cells. We tested three different types of lysis buffers (the composition of the solutions is given under the section Material

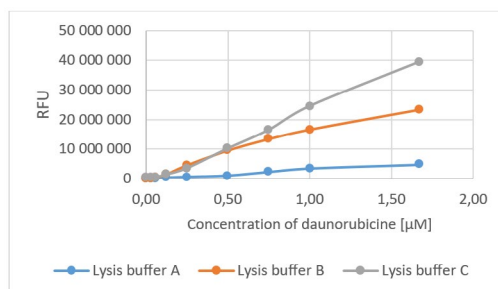


Figure 30.2 Calibration curve of daunorubicine in lysis buffer A, B, C (transferred by Echo). Fluorescence was detected using a Typhoon FLA 9000.

and Instrumentation, different volumes of lysis buffers and optimal time for cell lysis. Fig. 30.2 shows the fluorescence signal of daunorubicine depending on its concentration in 3 lysis buffer. Lysis buffer A (0.2 % Triton X-100/ 0.01 M NaOH) enhanced the fluorescence. Lysis buffer C seemed to be the best choice for our screening method. The optimal volume of lysis buffer was 10 μ l and optimal lysis time was 5 minutes. Higher volume of lysis buffer and a longer time of cell lysis reduced the fluorescent signal of daunorubicine.

We used phenol red-free cell culture medium to reduce the background signal and prevent the interference of phenol red with daunorubicine fluorescence (Fig. 30.3).

Daunorubicin is transported out of the cell by P-gp. Therefore, we wanted to determine whether inhibition of Pgp by cyclosporine A alters the transportation of daunorubicine in K562-TAX. In the final step of experiment, cells were lysed to release the cellular content and the

fluorescence in the wells determined using an EnVision Plate Reader, Typhoon FLA 9000 and ViewLux™ ultra HTS Microplate Imager.

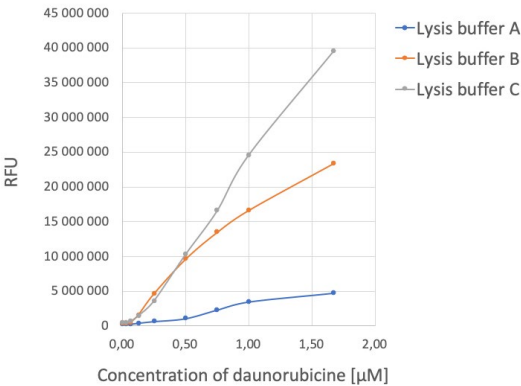


Figure 30.3 Fluorescence of RPMI and DMEM medium with and without phenol red measured by Envision (excitation wavelength: 473, emission: 588 nm) in 384 well plates (30 μ l per well).

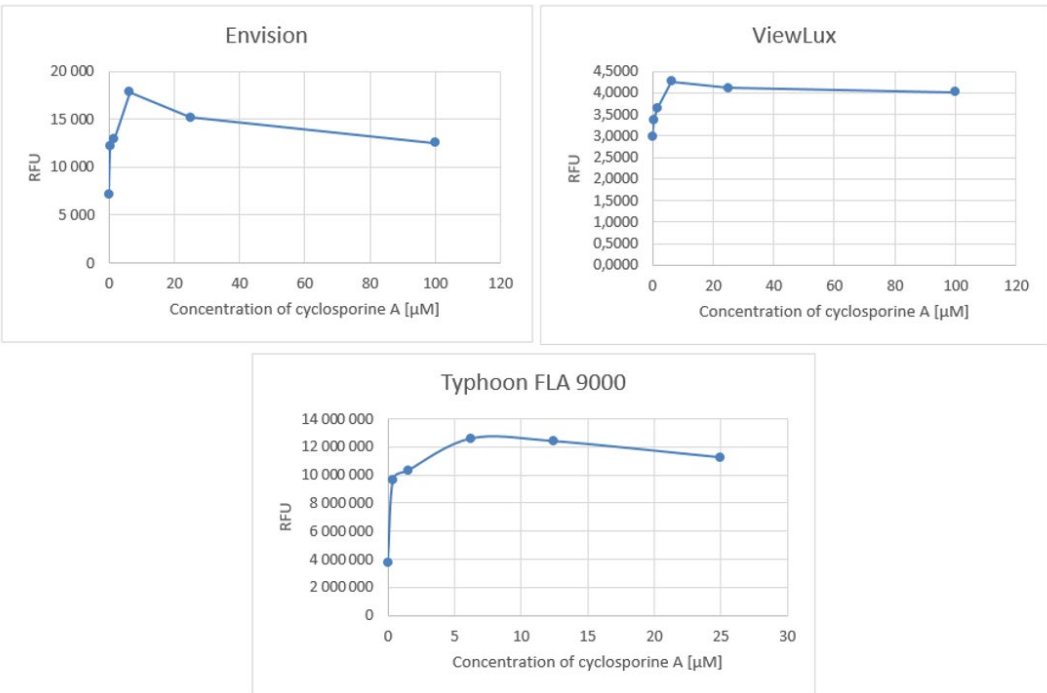


Figure 30.4 Figure 30.4. Analysis of Pgp inhibition in K562-TAX using cyclosporine A. Detection of daunorubicine fluorescence in cell lysate with lysis buffer C (1% Nonidet P-40, 1% sodium deoxycholate, 40 mM KCl, 20 mM Tris) using an EnVision Plate Reader, Typhoon FLA 9000 and ViewLux™ ultra HTS Microplate Imager.

V. Pitfalls

Problem	Cause and cure
Too high fluorescence in blank samples	• Cultivation media contains phenol red and its fluorescence interferes with the fluorescence of daunorubicine. • To decrease fluorescence of the background use phenol red-free media and pay attention to washing steps
Uneven fluorescence in the well	• The non-uniformity of the fluorescence signal is across wells due to incomplete lysis of cells. Extend the lysis time to 10 minutes. • Note: When quantifying the fluorescence intensity, TyphoonFLA software selects a few points from well but not the whole are. If cells were not completely lysed in a well, there can be darker and lighter spots in the well that can result in higher standard deviation.
Low fluorescence signal	• The low signal is probably due to the presence of low number of cells. Gently wash the cells as vigorously washing can result in cell loss. • If the plates are left in the light for too long, the signal may be reduced. Perform the experiments in low light.
No increase in fluorescence after the addition of the inhibitor	• To verify the functionality of the method, you need to add positive control to each plate. Positive control for K562-TAX is cyclosporine A and for A549-VCR probenecid. • If you don't detect an increase in the fluorescence according to the concentration of these compounds, there may be a problem in the washing steps. After the first incubation when the media with daunorubicin and inhibitor is removed, wash cells and apply new media with inhibitors as fast as possible. MDR transporters may not stay inactivated for long, and daunorubicine may be transported out of the cells

VI. Acknowledgement

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Methods for quantification of oxygen radicals

Juan B. De Sanctis

I. Introduction

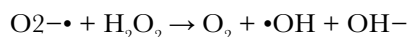
Oxygen radicals are usually formed by cells as a response to several reactions. Mitochondrial oxygen radical production is usually counteracted the superoxide dismutase and the peroxide formed is catabolized by catalase (1-3). Oxygen radicals have also been valuable tools to hamper bacteria and other microorganisms either alone or combined with halides (4-5). Oxygen radical production seems to be critical also against tumor growth (6) and aging (7). Several reactions involving oxygen radicals are illustrated in Fig. 1.

One of the critical issues is the formation of oxygen radicals by the Fenton reaction. Iron, Fe(II), participates in the Fenton reaction generating highly reactive hydroxyl radicals ($\bullet\text{OH}$)

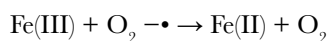
$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \bullet\text{OH} + \text{OH}^-$ (Fenton reaction)

The Fenton reaction has *in vivo* significance mainly in organisms overloaded by iron (as in the conditions of hemochromatosis, β -thalassemia, hemodialysis). Thus, high amounts of "free available iron" can induce the generation of oxygen radicals and cell death (ferroptosis).

Moreover, the superoxide radical participates in the Haber-Weiss reaction



which is a combination of Fenton reaction and the reduction of Fe(III) by superoxide:



The hydroxyl radical is highly reactive with a half-life in an aqueous solution of fewer than 1 ns. When produced *in vivo* it reacts close to its site of formation. Production of $\bullet\text{OH}$, close to DNA, could lead to this radical reacting with DNA bases or the deoxyribose backbone of DNA to produce damaged bases or strand breaks (Fig. 1). It is assumed that the most abundant *in vivo* production of hydroxyl radical according to the Fenton reaction occurs when Mn^{2+} is combined with iron and copper. One of the most common inductors of oxygen radical pathological effects is acrolein, a toxic metabolite produced by cigarette smoke and metabolism of cyclophosphamide and ifosfamide (Fig. 1).

Myeloperoxidase (MPO) is a heme-containing peroxidase expressed mainly in neutrophils and to a lesser degree in monocytes. Eosinophil

peroxidase (EPO) is a similar enzyme as MPO expressed in eosinophils. In the presence of hydrogen peroxide and halides, MPO/EPO catalyze the formation of reactive oxygen intermediates, hypochlorous acid (HOCl) by MPO and hydrobromic acid by EPO. Chlorotyrosine and bromo and dibromotyrosine can be detected by antibodies or by HPLC/MS or Malditoff assay. Detection of modified tyrosine by peroxynitrite completes the assay of the modified amino acids in the inflammatory process (20-26).

There are several methods to assess oxygen radical production. One of the most common is nitroblue tetrazolium which forms some yellow/brown formazan crystals (8). As described in the nitric oxide and metabolites chapter, oxygen radicals react with nitric oxide-forming several products, one of the most common peroxynitrite. This radical can also bind NBT; however, the reaction is partial and less efficient than

nitrotyrosine formation (9).

Fluorochromes are commonly used to assess peroxide production in the cell (10-11). Phagosome vesicles of some cells contain NADH reductase that generates superoxide and consequently hydrogen peroxide in immune cells. However, some fluorochromes are unspecific (12). Generally, 2',7'-dichlorofluorescein diacetate (DCFH-DA) (13-14) is one of the inexpensive and usually used fluorochromes which is not highly specific, rhodamine 123 and other probes to detect mitochondrial function (14).

Chemiluminescence is also used to detect oxygen radicals depending on the system. Luminol and Lucigenin are the most commonly used.

Superoxide and peroxide production can also modify fatty acids, free, phospholipid bound or in other lipid moieties (15). The thiobarbituric acid assay is one of the most common methods to

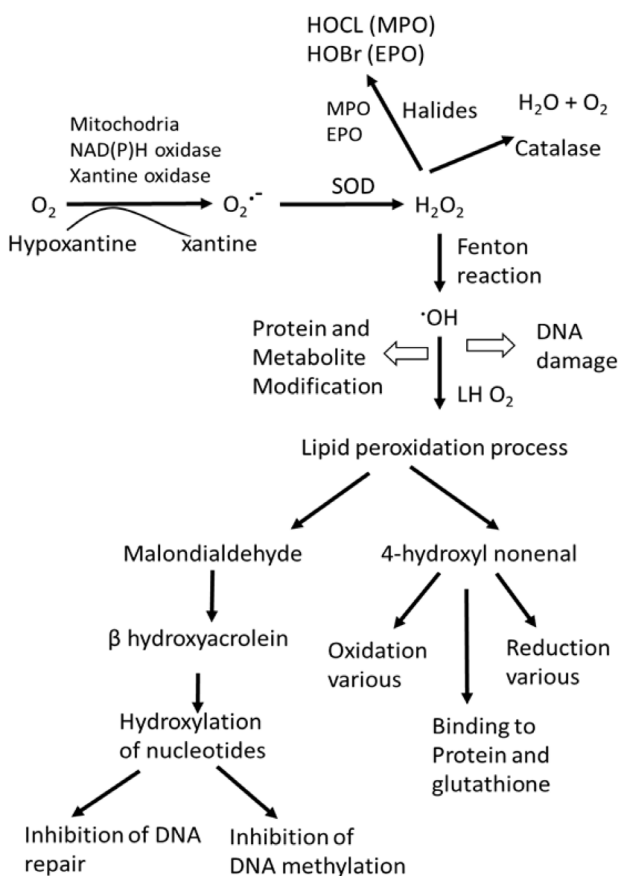


Figure 1. Reactions involving oxygen radicals.

detect fatty acid oxidation (malondialdehyde) which is related to inflammation and oxygen radical production (16-17). C11-BODIPY(581/591) can also be used to analyze lipid oxidation. Its binding is simple and more specific than cis-parinic acid.

Finally, modified nucleotides can also be assessed since damage to DNA may be crucial for cell function and epigenetics (27). There are kits to detect important 8-oxoguanine; however, 2,6-diamino-4-hydroxy-5-formamidopyrimidine can be detected using GC/HLPC/Mass spectrometry.

In the present chapter, we present the generally used methods for detection of the radicals and/ or its products.

II. Materials and instrumentation

Luminol and Lucigenin

For a general assessment of peroxide production Luminol and Lucigenin (Table 1). Luminol (Sigma-Aldrich, A8511; Pierce/Thermo Scientific, 32106). Lucigenin (Sigma-Aldrich, M8010; Cayman Chemical 2315-97-1). Use as specified by the manufacturer. Not recommended for specific assays or significant cell assays.

Prepare a stock solution of Lucigenin (1 mM), a or Luminol (10 mM) in DMSO (this can be stored at -20 °C); then prepare a fresh stock solution diluting the probe 1:100 (v/v) in PBS. For typical measurements in 96 well plates, 1×10^6 cells/ ml are resuspended in PBS including 5 mM glucose, 1 mM Mg^{2+} , 0.5 mM Ca_2^{+} and 0.05% BSA. Then, 0.1 ml of the cell suspension are added to the well plate which already contains 10 μ l of a freshly diluted probe. Incubate for 5 minutes in the dark at

37 °C before the addition of the stimulus. Use 20 μ l of 200 ng/ml PMA (diluted in PBS) as a positive control. Monitor reading from 5 up to 30 min at excitation emission 455/505 using the automatic luminescence analyzer EnVision (Perkin Elmer analyzer), Varioskan Flash (Thermo Scientific) at 37 °C. The assay was stopped either by the addition of superoxide dismutase 10 μ l solution (300U/ml) or by cooling the plate immediately at 4 degrees for 30 min. Use a standard curve prepared with fresh hydrogen peroxide in PBS from 1 μ M to 100 μ M. Prepare as follows use 30 % unopened hydrogen peroxide which is 0.88 M and take 10 μ l and dilute it in 870 μ l of PBS to prepare a 10 mM solution and then dilute accordingly (1:100 on in PBS).

Nitroblue tetrazolium (NBT) assay (Cayman Chemical, 298-83-9; Sigma-Aldrich, T8877).

It is recommended to use the compound to assess oxygen radicals in fresh samples (less than 2 hr). The principle of the assay is simple when NBT is oxidized; it generates yellow formazan crystals which then can be quantified by lysing the cells. The amount of color is proportional to the superoxide/peroxide produced.

A stock solution of NBT is generally prepared at 10 mg/ml in distilled water and kept at 4 °C in the dark. This assay refers to superoxide/peroxide production by cells in culture for a minimum of 60 minutes. Cells 1×10^5 /in 100 μ l well are incubated in their regular media and 30 μ l NBT solution is added to the culture along with the stimulus. As a positive control use 0.1 ml of hydrogen peroxide 30 %, as a negative control use glutathione 2 mg/ml solution. To induce peroxide production, depending on the cells or system to be assayed, PMA 10 ng/ml can be

TABLE 31.1 Comparison between Luminol and Lucigenin

Luminol	Lucigenin
1. It works through one electron oxidation	1. It works through one electron reduction
2. Measures both intracellular as well as extracellular ROS	2. It measures only the extracellular ROS
3. Hydrogen peroxide radical and oxygen radicals are involved	3. It involves measuring the superoxide anion
	4. Sensitive to chloride concentrations.

used to stimulate peroxide production. After the incubation, the cells are washed with PBS, and the intracellular formazan is solubilized with 2 M KOH, and dimethyl sulphoxide (DMSO) (Sigma-Aldrich, 276855). The crystals were resuspended and measured spectrophotometrically on a microplate reader at 630 nm. Alternatively, the precipitation of formazan can be observed in the cells in culture (23).

Probes for oxygen radicals for flow cytometry

The most common probes are:

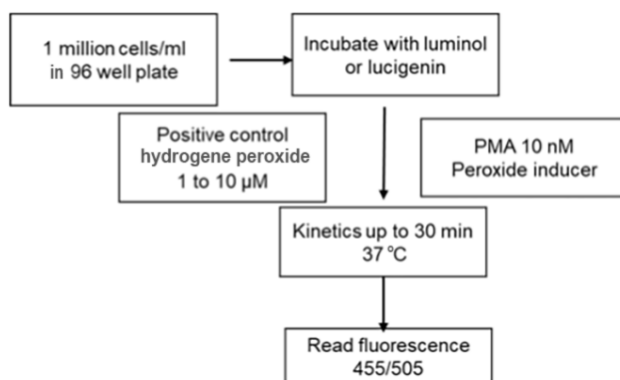
- Rhodamine 123 (Sigma-Aldrich, R8004; ThermoFisher, R302). The fluorochrome can be dissolved in ethanol or DMSO at a concentration of 50 μM for the stock solution. Use 1 μl of the stock solution per 2 million cells.
- 2',7' dichlorofluorescein diacetate (DCFH-DA). (Sigma-Aldrich, D6883; ThermoFisher, D399; Cayman Chemical, 2044-85-1). The fluorochrome can be dissolved in ethanol or DMSO at a concentration of 20 μM for the stock solution. Use 2 μl of the stock solution per 2 million cells.

Thermo Fisher (Molecular Probes) has an interesting discussion on the fluorochromes to detect oxygen radicals (<https://www.thermofisher.com/cz/en/home/references/molecular-probes-the-handbook/probes-for-reactive-oxygen-species-including-nitric-oxide/generating-and-detecting-reactive-oxygen-species.html#head2>); however, as it is stated in the page and the literature, it is recommended

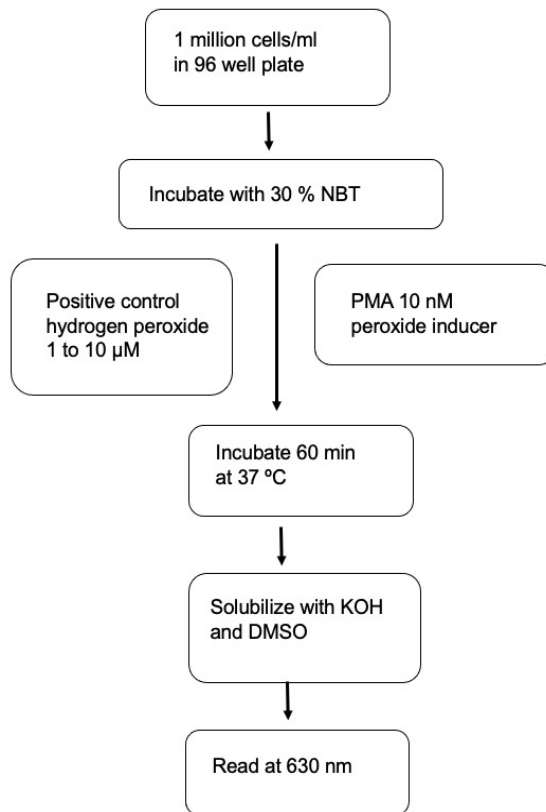
to use the classic Rhodamine 123, and 2,7 dichlorofluorescein diacetate.

III. Protocol for detection of oxygen radical by flow cytometry

Recall that the cells for flow cytometry should be in solution to do the assay. If you have tissue cells or adherent cells, make sure that you are using buffers which would avoid aggregation. The most used buffer is PBS 2 mM EDTA 0.1 % gelatin. Two million cells/ml in PBS are labeled with 5 μl of any of the two probes above prepared in ethanol. The cells are incubated at 37 degrees in the dark for 30 minutes. Then, a tube containing 0.1 ml (2×10^5 cells) is put on ice for basal fluorescence. The rest of the cells are incubated with different stimuli, and a kinetic is performed up to 30 min. For positive controls, cells incubated with hydrogen peroxide, at different concentrations from 1 to 10 μM are used as a control. Also, cells can be stimulated with PMA 10 nM to induce peroxide production. The reaction is stopped by incubating the cells at 4 degrees. The cells are then read by flow cytometry according to standard protocols 488 excitations and reading 525 nm for any of the two fluorochromes. It is recommended to set the tube with basal reading as the background fluorescence and tubes with labeled but untreated cells as controls of peroxide production. To compare the effect of the compounds are inductors or inhibitor of peroxide production, the fluorescence recorded is compared with basal activity and positive controls.



Scheme 31.1.

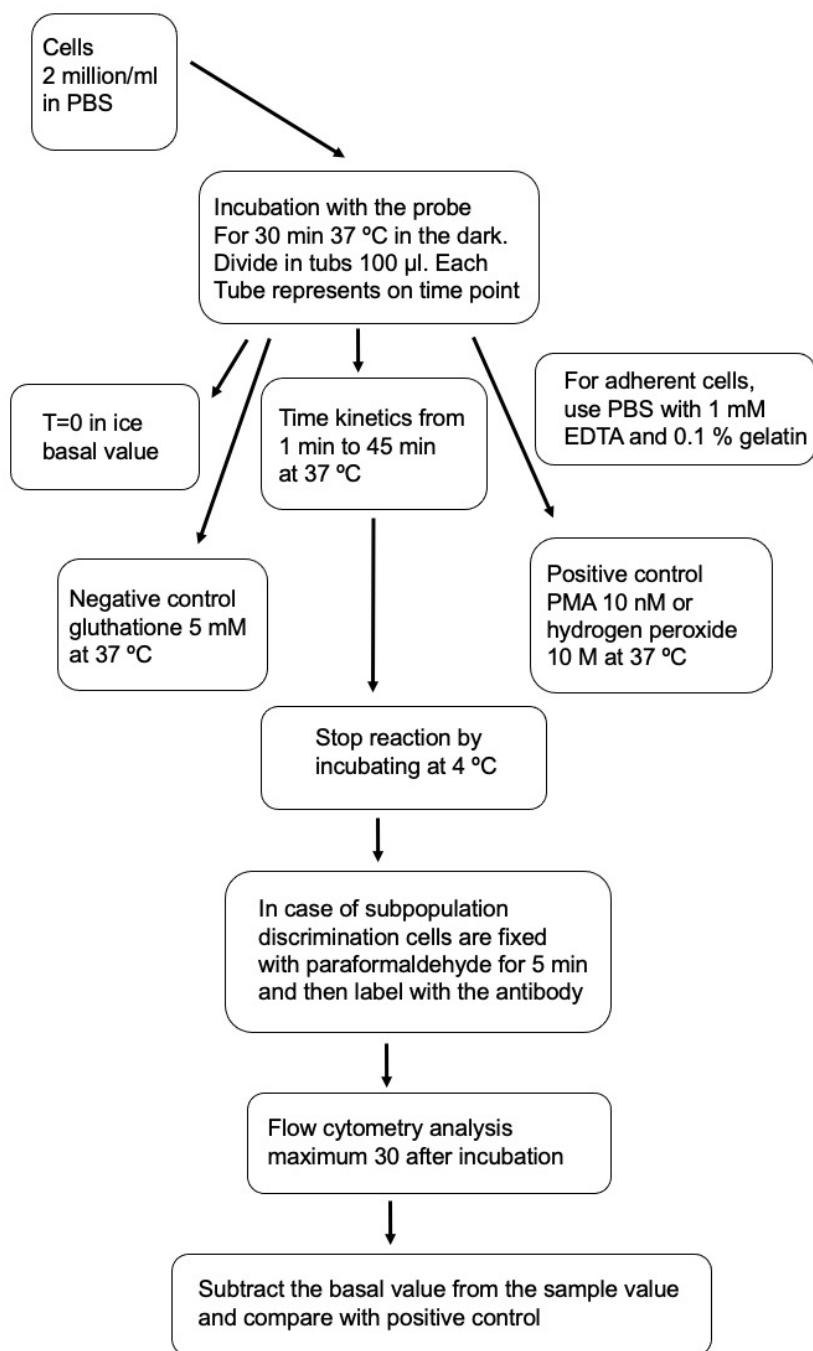


Scheme 31.2. Scheme of NTB assay

Measurement of lipid oxidized moieties by flow cytometry

C11-BODIPY (Thermo Fisher, D3861) is an oxidation-sensitive fluorescent lipid peroxidation probe. It is lipophilic and quickly enters membranes; once oxidized, C11-BODIPY(581/591) remains lipophilic and does not spontaneously leave the lipid bilayer. Low labeling concentrations can be used, ensuring minimal perturbation of the membrane while retaining favorable signal to noise ratios. It has good photo-stability and displays very few fluorescence artifacts. The fluorophore is insensitive to pH or solvent polarity. The scope of the probe is that there is a shift in the emission before oxidation at 590 nm and it decreases at 510 nm (from orange to green) when it is oxidized.

The tubes can be then incubated with the stimulus for 30 minutes. As a positive control of oxidation hydrogen peroxide, 1 and 10 µM are used as controls. Usually, glutathione is used to reduce lipid membrane oxidation at a concentration of 2 mM. An assay kit (recommended if you have a small number of samples which are critical) is commercially available from Abcam in colorimetric or fluorogenic setup (ab118970; ab233471). Essentially, the samples are heated and mixed with the reagents to generate a fluorogenic or colorimetric reaction. The reaction is read in either a fluorimeter or an ELISA reader at 530 nm in a quantitative way. Usually the values of the lipid-modified moieties in plasma, serum are around 200 nM and in urine 1000 nM.



Scheme 31.3. Flow cytometry assay

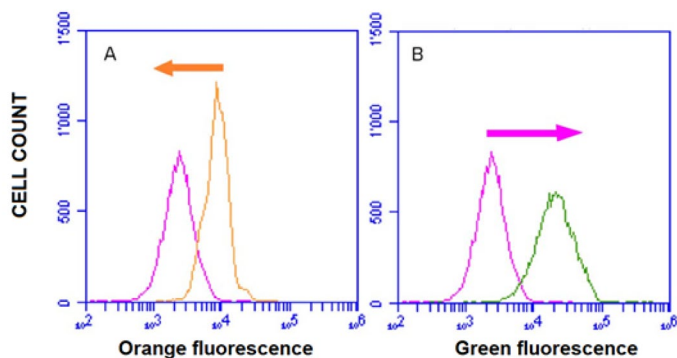


Figure 31.4 Effect of C11-BODIPY on oxidized lipids. Effect of C11-BODIPY on oxidized lipids. At the initial incubation, the fluorescence is orange, and then it turns green as the amount of oxidized lipids increase.

IV. Protocol samples for cell homogenates

One million cells are washed with PBS and then homogenized in 400 μ l of 0.5% Triton X-100. Then, 100 μ l cell homogenate is mixed with 1 ml of 0.67% Thiobarbituric acid (prepared in glacial acetic acid), 1 ml 20% trichloroacetic acid, and 0.1 ml 1 % butyl hydroxyl toluene (glacial acetic acid) glass test tubes. The mixture are incubated at 65 $^{\circ}$ C bath for 45 min. After cooling to room temperature, the reaction mixture is centrifuged at 4000 g for 10 min, and the absorbance of the supernatant is measured at 532 nm using a UV-Visible Spectrophotometer. The concentrations of TBARS are calculated using MDA as a reference standard commercially available. Make 1 mM, 10 ml MDA solution in PBS/ Homogenizing buffer. (Add 3.14 mg MDA in 10 ml). Make a serial dilution. Protein content can be determined for the same cell homogenate.

Protocol for serum, plasma or other biofluids. The protocol is similar to the one mentioned above, use 100 μ l of serum or plasma for assessment.

V. Antibodies and detection of modified amino acids

The anti chlorotyrosine polyclonal antibody is the only one available (Hycult Biotech, HP5002), chlorotyrosine standard can be purchased from Sigma Aldrich (512443). Several options of anti dibromotyrosine are available from Sigma (<https://www.sigmaaldrich.com/catalog/search?term=dibromotyrosine&interface=All&N=0&mode=match%20partial-max&lang=en&region=CZ&focus=product>).

For modified DNA

For detection of Human 7,8-dihydro-8-oxoguanine triphosphatase, there are two ELISA kits available. One is from Signalway (EK4571) detecting the nucleotide in serum, plasma, tissue homogenates, and other biological fluid and second by Abbexa LTD (abx251602). Quantitation by HPLC/MS can be done (27); however, the detection by ELISA in samples is preferred. The other most common metabolite 2,6-diamino-5-formamido-4-hydroxypyrimidine (28) is usually analyzed by HPLC/GC MS.

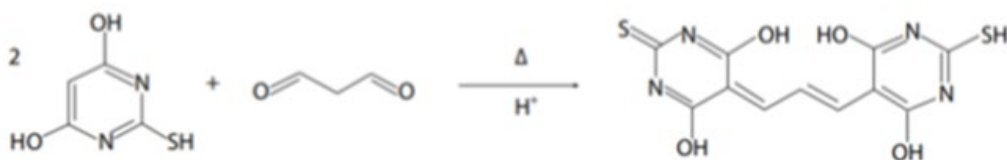
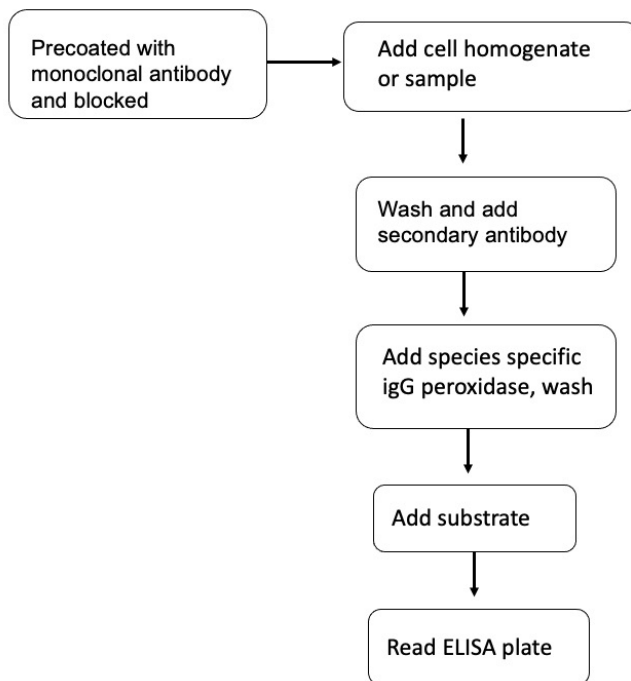


Figure 31.2 Thiobarbituric acid assay



Scheme 31.5 ELISA

TABLE 31.2 Summary. Modification of oxygen radicals and its method of detection

Type of reaction	Metabolite	Method of detection
ROS	Protein carbonyls	HPLC/MS/MALDI-TOF/2D electrophoresis [20]
ROS/RNS	3-nitrotyrosine	Antibody/ELISA/ HPLC/MS [20-22]
ROS/HCLO	chloro tyrosine	Antibody/HPLC/MS [20, 22]
ROS/HBrO	Bromotyrosine/ dibromotyrosine	Antibody/ELISA/ HPLC/MS [23]
ROS/glycosilation	Glycosylation adducts (RAGE)	Boronate affinity chromatography, HPLC/MS [25-26]
ROS/Lipid oxydation	Lipid peroxidation adducts (ALE)	Malondialdehyde (MDA), 4-hydroxy-nonenal (HNE) and F2-isoprostane 15(S)-8-iso- prostaglandin F2α (15(S)-8-iso-PGF2α). TBARS assay, HPLC/MS, ELISA (isoprostane) [21, 27]

IV. Comments

Oxygen radical production can be assessed with different assays; however, depending on the specific aim and the cell type, different protocols can be used. Many compounds that can inhibit radical production or affect signal transduction related to peroxide production are assessed in cell types with high radical production as eosinophils, neutrophils or macrophages. Nonetheless, these protocols may also be valuable in other cells are endothelial cells. *In vivo* assessment of oxygen radical production continues to be a difficult task.

V. Pitfalls

Problem	Cause and cure
False positivity or false negativity	• The absence of proper controls, positive and negative. • Slow analysis. Cell homogenates, if not quickly tested, can lead to a misdiagnosed analysis of metabolites due to several enzymatic reactions that may occur. • Wrong sample analysis with very reactive fluorophore, nitric oxide vs hydrogen peroxide.

VII. Acknowledgment

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Detection of nitric oxide enzymes and its metabolites

Juan B. De Sanctis

I. Introduction

Nitric oxide (NO) is an essential mediator in several biological functions (1). Measurement of NO has been widely used to ascertain vasodilatation, inflammation, and elimination of bacteria, parasite, and tumor cells (1). Three enzymes, 2 constitutive, neuronal or endothelial, and one inducible are responsible for generating NO from arginine. Arginine can also be metabolised by arginases (1) leading to a decreased protecting inflammatory response and tolerance. Several inhibitors of the nitric oxide synthases, N-acetyl-arginine, NG-Methyl-L-arginine, aminoguanidine, and dimethylarginine, have proven the importance of NO in the balance among inflammation and tolerance.

Since NO is a reactive gas, it may readily react with: 1) oxygen (generating nitrites and nitrates), 2) superoxide (protein nitration), 3) hydrogen peroxide generated upon superoxide radical or myeloperoxidase (MPO) and eosinophil peroxidase (EPO) activity inducing protein nitration and/halogenation, and 4) reacting with thiol groups generating S-nitro thiols (Fig. 1). It is important to stress that in the

absence of radicals, NO readily reacts with oxygen and generally nitrites. Nitrites, but not nitrates can be readily quantified by the Greiss reagent. Griess Reaction was first described in 1879 (2-3). Because of its simplicity, it has been used extensively in the analysis of various biological samples serum, plasma, urine, tears, CSF, saliva, and cell culture media (4).

Nitrates can be transformed into nitrites and consequently analyzed as such. The reduction of nitrate to nitrite is accomplished by using chemical reductants (cadmium, zinc, or vanadium chloride) or by the enzyme nitrate reductase (NR). Cadmium metal is the preferred option since Zinc is very reactive and it can also reduce nitrite. Vanadium chloride also represents a good option: however, the reagent may be unstable after 72 hrs (4). Usually, incubation of the sample with metal should be for short term (1 hr).

The measurement of nitric oxide gas has been used useful in the clinic. The fractional exhaled nitric oxide (FeNO) refers to the amount of nitric oxide in parts per billion (ppb) in the exhaled air. Increased NO is related to inflammation of

Scheme 32.1 for general reactions 1

Nitrite oxidation (oxyhemoglobin)



Nitrite reduction (deoxyhemoglobin)



Nitrate reduction

Nitrate reductase



Scheme 32.1

the airways which usually refers to levels above 20 ppb in children and adults (5). The levels of FeNO refers to inflammation of the airways due to Type 2 immune responses; however, other lung diseases like chronic obstructive pulmonary disease (COPD) and lung cancer can also be screened. Nitric oxide production is higher in the sinonasal area, and consequently, patients with allergic rhinitis may have increased levels of FeNO despite low bronchial restriction (5).

Fluorescent assessment of nitric oxide has been used to try to assess the immediate production of the radical. Several protocols have been used in different systems (6-8). There have been some concerns in the literature about the specificity of the probes and the meaningless results obtained using different methods (9). It is recommended, therefore, to assess nitric oxide metabolites using general methodology first and compared it with these new approaches (9).

Nitrotyrosine (fig. 1) is an essential metabolite, easily detectable with specific antibodies (10) or by other methodology as HPLC/MS (11). The other exciting metabolite is the formation of nitro thiols which have been involved in several reactions and cell death (12). S-nitrosothiols (RSNOs) are stable products of NO *in vivo* (12). RSNO act as NO donors to specific thiol-regulatory effector sites including enzymes and signaling proteins by trans S-nitrosylation (12). RSNOs bind covalently to SH groups of proteins and signal through the modification of cysteine residues, which regulates the function of a broad spectrum of proteins in cells (12). The reaction of NO with the free thiols of serum albumin, cysteine and, glutathione results in the formation of the respective S-nitrosothiols *in vivo* (13). RSNOs are reduced to NO and nitrite (13).

Nitric oxide reacts with fluorine, chlorine, and bromine to form the nitrosyl halides, such

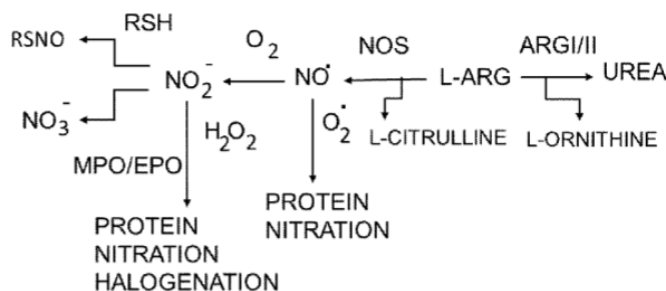


Figure 32.1 Reactions of NO radical

as nitrosyl chloride: $2 \text{ NO} + \text{Cl}_2 \rightarrow 2 \text{ NOCl}$. These compounds are highly toxic, and gas compounds reacting with amino acids as glycine converts them into chloroacetic acid. These metabolites are usually screened using HPLC/MS (13). However, little is known about their pathological role.

The assessment of nitric oxide synthases has usually been using RT-PCR, immune assays (ELISA), and homogenized cells or tissues using radioactive substrate. However, nitric oxide synthases can be phosphorylated modifying kinetic parameters (1, 14-17), generating misleading results. An interesting approach is to use the fluorogenic chromophore 4,5-diaminofluorescein (DAF-2 DA). DAF-2 DA crosses fast the plasma membrane, and it is then hydrolyzed by intracellular esterase to DAF-2. DAF-2 reacts with NO produced by NOS to form a fluorescent triazole-fluorescein. The fluorescent product can be quantitated using an excitation filter at 492 nm and an emission filter at 515 nm.

Both nNOS and eNOS have been found in tissues and cells different from neurons and endothelial cells (15-17). Post-transcriptional modifications can also be observed since they can be regulated by several kinases (Akt/PKB, AMPK, PKA, PKC, PKG, and CaMK-2). Namely, eNOS can be phosphorylated on serine 114, 615, 633, 1177 and threonine 495. The phosphorylation of threonine 495 decreases enzyme activity (18). It follows then that there is no direct and straightforward transcription, transcriptional modification/activity relationship. Product assessment rather than enzyme transcription kinetics or activity is highly recommended.

One of the useful tools to analyze nitric oxide and its possible role in a specific reaction is the use of nitric oxide donors. There are several well-characterized donors: sodium nitroprusside, well-known vasodilator, S-nitroso-N-acetylpenicillamine (SNAP), and diazeniumdiolate DETA/NO (1). They have been used in several models.

II. Materials and instrumentation

Greiss reaction

- Phosphoric acid (PA) (Sigma-Aldrich, 345245)
- sulfanilic acid (SA) (Sigma-Aldrich, 251917)
- N-alpha-naphthyl-ethylenediamine (NED) (Sigma-Aldrich, 33461).
- Sodium nitrite (Sigma-Aldrich, 237213)
- Nitrate reductase from *Aspergillus* species (Sigma-Aldrich, 1098124900).

Nitric oxide donors

There are different nitric donors. The donors release of NO depends on the media. However, the ones of the first group are recommended depending on the cell type and the experiment.

A. Nitric oxide donors that rapidly release NO (Diazeniumdiolate, pH and temperature sensitive).

- DEA NONOate (Sigma-Aldrich, D5431). Use for rapid release of NO. Short-acting (half-life of 2 min at pH 7.4, 37 °C).
- PAPA NONOate (Abcam ab145196). Half-life of 15 min at pH 7.4, 37 °C.
- Spermine NONOate (Abcam, #ab144522). Half-life of 40 min at pH 7.4, 37 °C.
- DPTA NONOate (Abcam, #ab145198). Half-life of 3 h at pH 7.4, 37 °C.
- DETA NONOate (Abcam, #ab144627). Use for prolonged release of NO. Half-life of 20 h at pH 7.4, 37 °C.

B. Enzymatic oxidation

- Organic nitrates: Nitroglycerin/GTN
- Inorganic nitroso compounds: sodium nitroprussiate (Abcam, ab145732).

C. Chemical reaction

- S nitrosothiol SNAP (Abcam, #ab120014). Requires Copper.
- S-nitrosoglutathione (SNOG) (Sigma-Aldrich, #N4148).
- Fluorogenic probe for NO. Fluorogenic detection of NO: The kit for a fluorogenic compound that binds NO is available from Sigma Aldrich (#FCANOS1).

Antibodies

Antibody against 3 nitrotyrosine, S nitrosylation. The most common monoclonal antibody clone for nitrotyrosine is 39B6 which can be obtained from several companies (<https://www.biocompare.com/pfu/110447/soids/345458/Antibodies/Nitrotyrosine>); however, for the ELISA the secondary polyclonal antibody (generally rabbit IgG) there are several options (<https://www.biocompare.com/Search-Antibodies/?search=Nitrotyrosine%2520polyclonal&said=1>). Usually, Thermo Fisher, Abcam, Santa Cruz, Cayman Chemicals are the most documented.

For inducible Nitric oxide synthase (NOS 2). The most convenient antibodies, sold by pair, are from MYBiosource, Cat. No. # MBS2090006. The antibodies can be used for ELISA, western blot and cell staining. EMC Biosciences also has a monoclonal and polyclonal, Cat. No. NM3981 / NP2131).

III. Procedures

Greiss reaction

Greiss reaction sulfanilic acid (SA) in the presence of acid medium (phosphoric acid) from the diazonium salt interacts with the azo dye agent, N-alpha-naphthyl-ethylenediamine (NAD), to form a pink color which is read at 540 nm. The preparation of the reagents is as follows: prepare a 0.1 % solution of NAD in double distilled water, reagent A, and then dissolve 0.2 g of SA in 5 ml of orthophosphoric acid 85 % and slowly add water up to 100 ml, reagent B. Prepare a fresh solution of sodium nitrite (0.068 g/100 ml of double distilled water, 10 mM stock solution). Prepare a standard curve from 1 to 100 μ M. Mix solutions A and B 1:1 (stable up to four weeks in the dark in the cold room) and use this reagent mix with the sample at a ratio 1:1.

There are commercial kits that are suitable for the analysis of nitrite and nitrate. Sigma-Aldrich (23479-1KT-F) has a kit that involves the

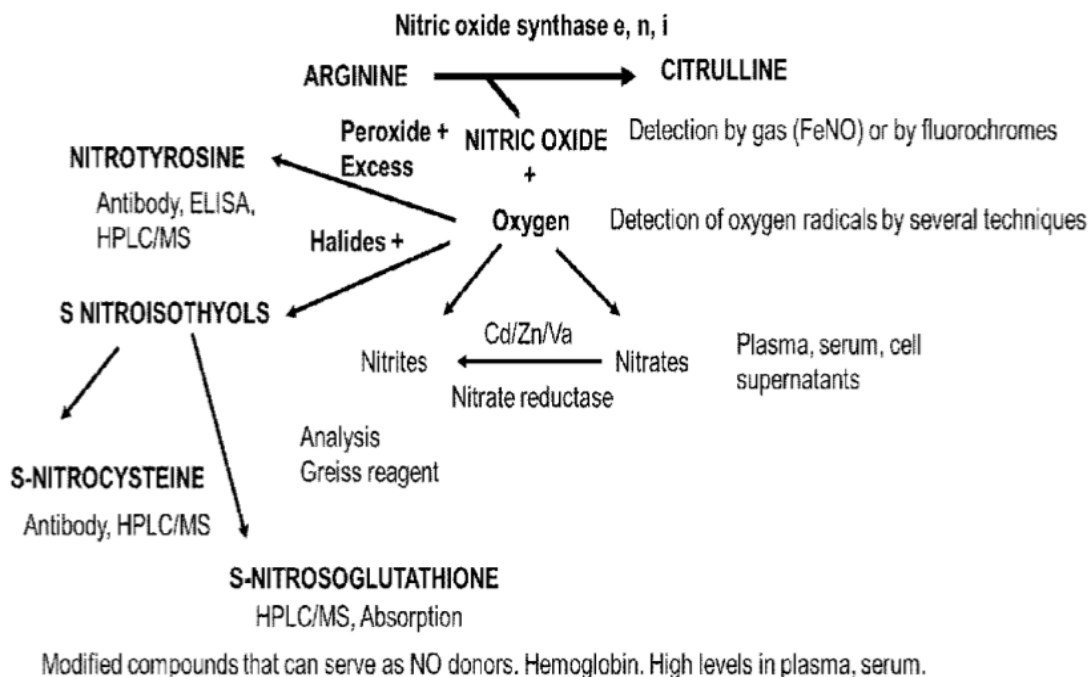


Figure 32.3 Reactions of Nitric oxide

reduction using the enzyme nitrate reductase.

Nitric Oxide Assay Kit (Flow cytometry - Orange) (ab219933) is a sensitive fluorometric assay to monitor intracellular nitric oxide (NO) levels in live cells using a flow cytometer. The assay uses an orange dye that can react with NO to generate a bright orange fluorescent product that can be easily detected at Ex/Em = 540/590 nm, using the same filter set as Cy3® or TRITC. This fluorescent reagent can also be used in fluorescence assay and read with the automatic luminescence analyzer EnVision (Perkin Elmer analyzer), Varioskan Flash (Thermo Scientific) at 37 °C after 30 minute incubation, excitation 540 nm/emission 590 nm.

Detection of nitrotyrosine and S-nitrosothiols

Nitrotyrosine can be detected by HPLC/MS or fluorogenic substrates; however, it is not recommended since protocols require validation in order to quantitate.

General protocol for nitrotyrosine detection by ELISA

Generally (33-38), the sandwich ELISA the anti nitrotyrosine antibody clone 39B6 is used to coat the 96 well plate at 1 µg/well in carbonate buffer pH 8.0 overnight. The plates are then blocked with 5 % BSA for 1 hr at 37 °C, then washed and the samples are added. The supernatant, tissue homogenate or other fluids are added to a maximum amount of 20 µg protein/well and incubated for 1 hr at 37 °C. Then the plates are washed with PBS 1 % Tween 20, five times. Then, the secondary antibody usually IgG anti tyrosine from rabbit at a dilution 1:100 from Cayman/Thermo Fisher is incubated for 1 hr at 37 °C or 4 hr at 25 °C. Then, the plates are washed as described before and incubated with anti rabbit IgG usually Thermo Fisher 1:100 dilution and incubated for 1 hr at 37 °C. Finally, after washing the plates, 3,3',5,5'-Tetramethylbenzidine (TMB) in acidic solution is used. The reaction is stopped using 2 M sulphuric acid. The standard for nitrotyrosine Oxford Biomedical Research has nitrated carbonic anhydrase (Mr=29kDa), number NT60. Cell Biolabs have nitrated albumin, 230503. Alternatively, a modification

of any protein is possible by using the protocol of Ischiropoulos et al. [32] in which tyrosine containing proteins can be mixed with peroxynitrite formed from mixing hydrogen peroxide with sodium nitrate. The absorption at 450 nm of nitrotyrosine is usually used to assess the amount of the modified amino acid; however, absorption depends on pH and the type of buffered solution.

S-nitrosylation is one of the common modifications by nitric oxide. Usually, S-nitrosocysteine is detected using antibodies. The antibody clone HY8E12 (Abcam, #ab94930) is the most documented reagent to detect S-nitrosocysteine. The modification may generate conformational changes of different proteins, and as a consequence alter cell signal transduction. S-nitrosylation analysis of proteins in western blot and cells can be performed using the kit of Cayman Chemical, 10006518. Most of the methods for detection of these modified molecules is simple Western Blot analysis using the standard methodology illustrated in other chapters of this book.

Nitric oxide synthases

It is important to note that the commercial kits are highly recommended since they contain the recombinant protein or peptides for the assay which are difficult to purchase commercially as not part of the kit. Since enzymes may modify their kinetic activity depending on phosphorylation, it is recommended that detection of the enzymes as a whole and as phosphorylated target should be performed. The reason is the high amount of pharmacological compounds which affects signal transduction pathways which also affect these enzymes. Detection is usually performed by Western blot.

For the enzymes, there are several options for total and phosphorylated constitutive enzymes (<https://www.biocompare.com/Search-Antibodies/?search=Nitric%2520oxide%2520synthase&said=1>). Antibodies from Merck, Invitrogen, Enzo Life Science, Biorad, US Biological have been widely used in the literature; however, there are only a few companies that sell antibodies against the phosphorylated enzyme. The R&D systems, Merck, Invitrogen, US Biological and EMC Biosciences are the most common (<https://>

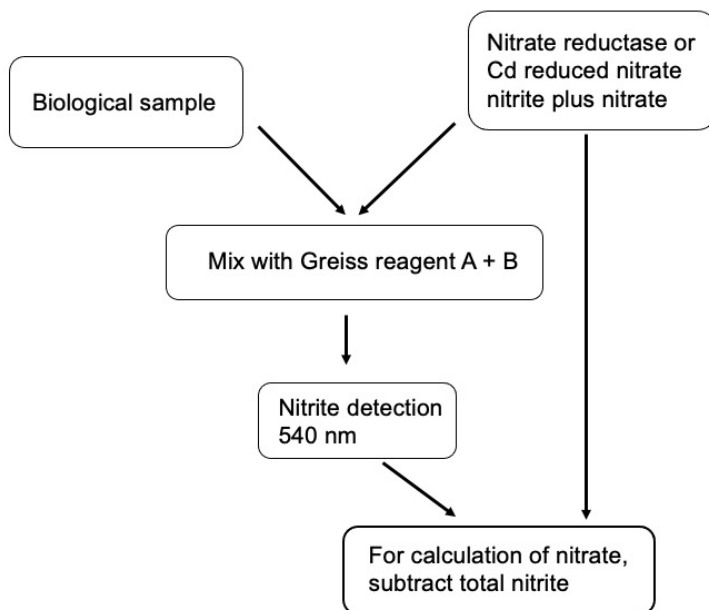
www.biocompare.com/Search-Antibodies/?search=phospho%2520Nitric%2520oxide%2520synthase&said=1).

Cell signaling also has a broad range of antibodies and even an ELISA kit for Phospho-eNOS (Ser1177) Cat. No. #7980.

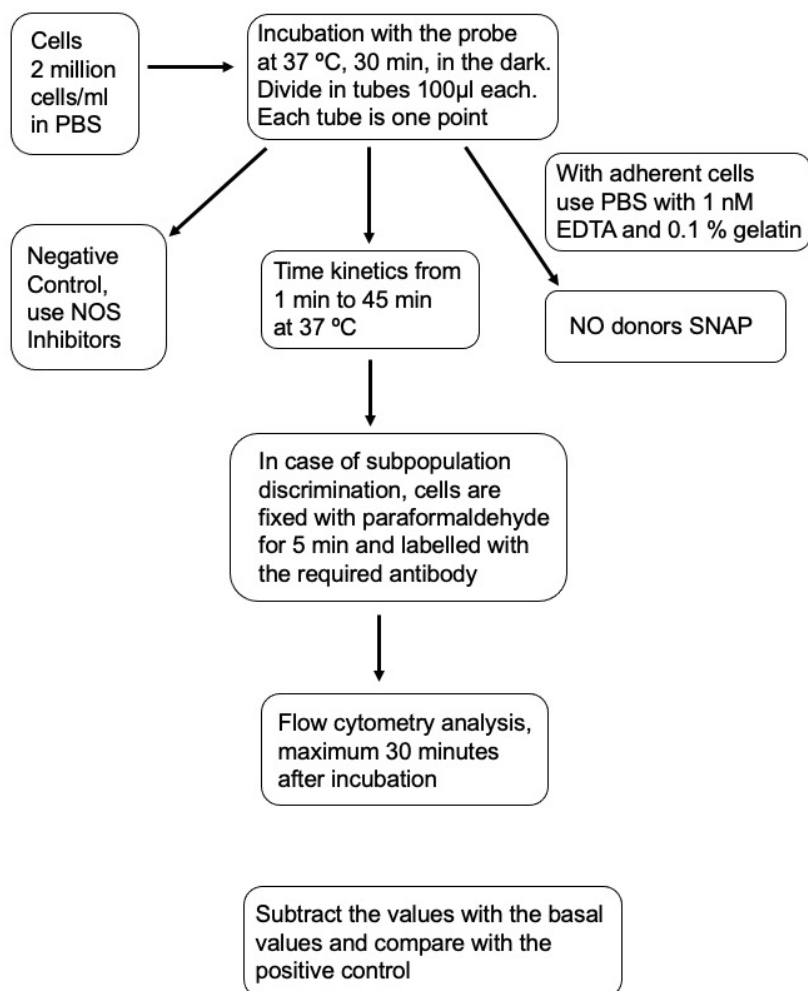
Santa Cruz Biotechnology also has several options (https://www.scbt.com/scbt/browse/nos-Antibodies/_/N-1z0cjrN?Nr=AND%28product.siteId%3AAscbtSite%2COR%28product.catalogId%3AAscbtCatalog%29%2Cproduct.webDisplayFlag%3A1%2Cproduct.suppressDisplayFlag%3A0%29).

Molecular Biology of NOS

NOS siRNA, shRNA Plasmid and shRNA Lentiviral Particles gene silencers are available from Santa Cruz biotechnology as well as CRISP constructed plasmids (https://www.scbt.com/scbt/browse/nos-Antibodies/_/N-1z0cjrN?Nr=AND%28product.siteId%3AAscbtSite%2COR%28product.catalogId%3AAscbtCatalog%29%2Cproduct.webDisplayFlag%3A1%2Cproduct.suppressDisplayFlag%3A0%29).



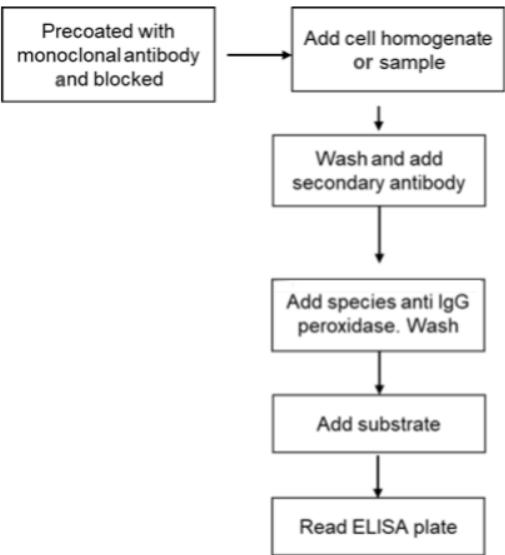
Scheme 32.2 Nitrite and nitrate detection



Scheme 32.3 Analysis of nitric oxide in cells

IV. Comments

- Nitric oxide is a reactive radical that generates several products. Low concentrations of nitric oxide are physiologically relevant for smooth muscle relaxation, and protective effects on cells; however, when inflammation occurs and more radical is formed, inducible nitric oxide synthase is responsible for it, it can react with oxygen from either NADH oxidase, myeloperoxidase, eosinophil peroxidase and halides released from cells. The generation of nitrotyrosine induces major changes on protein function and consequently signal transduction effects. On the other hand, production of S nitrosothiols is naturally occurring and detected in plasma and serum. Haemoglobin can also store part of these compounds for immediate release in the vasculature. Thus, the detection of nitric oxide is important for physiological and pathological responses from cells, tissues, and organisms.
- Several reports from our group are available in different systems [19-26].
- Macrophage and neutrophils from mouse and rat origin produce more nitric oxide than the human counterparts and consequently more metabolites [26]. This point should be analysed cautiously.



Scheme 32.4

V. Pitfalls

Problem	Cause and cure
Lack of nitric oxide production	• Cell death or mitochondrial death. • Inhibition of NOS • Check the pH of the sample. • Check for oxygen presence in the tissue/organ/cell culture.
Excess of nitric oxide	• More than one enzyme involved? Phosphorylation?
Negative control is not working	• No inhibition of NOS
Positive control is not working	• Adjust pH of the sample; NO donor is time and pH dependent.

VI. Acknowledgment

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Western blot analysis

Jana Václavková

I. Introduction

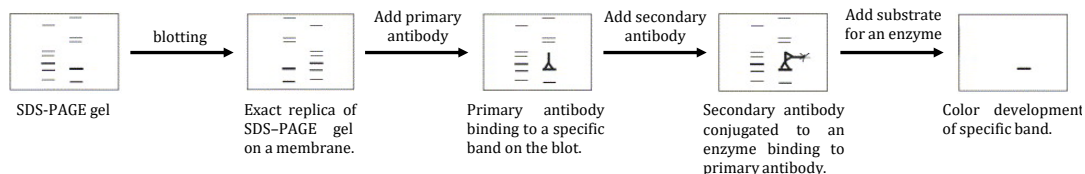
Western blot is a method which is often used to separate and identify proteins. A mixture of proteins is separated by gel electrophoresis based on molecular weight. Separated proteins are then transferred to a membrane which is incubated with an antibody to detect the specific protein of interest (1).

Prior to Western blot analysis the separated proteins in the gel matrix were challenging to access with molecular probes. Transfer of proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) to an adsorbent membrane is a powerful tool to detect and characterize a broad spectrum of proteins including low abundant proteins (2). Electrophoretic transfer of proteins to nitrocellulose membrane was first described in 1979 by Harry Towbin (3). In 1981, W. Neal Burnette published very similar technique as Towbin and first used the name Western blotting in honor of Edwin Southern and the West Coast location of its invention (4, 5). They both described the denaturing electrophoresis coupled with Western blot. In 1986, the native electrophoresis coupled with Western blot was described. They use a system with very low

(0.1%) SDS concentration to keep the protein in the native state and they successfully blotted and localized discontinuous epitopes of herpes simplex virus glycoprotein D (6). The blue native PAGE can be performed as well, followed by gel incubation in a transfer buffer supplemented with low SDS concentrations (such as 0.037%) before normal Western blot transfer (7).

Various lysis buffers can be used according to individual experiments. Here, the cell lysis using RIPA lysis buffer is described as the most common approach in our labs.

To transfer proteins from gel to membrane following techniques were developed: simple diffusion, vacuum blotting, and electroblotting. Electroblotting is the most commonly used procedure to transfer proteins from a gel to a membrane. Compared to simple diffusion or vacuum blotting, the electroblotting is fast and the transfer is efficient and quantitative. Basically, there are three approaches in electroblotting: complete immersion of a gel-membrane sandwich, wet blotting, and semi-dry blotting. Complete immersion of a gel-membrane sandwich is performed that the transfer membrane is sandwiched



Scheme 33.1 The Western blot and detection procedure, adapted according to Kurien and Scofield (2).

between the gel, filter papers, and support pad. The transfer membrane is on the anode side. The cassette containing the assembled gel-membrane sandwich is inserted into a Trans-blot apparatus containing cold transfer buffer (2). In the procedure of wet transfer, the sandwich is placed in a buffer tank, connected to the power supply and performed on ice (1, 2). Semi-dry or horizontal blotting uses two plate electrodes for a uniform electrical field over a short distance. Sandwiches contain up to six gel/membrane/filter paper assemblies, all the assemblies are soaked by transfer buffers (2). As well there are multiple modifications of electroblotting which could be used (2). Here, I described the semi-dry Western blot as an approach typically used in our labs.

There are many ways how to detect an antigen on a membrane. In principle, the primary antibody binds to a specific band on the blot. The secondary antibody is conjugated to a label and binds to the primary antibody. Signal for a specific band is developed. The whole process is summarized in scheme 33. 1 (2).

In this chapter, I focused on denaturing SDS-PAGE followed by Western blot with chemiluminescent detection using horseradish peroxidase secondary antibodies.

II. Materials and instrumentation

Water (MiliQ® ultrapure water, Merck), NaCl (cat. no S5886; Sigma-Aldrich), KCl (cat. no P5405; Sigma-Aldrich), $\text{Na}_2\text{HPO}_4 \times 12\text{H}_2\text{O}$ (cat. no 30061-AP0; Lach-Ner), KH_2PO_4 (cat. No 30016-AP0; Lach-Ner), HCl (cat. no 19360; Penta), Tris base (cat. no 37180; Serva), NP-40 (cat. no 74385; Sigma-Aldrich), sodium dodecylsulfate (SDS; cat. no 71729; Sigma-Aldrich), sodium deoxycholate (cat. no 30970; Sigma-Aldrich), EDTA (cat. no

11280; Serva), Pierce™ BCA Protein Assay Reagent A (cat. no 23223; Thermo Scientific), Pierce™ BCA Protein Assay Reagent B (cat. no 23224; Thermo Scientific), glycerol (cat. no G5516; Sigma-Aldrich), dithiothreitol (DTT; cat. no D9779; Sigma-Aldrich), bromophenol blue (cat. no B0126; Sigma-Aldrich), 30% acrylamide 29:1 (cat. no 161-0156; Bio-Rad), Resolving Gel Buffer (1.5 M Tris-HCl, pH 8.8; cat. no 161-0798; Bio-Rad), ammonium persulfate (APS; cat. no A3678; Sigma-Aldrich), N,N,N',N'-tetramethylethylenediamine (TEMED; cat. no 161-0800; Bio-Rad), butanol (cat. no 22130, Penta), Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8; cat. no 161-0799; Bio-Rad), 10× TGS buffer (cat. no 161-0772; Bio-Rad), Spectra™ Multicolor Broad Range Protein Ladder (cat. no 26634; Thermo Scientific), glycine (cat. no 23390; Serva), methanol p.a. (cat. no 20038-AT0; Lach-Ner), Tween-20 (cat. no P2287; Sigma-Aldrich), bovine serum albumin (BSA; cat. no A7906; Sigma-Aldrich), sodium azide (cat. no 71290; Sigma-Aldrich), filter papers (cat. no 1650962; Bio-Rad), nitrocellulose membrane (cat. no 1620097 for 0.2 μm pore size, cat. no 1620094 for 0.45 μm pore size; Bio-Rad) or Immobilon®-PSQ Transfer Membrane (cat. no ISEQ00010 for 0.22 μm pore size, cat. no IPVH00010 for 0.45 μm pore size; Merck), primary antibody (depending on the protein detected), secondary antibody (depending on the primary antibody), Luminata™ Forte Western HRP Substrate (cat. no WBLUF0500; Merck).

Vi-CELL™ XR Cell Viability Analyzer (Beckman Coulter); centrifuge Rotina 420R (Hettich); sonicator Branson Digital Sonifier 450 (BRANSON Ultrasonic corporation); Envision multimode plate reader (PerkinElmer); Mini-PROTEAN™ Tetra Cell Casting Stand with Clamp Kit for Single Core System (cat. no 1658052;

Bio-Rad); combs: Mini-PROTEAN® Comb, 10-well, 1.0 mm, 44 µl (cat. no 1653359; Bio-Rad), Mini-PROTEAN Comb, 15-well, 1.0 mm, 26 µl (cat. no 1653360; Bio-Rad), Mini-PROTEAN® Comb, 10-well, 1.5 mm, 66 µl (cat. no 1653365; Bio-Rad) or Mini-PROTEAN® Combs, 15-well, 1.5 mm, 40 µl (cat. no 1653366; Bio-Rad); plates with spacers: Mini-PROTEAN® Spacer Plates with 1.0 mm Integrated Spacers (cat. no 1653311; Bio-Rad) or Mini-PROTEAN® Spacer Plates with 1.5 mm Integrated Spacers (cat. no 1653312; Bio-Rad); Mini-PROTEAN® Short Plates (cat. no 1653308; Bio-Rad); Mini Cell Buffer Dams (cat. no 1653130; Bio-Rad); Mini-PROTEAN® Tetra Electrode Assembly (cat. no 1658037; Bio-Rad); Mini-PROTEAN® Tetra Companion Running Module (cat. no 1658038; Bio-Rad), Buffer Tank and Lid (cat. no 1658040; Bio-Rad); PowerPac™ Basic Power Supply (cat. no 1645050; Bio-Rad);

Trans-blot®Turbo™ Transfer System (cat. no 1704150; Bio-Rad), LI-COR Odyssey® Fc Imaging System (LI-COR Biotechnology).

III. Procedures

A. Cell lysate preparation

Solutions

- **10× phosphate buffered saline (PBS)**

1.37 M NaCl

27 mM KCl

100 mM Na₂HPO₄ × 12H₂O

18 mM KH₂PO₄, adjust the pH to 7.4 with HCl. Sterilize the solution by autoclaving. The working solution is 1× PBS. To prepare the working solution, dilute 100 ml of 10× PBS in 900 ml of water.

- **RIPA lysis buffer**

50 mM Tris base, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, adjust the pH to 8.

- **5× SDS loading buffer**

250 mM Tris base, 10% SDS, 30% glycerol, 0.5 M DTT, adjust the pH to 6.8 and add bromophenol blue to the buffer to be blue enough.

Steps

1. Scrape the adherent cells or collect the suspension cells and count the cells (Vi-cell). Perform all the following steps at 4 °C or on ice.
2. Spin down the cells in a pre-cooled centrifuge at 1000× g, 5 min, 4 °C.
3. Remove the supernatant and add ice-cold 1× PBS and spin down the cells in a pre-cooled centrifuge at 1000× g, 5 min, 4 °C. Repeat this step three times to wash the pellet well.
4. After three rounds of washing with 1× PBS remove the supernatant. Continue with a procedure with a step 5 or store the pellet in -80 °C and then continue with a step 6.
5. If it is needed to continue immediately, it is recommended to put the pellet in liquid nitrogen for 10 min, and proceed to step 6.
6. Add 1 ml of RIPA lysis buffer per 100×10⁶ cells, suspend the pellet and incubate 30 min on ice.
7. Sonicate the lysate according to the manufacturer instructions.
8. Spin down the lysate at 14 000× g, 5 min, 4 °C.
9. Pipette the supernatant into new Eppendorf tubes and measure the protein concentration using BCA assay. Perform the BCA assay in 96-well format. Prepare the BCA reagent, pipette the standards and samples according to the product datasheet. The maximum concentration which can be measured by BCA assay is 2 mg/ml; it is recommended to dilute the samples prior the assay, 2×, 4× and 8× dilution can be used (usually the protein concentration in protein lysates is around 4 mg/ml). Incubate the samples at 37 °C for 30 min. Measure the absorbance at 562 nm using plate reader (Envision multimode plate reader). Construct the calibration curve with standards and calculate the protein concentration in the samples (Microsoft Excel). An amount of 10 to 50 µg of proteins (depending on an abundance of protein which is visualized by Western blot) of each sample can be used for Western blot analysis.
10. Add 5× SDS loading buffer to the samples to the final concentration of 1× SDS loading buffer, boil the samples at 95 °C for 5 min. Store the samples in the freezer or continue directly with SDS-PAGE.

B. SDS-PAGE

Steps

1. Wash the electrophoretic glass properly.
2. Put together the chambers for gel preparation. Use the glass with spacers (usually 1 mm or 1.5 mm spacers are used) and put the short plates on them. Put the glass into a casting frame, snap the glass with both clamps together.
3. Put the casting frame into a casting stand with gaskets and pour the water between the glass, after a few minutes check if the system seals well (the water do not flow out). If the water flows out, cast up the system again. If the system seals well, pour out the water and let the rest of the water soak into a paper towel.
4. Choose the separation gel according to the Tab. 1 at the end of the section B. Prepare the separation gel solution (according to Tab. 2), ammonium persulfate (APS) and TEMED are added as the last reagents.
5. Pipette the separation gel solution between the glasses approximately 2 cm under the short plate margin, pipette butanol carefully on the top of the gel solution to form a thin layer. Let the gel polymerize for at least 30 min.
6. Pour out the butanol, wash the remaining butanol with distilled water and let the remaining water soak into a paper towel.
7. Choose 10 or 15 well comb according to sample count and amount of samples you want to pipette. See well capacity in a Tab. 4 at the end of section B. An amount of 10 to 50 μg of proteins (depending on an abundance of protein which is visualized by Western blot) of each sample can be used for Western blot analysis. Usually, 30 μg of proteins per well is loaded.
8. Prepare the 4% stacking gel solution (according to Tab. 3), ammonium persulfate (APS) and TEMED are added as the last reagents.
9. Pipette the stacking gel solution on the separation gel towards the top margin of the short plate. Add a comb to make wells for samples. Let the gel polymerize for at least 30 min.
10. Put the gels into the electrode assembly. The short plates must be oriented inside; one short plate is oriented towards each other. In the case of 1 gel, put there one gel and one buffer dam. In the case of 2 gels, put them both into the electrode assembly. In the case of 3 and 4 gels, use companion running module. Put the electrode modules into the buffer tank. The red color label must fit into another red color label; black color label must fit another black color label.
11. Prepare 1 $\times$ TGS running buffer by diluting 100 ml of 10 $\times$ TGS buffer in 1 l of water.
12. Pour the 1 $\times$ TGS running buffer between the gels. If the running buffer soaks out, set the gels in the electrode assembly again more tightly. Pour the 1 $\times$ TGS running buffer to the buffer tank towards the guideline. There are two guidelines depending on gel number, pour the amount of 1 $\times$ TGS running buffer corresponding to a number of gels used.
13. Put the combs out of the gels.
14. In case that the electrophoresis is performed the same day as sample preparation, continue with step 15. In case that the samples are taken out of the freezer, let the samples thaw and then boil them at 95 $^{\circ}\text{C}$ for 5 min.
15. Load the molecular weight marker (Spectra™ Multicolor Broad Range Protein Ladder; 5 μl) and the samples.
16. Close the electrophoresis chamber with a lid and connect it to the power supply. Again, the red color label must fit another red color label; black color label must fit another black color label.
17. Set the constant voltage to 100 V and run the separation. When the dye front reaches the stacking – separation gel interface, set the constant voltage to 120 V and let it run until the dye front reaches the tail of the gel. Then stop the power supply.
18. Decompose the electrophoresis chamber. Carefully separate the short plate from the spacer plate and remove the stacking gel. The gel is now ready for subsequent Western blotting procedure.

TABLE 33.1 Choose the proper acrylamide concentration according to molecular weight (MW) of your protein of interest.

Final concentration of acrylamide	MW (kDa) of proteins separated on this gel (linear separation)
15 %	3 - 100
12 %	10 - 200
10 %	20 - 300
8 %	50 - 500

TABLE 33.2. Separation gel preparation – choose the proper acrylamide concentration in Table 33.1., decide how many gels are needed and pipette the reagents according to this table.

Volumes (ml) for 2 gels								
Reagent	8% 1 mm	10% 1 mm	12% 1 mm	15% 1 mm	8% 1.5 mm	10% 1.5 mm	12% 1.5 mm	15% 1.5 mm
30% acrylamide/Bis 29:1	4	5	6	7.5	5.6	7	8.4	10.5
H ₂ O	6.95	5.95	4.95	3.45	9.73	8.33	6.93	4.83
1.5 M Tris-HCl, pH 8.8	3.75	3.75	3.75	3.75	5.25	5.25	5.25	5.25
10% SDS	0.15	0.15	0.15	0.15	0.21	0.21	0.21	0.21
10% APS	0.15	0.15	0.15	0.15	0.21	0.21	0.21	0.21
TEMED	0.009	0.006	0.006	0.006	0.0126	0.0084	0.0084	0.0084

TABLE 33.3 Stacking gel preparation – stacking gel is always 4% in our lab, decide how many gels are needed and pipette the reagents according to this table.

Reagent	Volumes (ml) for 2 gels	
	1 mm	1.5 mm
30% acrylamide/Bis 29:1	0.85	1.7
H ₂ O	3.4	6.8
0.5 M Tris-HCl, pH 6.8	0.625	1.25
10% SDS	0.05	0.1
10% APS	0.05	0.1
TEMED	0.005	0.001

TABLE 33.4 Choose the proper comb according to the well capacity.

Comb	Well capacity (μl)
1 mm, 10 wells	30
1 mm, 15 wells	20
1.5 mm, 10 wells	50
1.5 mm, 15 wells	30

C. Western blot transfer and immunodetection of proteins on a membrane

Solutions

- **Cathode buffer**

25 mM Tris base, 192 mM glycine, 10% methanol, adjust the pH to 9.4 with HCl.

- **Anode I buffer**

0.3 M Tris base, 10% methanol, adjust the pH to 10.4 with HCl.

- **Anode II buffer**

25 mM Tris base, 10% methanol, adjust the pH to 10.4 with HCl.

- **10× Tris-buffered saline (TBS) buffer**

0.2 M Tris base, 1.5 M NaCl, adjust the pH to 7.6 with HCl.

- **1× TBS/T buffer**

Dilute 100 ml of 10× TBS to 1 l of water, add Tween-20 to 0.1% final concentration.

- **Blocking buffer**

5% bovine serum albumin (BSA) in 1× TBS/T buffer.

- **Primary antibody buffer**

5% bovine serum albumin (BSA), 0.1% sodium azide in 1× TBS/T buffer.

Steps

1. After electrophoresis, put the gel in the cathode buffer for 10 min for equilibration.
2. Choose the membrane for Western blot. It is possible to use PVDF or nitrocellulose membrane (see differences in Tab. 5) and choose the pore size. Membrane with 0.45 µm pore size is used for general Western blotting application but not low molecular weight proteins. Membrane with 0.22 µm pore size is used for blotting applications of proteins with molecular weight less than 20 kDa. Cut the membrane to the same size as a gel.
3. If using nitrocellulose membrane, equilibrate it in anode II buffer for 5 min and proceed to step 5.
4. If using PVDF membrane, activate it in

100% methanol for 15 s, the membrane will change from an opaque white to a translucent grey. Immerse the membrane in water for 1 – 2 min to replace the methanol, keep the membrane submerged under the water table. Equilibrate the membrane in a transfer buffer (anode II) for 5 min.

5. Cut the filter papers to the same size as a gel. Submerge 3 filter papers into anode I buffer, 2 filter papers into anode II buffer, 3 filter papers into cathode buffer.
6. Take out a cassette of Trans-blot®Turbo™ Transfer System, open it and assemble the components on the cassette. In case of one gel per cassette, place a gel in the middle of the cassette. In the case of two gels per cassette, they should be placed side by side towards the center. There are two cassettes, and it is necessary to have the same number of gels in both cassettes.
7. Bottom of the cassette is anode; the top is the cathode. Assemble the blotting sandwich in the following order. Place: 3 filter papers from anode I buffer on the bottom of the cassette, then 2 filter papers from anode II buffer, then blotting membrane, then gel and finally 3 filter papers from cathode buffer. After putting of each layer, remove air trapped between the layers using the roller.
8. Close the cassettes with lid and lock the cassette by turning the knob clockwise. Insert the cassettes into the instrument. Select the protocol for transfer. In our lab, STANDARD SD protocol (30 min; up to 1 A; 25 V constant) was successfully used for transfer of proteins from 120 kDa to 10 kDa. However, it is possible to choose special protocol for high or low MW proteins as well. Choose the number of gels per cassette. Press A:RUN and/or B:RUN (depending on the fact which cassettes are used) to start the transfer.
9. At the end of the transfer, the instrument will signalize the end by sound and the message RUN COMPLETE! will appear on display. Remove the cassettes and unlock the lid.
10. Disassemble the blotting sandwich and take out the membrane for subsequent immunodetection.
11. After the blotting, wash the membrane with 1× TBS/T buffer on a shaker twice.
12. Submerge the membrane into the blocking

buffer and incubate at room temperature for 1 h on a shaker.

13. Prepare the primary antibody buffer in the 50 ml Falcon tube, 5 ml of the solution is sufficient for one membrane. Dilute the primary antibody according to the primary antibody datasheet. The dilution is different for individual primary antibodies; optimal dilution is always written in the datasheet.
14. After 1 h incubation, take out the membrane from the blocking buffer and place it the 50 ml Falcon tube with primary antibody solution. Incubate the membrane overnight at 4 °C on a tube roller mixer.
15. The next day, take out the membrane of the tube. Wash the membrane with 1× TBS/T buffer on a shaker five times.
16. During the last washing step dilute the secondary antibody in the blocking buffer. The recommended dilution for most secondary antibodies is 1:10,000 or see the datasheet for the individual secondary antibody. Use the secondary antibody against the organism from which comes the primary antibody (anti-mouse secondary antibody against mouse primary antibody etc.). Horseradish peroxidase (HRP) labeled secondary antibodies are used.
17. After washing, incubate the membrane with a secondary antibody at room temperature for 1 h on a shaker.
18. Wash the membrane with 1× TBS/T buffer on a shaker three times.
19. Incubate the membrane with HRP substrate for 5 min in the dark, then perform the chemiluminescence detection.

20. Detection is done on a LI-COR Odyssey® Fc Imaging System. Set the acquisition to 10 min exposure for chemi channel and 0.5 s for 700 nm channel, 700 nm channel is set to see the protein molecular weight marker.
21. After acquiring, rotate, edit and save the image. Interpret your Western blot result.

IV. Comments

- When cultivating cells, 10×10^6 cells is sufficient, lyse it in 100 µl of lysis buffer.
- RIPA lysis buffer is used in this protocol, but it is possible to use another lysis buffer which is sufficient for your application, i. e. for extraction of membrane proteins.
- As a blocking buffer you can use 5% nonfat dry milk instead of 5% BSA, but then, it is needed to wash the membrane in 1× TBS/T buffer three times prior to incubation with primary antibody.
- Concerning the optimal dilution of primary and secondary antibodies, there is always a range of optimal dilutions in the datasheet, you have to test which dilution will provide the best signal.
- The primary antibody solution can be stored in the freezer and be reused at least twice more.
- Horseradish peroxidase (HRP) labeled secondary antibodies are frequently used but it is possible to use any other secondary antibodies, i. e. fluorescently labeled.

V. Pitfalls

Problem	Cause and cure
Unusual or unexpected bands	• Protease degradation – keep the samples on ice during sample preparation. • Unspecific antibody – try another antibody. • If the detected bands are heavier than expected, it could be caused by quaternary structures - reheating the sample can help. Alternatively, it could be protein complexes – for further study.

Blurry bands	• High voltage or air bubbles present during transfer – run the gel at a lower voltage and cast the transfer sandwich properly.
Non-flat bands	• Too fast travel through the gel.
White (negative) bands	• Too much protein or antibody – use the lower amount.
No bands	• The improper antibody is used – check it; some antibodies are not to be used for Western blot. • Too low protein concentration (under the detection limit) or antibody concentration. • Absence of the antigen, it is not there or it was not extracted – try another sample preparation protocol (changing lysis buffer by more efficient can help). • Try to use antigen from another source to confirm whether the problem lies with the sample or with other reagents, such as the antibody. • Long washing can decrease the signal. • Contaminated buffers – try to prepare fresh ones.
Weak signals	• Low protein or antibody concentration. • Long washing steps.
High background	• Too high concentration of the antibody. • Too old buffers. • Too short washing – increase the washing time. • Too high of an exposure – try different exposure time to choose the optimal.
Patchy and uneven spots	• Improper transfer, air bubbles during the transfer. • It is important to use a shaker for incubation and washing steps. • Antibodies binding to the blocking agents - another blocking agent should be tried. • Contaminants in blocking buffer – filter it. • Aggregation of the secondary antibody – centrifuge or filter the secondary antibody solution to remove the aggregates.

**Adapted according to Mahmood T and Yang PC (1) and supplied by other pitfalls from our lab.*

TABLE 33.5 PVDF vs. Nitrocellulose Membranes (adapted according to citations 1, 2, 8, 9).

Nitrocellulose membrane		PVDF membrane	
+/-	Comment	Comment	+/-
+	Has a high affinity for proteins and high retention		
+	Does not require activation, easily wetted in water or transfer buffer.	Requires methanol activation.	-
+	Less expensive.	More expensive.	-
+	Lower nonspecific background binding of antibodies.	Higher nonspecific background binding of antibodies.	-
-	Lower binding capacity.	Higher binding capacity.	+
-	Unsupported nitrocellulose is not recommended for reprobing. We use supported nitrocellulose which can be used for reprobing.	Better mechanical support, the membrane is good for reprobing and storage.	+
-	Methanol in transfer buffers has the effect of reducing the pore size of the gel, which restricts the transfer of some molecules.		
-	Proteins are not covalently bound; nitrocellulose is brittle when dry. Small proteins tend to move through nitrocellulose, using membranes with smaller pores can obviate this.		
		Retains proteins during exposure to acidic or basic conditions and in the presence of organic solvents.	+
		Better binding efficiency in the presence of SDS in the transfer buffer.	+

VI. Acknowledgment

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Proteomic analysis of complex samples by mass spectrometry

Dušan Holub

I. Introduction

Mass spectrometry-based proteomics is today most widespread tool in biology due to its ability to identify and quantify thousands of proteins from different samples (e.g., cell culture, cerebrospinal fluid, plasma, serum, tissue, and urine) (1). In proteomics, sample preparation, instrumentation and software are critical parts required to accomplish a successful experiment (2).

In this chapter, we will focus on proteomic sample preparation for mass spectrometry analysis. In general, several sample processing steps are required as proteins extraction, reduction, alkylation, enzymatic digestion of protein to peptides and peptide purification (Scheme 34.1). Many options for sample preparation were introduced in proteomics like iST (3), filter-assisted sample preparation – FASP (4), urea sample preparation, in-solution sample preparation with acetone precipitation, and in-gel digestion (2). Each of these methods has advantages and disadvantages, and all of these methods are capable of providing a peptide sample compatible with mass spectrometric

analysis (2). It is not within the scope of this chapter to offer an exhaustive description overview of many options for proteomic sample preparation. The goal of this chapter is to describe a fast and straightforward protocol with minimal sample manipulation used to prepare samples for mass spectrometry-based proteomic analysis.

II. Materials and instrumentation

Reagents

Trypsin/Lys-C Mix was obtained from Promega (V5071, Promega, East Port Praha). TRIS was obtained from SERVA (37180.04, SERVA, BioTech). Tris[2-carboxyethyl]phosphine hydrochloride (TCEP), Sodium deoxycholate (SDC), 2-chloroacetamide (CAA), Trifluoroacetic acid (TFA), Formic acid (FA) and Ethyl acetate were purchased from Sigma-Aldrich (C4706, 30970, 22790, 14264, 56302 and 34972, Sigma-Aldrich, Merck). Acetonitrile (ACN) and water were purchased from Millipore (100029 and 115333, Millipore, Merck).

Equipments and consumables

- Pipettes (0.5 µl – 1000 µl) with corresponding tips (Eppendorf)
- Vortex mixer (REAX control, Heidolph)
- pH paper (Lach:ner)
- 15 ml and 50 ml plastic tubes (Eppendorf)
- 96-Well MACROSpin C18 plate (74-5657, The Nest Group, KRD, Harvard Apparatus)
- Benchtop centrifuge (Centrifuge 5810R, Eppendorf)
- Oven at 37 °C (MS Incubator, Major Science)
- Vacuum centrifuge (Concentrator 5301, Eppendorf)
- LC-MS vials and inserts (Santiago s.r.o.)
- Microcentrifuge (MiniSpin®, Eppendorf)

Solutions

• Lysis buffer

1 % SDC (v/v), 10 mM tris[2-carboxyethyl] phosphine, 40 mM 2-chloroacetamide, 100 mM Tris buffer pH 8.5

• Digestion buffer

2 µg Trypsin/LysC mix per 50 µl water

III. Procedures

A. Lysis of cells (1 – 100 µg of total protein per sample)

1. Add 50 µl SDC lysis buffer to 100 µg of total protein per sample (approximately, 0.3×10^6 HeLa cells) and place sample in a heating block at 95 °C for 10 min.
2. Spin down droplets in a microcentrifuge at

300 rpm for 10 sec.

3. Shear DNA in a sample using a sonicator (10 cycles 30 sec ON/OFF).

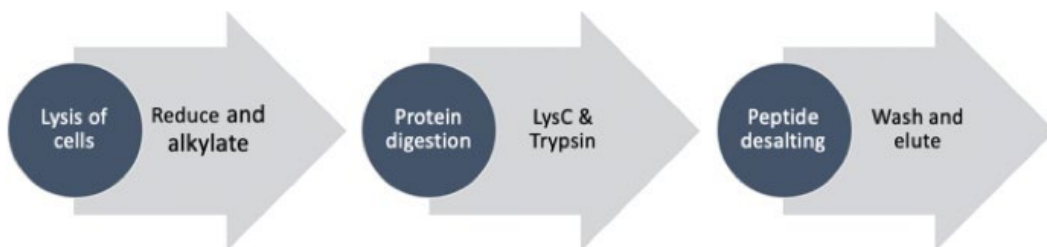
B. Protein digestion

1. Add 50 µl of digestion buffer (Trypsin/Lys-C mix in water) to protein sample and place it in a heating block at 37 °C for 3h. Note.: Digestion buffer contain respective amounts of the proteolytic enzyme to ensure a ratio of 1:50 (2 µg of the enzyme to 100 µg of protein).
2. Terminate digestion by adding 50 µl of 2% TFA to sample and mix. Check the pH (2 - 3).
3. For extraction of sodium deoxycholate add 50 µl of ethyl acetate into the sample and mix.
4. Centrifuge the sample at 13,200 rpm for 5 min and remove the organic phase with sodium deoxycholate.
5. Use the water phase with peptides for desalting on a C18 column.

C. Peptide desalting

Column equilibration

1. Place 96-well MACROSpin C18 plate on top of 96-well plate (waste plate) and remove the aluminum protection from the wells that will be used.
2. Add 200 µl of MeOH to each well, centrifuge at 1,000 x g for 2 min and discard flow-through.
3. Add 200 µl of 80% ACN with 0.1 % TFA to each well, centrifuge at 1,000 x g for 2 min and discard flow-through.



Scheme 34.1 General proteomic sample preparation workflow. Proteins are extracted from cell lysates and digested to peptides. The peptides are desalted before mass spectrometry analysis.

4. Add 200 μ l of 0.1% TFA to each well, centrifuge at 1,000 x g for 2 min and discard flow-through.
5. Repeat step 3-4 two more times.

Sample loading

1. Place 96-well MACROSpin C18 plate on a new 96-well plate.
2. Load samples on 96-well MACROSpin C18 plate, centrifuge at 1,000 x g for 2 min and do not discard flow-through.
3. Place 96-well plate (waste plate) under 96-well MACROSpin C18 plate.
4. Load flow-through from step 2 on 96-well MACROSpin C18 plate, centrifuge at 1,000 x g for 2 min and discard flow-through.
5. Add 200 μ l of 0.1 % TFA to each well, centrifuge at 1,000 x g for 2 min and discard flow-through.
6. Repeat step 5 two more times.

Elution and sample preparation

1. Place 96-well plate MACROSpin C18 plate on a new elution 96-well plate.
2. Add 200 μ l 50 % ACN with 0.1 % TFA, centrifuge at 1,000 x g for 2 min and keep flow-through in the elution 96-well plate.
3. Place elution 96-well plate in a SPEED-VAC at 45 °C, until completely dry.
4. Dissolve dried samples in LC solution to obtained peptide concentration of 1 μ g/ μ L.
5. Transfer samples to vials for LC-MS analysis or samples can be frozen at -20 °C.

D. Liquid chromatography and mass spectrometry

Analyze samples by LC-MS consisting of a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled via an EASY-spray ion source (Thermo Fisher Scientific) to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) with the following LC setting:

1. Setup LC condition for the preconcentration onto trap column (Acclaim PepMap 100, 100 μ m ID, C18, 5 μ m particles, 100 Å pore size; Thermo Fisher Scientific) in the nanoLC-MS system.

2. Separate purified peptides on 50 cm EASY-Spray column (75 μ m ID, PepMap C18, 2 μ m particles, 100 Å pore size; Thermo Fisher Scientific).
3. Use mobile phase A (water with 0.1% formic acid), B (acetonitrile with 0.1% formic acid) and loading buffer (1% acetonitrile with 0.1% formic acid in water).
4. Keep column temperature at 35 °C.
5. Inject approximately 1 μ g of peptides and load peptide onto trap column first 5 min by loading buffer at a flow rate of 6 μ l/min.
6. After 5 min, switch the valve (1_2 to 10_1) and for peptide separation on the column used the following gradient at 300 nL/min: 0 - 5 min (2% buffer B), 5 - 125 min (2 - 35% buffer B), 125 - 127 min (35 - 90% buffer B), 127 - 133 min (90% buffer B), 133 - 135 min (90 - 2% buffer B) and 135 - 155 min (2% buffer B). Switch the valve back (10_1 to 1_2) at 150 min.

For MS acquisition use following setting:

1. Spray voltage 2200 V.
2. Ion Transfer Tube Temperature: 200 °C
3. MS-OT: Orbitrap Resolution 120,000, the scan range of 300-1700 m/z, an AGC Target of 1.0×10^6 , a max injection time 35 ms.
4. Monoisotopic precursor selection filter: Peptide.
5. Charge Stage: 2 - 4.
6. Dynamic exclusion: 70 s.
7. Top12 data-dependent MS2 OT CID: Quadrupole isolation, a 2 m/z isolation window, CID activation, 35 collision energy, Normal scan range, 110 m/z fixed first mass, the ion target value of 5.0×10^4 , 100 ms max injection time.

E. Data and bioinformatics analyses

MS raw file(s) analyze by MaxQuant software (version 1.6.5.0) with the following setting:

1. Peptide list search against the human Uniprot FASTA database and common contaminants database by the Andromeda search engine with cysteine carbamidomethylation as a fixed modification and N-terminal acetylation and methionine oxidations as variable modification.

2. The false discovery rate set to 0.01 for both proteins and peptides.
3. Setup a minimum length of seven amino acids.
4. Use trypsin as protease and allow a maximum of two missed cleavages in the database search.
5. Allow initial precursor mass deviation up to 7 ppm and fragment mass deviation of 0.5 Da for peptide identification.
6. Perform matching between runs.
7. Perform LFQ with a minimum ratio count of 2.

The bioinformatics analyses are done by the Perseus software (version 1.6.5.0).

IV. Comments

The procedure described herein is optimized for 1 - 100 µg of cell lysate protein at 1 µg/µL concentration. However, the procedure may be used for 100 - 200 µg of cell lysate with an appropriate amount of reagents (TCEP, CAA, or Trypsin/Lys-C mix).

V. Pitfalls

Problem	Cause and cure
Low protein yield	An insufficient number of cells. → Increase the number of cells used for lysis.
Incomplete digestion	Inactive enzyme. → Prepare fresh enzyme solution.

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Sample preparation of formalin-fixed paraffin-embedded tissue for mass spectrometry-based proteomic analysis

Dušan Holub

I. Introduction

Proteins are the main players in the regulation of physiological and pathophysiological activities of mammalian tissues. Formalin fixation and paraffin-embedding (FFPE) are one of the main methods to preserve tissues for routine clinical diagnostics and archival purposes. As such, FFPE specimens represent a collection of clinically annotated samples that are stored for a long time at room temperature (1). Protein content varies considerably across different tissue types. Usually, 1 mm³ of tissue corresponds to roughly 100 µg protein. Based on the goals of the project, it is necessary to consider which cells from different anatomical regions (whole tissue or specific region) will be required and collected. For example, laser capture microdissection enabled region specific tissue analyses are critical to better understand complex multicellular processes. Mass spectrometry-based analyses of tissues have allowed gaining a deeper understanding of the proteome, including substantial differences in protein, its abundances, cell type-dependent and time-dependent protein expression changes (2, 3, 4). The goal of this chapter is to describe a fast and robust protocol to prepare samples from FFPE for mass spectrometry-based proteomic analysis to ensure deep proteome coverage (Scheme 35.1).

II. Materials and instrumentation

Reagents

Trypsin/Lys-C Mix (V5071, Promega, East Port Praha), TRIS (37180.04, SERVA, BioTech), Xylene (534056, Sigma-Aldrich), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (C4706, Sigma-Aldrich), Sodium deoxycholate (SDC) (30970, Sigma-Aldrich), 2-chloroacetamide (CAA) (22790, Sigma-Aldrich), Trifluoroacetic acid (TFA) (14264, Sigma-Aldrich), Formic acid (FA) (56302, Sigma-Aldrich), Ethyl acetate (34972, Sigma-Aldrich), Acetonitrile (100029, Millipore) and water (115333, Millipore).

Equipment and consumables

- Pipettes (0.5 µl – 1000 µl) with corresponding tips (Eppendorf)
- Vortex mixer (REAX control, Heidolph)
- pH papers (Lachner)
- 15 ml and 50 ml plastic tubes (Eppendorf)
- 96-Well MACROSpin C18 plate (74-5657, The Nest Group, KRD, Harvard Apparatus)
- Benchtop centrifuge (Centrifuge 5810R,

- Eppendorf)
- Oven at 37 °C (MS Incubator, Major Science)
- Vacuum centrifuge (Concentrator 5301, Eppendorf)
- LC-MS vials and inserts (Santiago s.r.o)
- Microcentrifuge (MiniSpin®, Eppendorf)
- Microtome
- Microscope glass

Solutions

• Lysis buffer

1 % SDC (v/v), 10 mM tris (2-carboxyethyl) phosphine, 40 mM 2-chloroacetamide, 100 mM Tris buffer pH 8.5

• Digestion buffer

2 µg Trypsin/LysC mix per 50 µl water

III. Procedures

Workflow for FFPE tissue sample preparation: Tissues are sectioned using a microtome. The slides with paraffin sections are placed on the microscopic glass surface to bond the tissue. Paraffin is removed from the slides by several washing steps. Whole tissue slide is collected. Cells are lysed, proteins are extracted and solubilized. The proteins are reduced and alkylated in a single step followed by digestion with Trypsin/Lys-C mix. Sample contaminants are removed to prepare the peptide sample for LC-MS analysis.

Sample Preparation

A. Prepare Sections on Slides

1. Cut the tissue block into 4-µm sections and place three sections onto a slide.
2. Incubate at 37 °C to secure sections on the slide.

B. Deparaffinization

1. Transfer the slide to a staining dish containing fresh xylene. Incubate for 10 min at RT.
2. Repeat step 1 twice, using fresh xylene each time.
3. Transfer the slide to a staining dish containing fresh 100% ethanol. Incubate for 10 min at RT.
4. Repeat step 3 using fresh 100% ethanol.
5. Transfer the slide to a staining dish containing fresh 96% ethanol. Incubate for 10 min at RT.
6. Repeat step 5 using fresh 96% ethanol.
7. Transfer the slide to a staining dish containing fresh 70% ethanol. Incubate for 10 min at RT.
8. Repeat step 7 using fresh 70% ethanol.
9. Remove the slide and air dry.

C. Cell lysis

1. Add 50 µl of lysis buffer to deparaffinized sample and place it in a heating block at 95 °C for 60 min.
2. Spin down droplets in a microcentrifuge at 300 rcf for 10 sec.
3. Shear DNA in a sample using a sonicator (10 cycles: 30 sec ON/OFF).

D. Protein digestion

1. Add 50 µl of digestion buffer (Trypsin/Lys-C mix) to protein sample and place it in a heating block at 37 °C for 3h.
2. Note.: Digestion buffer contains respective amounts of the proteolytic enzyme to ensure a ratio of 1:50 (2 µg of the enzyme to 100 µg of protein).
3. Terminate digestion by adding 50 µl of 2% TFA to sample and mix.
4. Check the pH (2 - 3).
5. For extraction of sodium deoxycholate add 50 µl of ethyl acetate into the sample and mix.



Scheme 35.1 FFPE tissue sample preparation workflow for proteomic analysis.

6. Centrifuge the sample at 13,200 rpm for 5 min and remove the organic phase with sodium deoxycholate.
7. Use water phase with peptides for desalting on a C18 column.

E. Peptide desalting

Column equilibration

1. Place 96-well MACROSpin C18 plate on top of 96-well plate (waste plate) and remove the aluminum protection from the wells that will be used.
2. Add 200 μ l of MeOH to each well, centrifuge at $1,000 \times g$ for 2 min and discard flow-through.
3. Add 200 μ l of 80% ACN with 0.1 % TFA to each well, centrifuge at $1,000 \times g$ for 2 min and discard flow-through.
4. Add 200 μ l of 0.1% TFA to each well, centrifuge at $1,000 \times g$ for 2 min and discard flow-through.
5. Repeat step 2 - 4 two more times.

Sample loading

1. Place 96-well MACROSpin C18 plate on a new 96-well plate.
2. Load samples on 96-well MACROSpin C18 plate, centrifuge at $1,000 \times g$ for 2 min and do not discard flow-through.
3. Place 96-well plate (waste plate) under 96-well MACROSpin C18 plate.
4. Load flow-through from step 2 on 96-well MACROSpin C18 plate, centrifuge at $1,000 \times g$ for 2 min and discard flow-through.
5. Add 200 μ l of 0.1 % TFA to each well, centrifuge at $1,000 \times g$ for 2 min and discard flow-through.
6. Repeat step 6 two more times.

Elution and sample preparation

1. Place 96-well plate MACROSpin C18 plate on a new elution 96-well plate.
2. Add 200 μ l 50 % ACN with 0.1 % TFA, centrifuge at $1,000 \times g$ for 2 min and keep flow-through in the elution 96-well plate.
3. Place elution 96-well plate in a SPEED-VAC at 45 °C, until completely dry.
4. Dissolve dried samples in LC solution to

obtain peptide concentration of 1 μ g/ μ L.

5. Transfer samples to vials for LC-MS analysis or samples can be frozen at -20 °C.

F. Liquid chromatography and mass spectrometry

Analyze samples by LC-MS consisting of a Dionex UltiMate 3000 RSLCnano system (Thermo Fisher Scientific) coupled via an EASY-spray ion source (Thermo Fisher Scientific) to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) with the following LC setting:

1. Setup LC condition for the preconcentration onto trap column (Acclaim PepMap 100, 100 μ m ID, C18, 5 μ m particles, 100 Å pore size; Thermo Fisher Scientific) in the nanoLC-MS system.
2. Separate purified peptides on 50 cm EASY-Spray column (75 μ m ID, PepMap C18, 2 μ m particles, 100 Å pore size; Thermo Fisher Scientific).
3. Use mobile phase A (water with 0.1% formic acid), B (acetonitrile with 0.1% formic acid) and loading buffer (1% acetonitrile with 0.1% formic acid in water).
4. Keep column temperature at 35 °C.
5. Inject approximately 1 μ g of peptides and load peptide onto trap column first 5 min by loading buffer at a flow rate of 6 μ l/min.
6. After 5 min switch the valve (1_2 to 10_1) and separate peptides on the column used the following gradient at 300 nL/min: 0 - 5 min (2% buffer B), 5 - 125 min (2 - 35% buffer B), 125 - 127 min (35 - 90% buffer B), 127 - 133 min (90% buffer B), 133 - 135 min (90 % buffer B) and 135 - 155 min (2% buffer B). Switch the valve back (10_1 to 1_2) at 150 min.

For MS acquisition use following setting:

1. Spray voltage 2200 V.
2. Ion Transfer Tube Temperature: 200 °C.
3. MS-OT: Orbitrap Resolution 120,000, the scan range of 300-1700 m/z, an AGC Target of 1.0e6, a max injection time 35 ms.
4. Monoisotopic precursor selection filter: Peptide.
5. Charge Stage: 2 - 4.

- Dynamic exclusion: 70 s.
- Top12 data-dependent MS2 OT CID: Quadrupole isolation, a 2 m/z isolation window, CID activation, 35 collision energy, Normal scan range, 110 m/z fixed first mass, the ion target value of 5.0×10^4 , 100 ms max injection time.
- Allow initial precursor mass deviation up to 7 ppm and fragment mass deviation of 0.5 Da for peptide identification.
- Perform matching between runs.
- Perform LFQ with a minimum ratio count of 2.

The bioinformatics analyses are done by the Perseus software (version 1.6.5.0).

G. Data and bioinformatics analyses

MS raw file(s) analyze by MaxQuant software (version 1.6.5.0) with the following setting:

- Peptide search against the human Uniprot FASTA database and common contaminants database by the Andromeda search engine with cysteine carbamidomethylation as a fixed modification and N-terminal acetylation and methionine oxidations as variable modification.
- The false discovery rate set to 0.01 for both proteins and peptides.
- Setup a minimum length of seven amino acids.
- Use trypsin as protease and allow a maximum of two missed cleavages in the database search.

IV. Comments

The procedure described herein is optimized for 1 - 100 µg of protein extracted from FFPE tissue sample. However, the procedure may be used for 100 - 200 µg of protein extracted from FFPE tissue sample with an appropriate amount of reagents (TCEP, CAA, Trypsin/Lys-C mix).

V. Pitfalls

Problem	Cause and cure
Low protein yield	Insufficient amount of tissue. → Increase the amount of tissue used for lysis.
Incomplete digestion	Inactive enzyme. → Prepare fresh enzyme solution.

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Methods of targeted mass spectrometry in proteomics

Tomáš Oždian and Martina Jakoubková

I. Introduction

Proteomics is one of the fields in molecular biology, which relies on analytical chemistry methods. Modern proteomics is based on mass spectrometry; precise analysis of protein and peptide mass and its identification. The principle of proteomic experiment and sample preparation is explained in Chapter 34. Briefly, proteins are digested into peptides which will be separated and analyzed by mass spectrometry. The masses are searched through the protein database and finally, the list of identified proteins will be created. Although this approach is very powerful in the discovery of protein composition, it is very costly and generates huge amount of data – from hundreds of megabytes to gigabytes per single sample. Moreover, due to the nature of the proteomic analysis, most of the peptides will not be identified in all samples. Targeted analysis is thus required for clinical and scientific routine. This can be done either using ELISA (1) or targeted mass spectrometry.

Mass spectrometry (MS) methods have undergone big developments since its beginnings in 19th century (2). This method proved its versatility in i.e. isotope determination of elements,

determination of organic compounds mass, and finally, in analysis of biologic samples. Hand in hand with the grow of the method versatility was the development of instrumentation. Ion sources were developed to provide optimized ionization with less ion fragmentation and mass analyzers to provide better resolution and higher sensitivity. Electrospray, which is widely used in the field of biochemistry currently. Such dominance is not in the case of mass analyzers. There are multiple choices of mass analyzers depending of instrument price and required parameters.

The only mass analyzer, that has ion isolation ability, is quadrupole mass analyzer (3). That is usually used for targeted analysis. However, quadrupole itself does not have a high mass resolution, and therefore there will be other compounds of the same molecular weight in the background which may interfere with the signal.

For even more precise filtering of a compound of interest, single quadrupole is not sufficient. However, it can be used as a mass filter in more complex, hybrid mass spectrometers. Such instruments usually consist of two quadrupoles at the beginning – one serving as a mass filter and second one as a collision cell for a collision of ions

with inert gas, usually helium or nitrogen. This collision will lead to the destruction of analyzed ion and its fragmentation to smaller parts – fragments. Those fragments are unique for the compound and instrument used. For analysis of fragments, the last part of the hybrid mass spectrometer is analyzer of fragment masses. The analyzer of choice is the third quadrupole, set as a filter for specific fragment mass. Thus, selectivity for the compound of interest is raised not only to the mass of the whole ion but to the mass of specific fragment as well. The one setting of parent mass and corresponding fragment mass is called transition. The probability that any other interfering compound with a similar molecular mass will be fragmented to a similar fragment is much lower but still is there. To even minimize this probability, there are usually more transitions monitored for one compound. This leads to a unique signature of a compound of interest (4).

Nowadays, there are another hybrid mass spectrometers as well. Instead of analyzing fragment ions in the third quadrupole, they offer high-resolution mass analyzers such as time-of-flight or Orbitrap analyzer. This leads to a much higher precision of analysis. On the other side, those analyzers do not offer filtering ability. Complete fragmentation spectrum is thus recorded and fragment ions of interest are extracted in-silico after analysis. This has one advantage that any other fragment of interest can be extracted at any time after analysis (5).

The procedure described here is known as selected or multiple reaction monitoring (SRM/MRM) and is the nowadays standard method of targeted analysis of compounds. With modern electronics, the set-up of mass spectrometer quadrupoles can change very quickly, allowing to monitor tens to hundreds of transitions during the same analysis. Effectivity of this process will be dependent on the synchronization of chromatographic separation with the setup of the mass spectrometer.

The mass spectrometer itself does not allow to analyze very complex matrices. It is thus beneficial to couple mass spectrometer with some separation technique. Today, the most versatile separation system is liquid chromatography (LC) using a reverse phase column. This system allows separating most of the compounds with sufficient effectivity. The other modes of liquid chromatography can be used in specific cases,

usually when the reverse phase is not suitable for sample separation. The reverse phase based LC-MS is the method of choice also for proteomics. It is common that proteome complexity is reduced different ways, but if the final analysis is done on a mass spectrometer, LC with reversed phase separation is used almost exclusively.

II. Materials and instrumentation

Liquid chromatograph

For connection with MS, the best options are high-performance LCs with flow up to approximately 500 $\mu\text{L}\cdot\text{min}^{-1}$. This limitation lays in the effectivity of electrospray to ionize larger flows effectively. For LC-MS connection, there can be used even smaller scales of chromatography like a capillary or nano-flow chromatography. Those variants offer a lower flow of solvents and higher sensitivity. On the other side, those techniques require specific pumps and columns and are more challenging to optimize. In this chapter, there will be showed Thermo Ultimate 3000 LCs as they are standard instrumentation in our institute. However, the steps described applies to different LCs as well.

Mass spectrometer

There are multiple mass spectrometers suitable for targeted mass analysis. The golden standard of such instruments are triple quadrupoles, eventually with linear ion trap instead of the last quadrupole. Those instruments are also available from big amount of vendors (AB Sciex, Agilent, Shimadzu, Thermo etc.). The higher level of instruments consists of a high-resolution detector like time-of-flight or Orbitrap at the end of the ion path. The instrument used for this chapter is quadrupole – linear ion trap AB Sciex Qtrap 5500.

Chemicals

All chemical needed in this experiment has to be the highest purity. This purity is usually marked as HPLC or LC-MS grade. The lack of impurities in those chemicals will provide a clear signal without elevated noise.

The basic solvents for reversed phase LC are water and organic solvent. The organic solvent may vary depending on sample polarity. The solvent of choice for proteomics is acetonitrile.

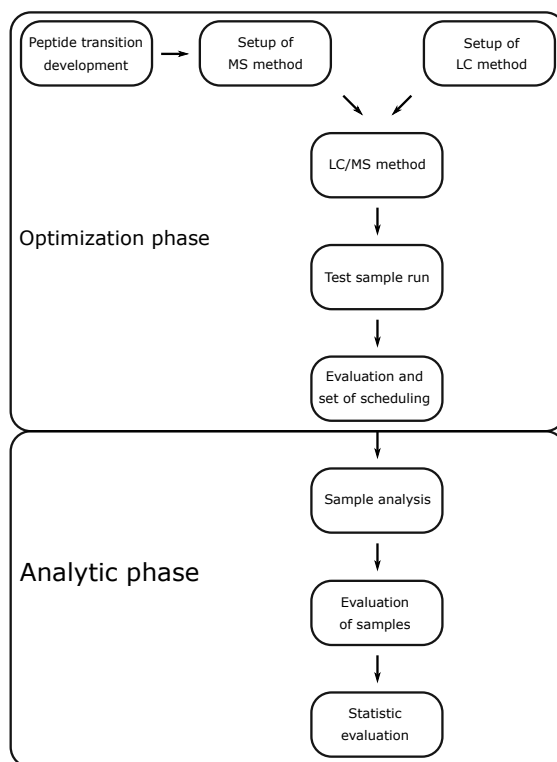
The solvents for LC has to be often modified to enhance separation and ionization efficiency. pH is the most often modified property by a variety of buffers. The specific of LC-MS is that those buffers have to be volatile. During the process of evaporation, the buffers have to decompose to inert gases like ammonia or carbon dioxide. The formic acid in very low concentration (below 0.1%) is typically used in proteomic experiments.

For sample optimization, it is highly recommended to use synthetic peptides corresponding to target sequences. Those peptides could be of two types – regular, which will be used for SRM assay development, method optimization and check and isotopically labeled. Those peptides are then added into samples as an internal standard for quantification and peptide identity verification.

Software

Software used in this experiment is Analyst v. 1.6.2 for mass spectrometer control and Chromeleon v. 6.80 (DCMSlink v. 2.10 respectively) for LC control. Those softwares are instrument specific and will differ across the vendors.

The Skyline software (6) is an academic free program for optimization of targeted proteomics, sample processing and statistical evaluation.



Scheme 36.1 Typical workflow of targeted proteomic experiment

The most complicated part of targeted proteomics is the optimization of the method. In the beginning, it is necessary to set-up peptide transitions into the mass spectrometer and set-up LC program including sample draw and pumps gradient. Then, test samples could be run. On those samples, the correctness of the LS and MS method is checked. Optionally, based on test runs, a MRM scheduling can be set. Thus each transition is set up only in time frame around retention time of peptide and saves instrument time during the rest of the experiment.

A. Peptide transition development

1. Open new document in Skyline
2. Input target proteins. This can be done by inputting FASTA files from the database (e. g. Uniprot.org), or directly copy and paste proteins or sequences of interest. The protein sequences are automatically digested, and peptides with the best criteria for targeted proteomics are shown including fragment masses. There are several conditions for peptides to be suitable for SRM. There should not be present amino acids suitable to modifications, both-post-translational or created during sample processing. The peptide length should not be shorter than 6 amino acids (those peptides are not unique) or longer than 20 amino acids (those peptides have poor ionization efficiency). Those parameters should be set in Skyline in Settings/Peptide settings... The last property of the peptide is that it has to be proteotypic. This means that it should be present only in the protein of interest. This parameter is checked by comparing candidate peptide against human proteome by BLAST (7). Peptides, which are present in candidate proteins can only be used in the analysis. Another, Skyline independent way of selecting peptides is SRM atlas, which provides SRM spectra experimentally obtained for different proteins (8).
3. The list of transitions satisfactory to experiment aim and instrument can be directly exported by menu File > Export > Transition list. This transition list can be created in a format specific to different instruments.

B Creation of MS method

1. Following part is illustrated at Analyst 1.6.2 used for AB Sciex Qtrap 5500. For different instruments, software environment may differ.
2. Choose the proper hardware including LC separation. At the left side of the Analyst screen, there are multiple choices of commands. Choose Configure/Hardware Configuration and select your hardware profile containing both LC and MS. This setting is usually created during instrument

installation. Selection will be confirmed by button Activate profile. If any other hardware configuration is selected, it has to be deactivated first. If the LC/MS profile is activated properly, a program DCMS link will be started immediately (it applies for connection with Thermo/Dionex LCs).

3. In the command screen, choose the Acquire/Build Acquisition method. A new window will open. In this window, there will be two folders, LC and MS method. It is important to leave the LC method empty at this stage and focus on the MS method only. The MS method window overview is shown in Fig. 36.1. In the Experiment window, there is a number of a particular experiment. For simplicity, there will be only one MS experiment used. In Scan type window, there are multiple choices of scan types, which are allowed by Qtrap 5500. Select MRM (MRM) there. The Polarity window is always Positive for proteomics. The next field which has to be filled is a Period Summary, where Duration means the total duration of MS method and Delay Time is a delay with MS recording after the start of the run. Duration is recommended to fill for a total time of LC run and Delay is optional, usually for the time of dead retention time. Then there is a window Scheduled MRM, which will not be enabled this time. The last two parts are the table of transitions and Import button. The table can be filled manually, but the list of transitions created in sub-chapter A above can be imported by the Import button. If done correctly, the table will be filled with transition list corresponding to target proteins filtered by Skyline.
4. When the method is saved, the dialogue from the DCMS link asking for LC method creation will open. Continue with this dialogue only when MS method is finished.
5. Close the Acquisition method window before continuing to the LC method. Otherwise, the LC method will not be saved correctly.

C. Creation of LC method

1. Following part is illustrated at Dionex chromatography MS Link 2.10 used for Thermo/Dionex instruments in connection

with mass spectrometers. For different instruments, software environment will differ. The LC methods described there are standard in our proteomic facility.

2. Continue with DCMSlink dialogue window after saving MS method. The LC method creation wizard will open.
3. The first window of the wizard is for the setting of column department temperature. This temperature should be set to 35 °C. Further, it allows to set upper and lower limits of this temperature – the more relaxed limits will allow the instrument to start faster; whereas stricter limits forces instrument to wait until the limits are fulfilled.
4. The next window allows setting valves in column department. Those valves are important if pre-column is used. Otherwise, this field can be left empty. If the pre-column is used, turn the valve to flow from sampler as described in Table 36.1. For the rest of the run, pre-column will be turned in flow from nano pump.
5. Wizard will follow in setting the autosampler temperature. This is recommended to keep

low, e.g. at 5 °C, to enhance sample stability. The sample should be in autosampler for the maximal time of 24 h; longer storage is recommended in the freezer.

6. The set-up for loading pump follows. Loading pump is used in a pre-column configuration only. In such case, set isocratic gradient of 100% A (water with 1% acetonitrile and 0.05% trifluoroacetic acid) with flow 200 $\mu\text{L}\cdot\text{min}^{-1}$. Pressure limit can be kept at 500 bar.
7. Nano pump settings are in the next wizard window. For peptide separation in proteomics, the Multi-step gradient is the best option. Multi-step is set-up in the separate window, which follows only in case of setting it in the main wizard window. Multi-step gradients used in IMTM for peptide separation are shown in Table 36.1. Solvents used are water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) with flow 300 $\text{nL}\cdot\text{min}^{-1}$.
8. The following window will ask for the volume of the injected sample. For set-up without pre-column, as low as 1 μL or 2 μL

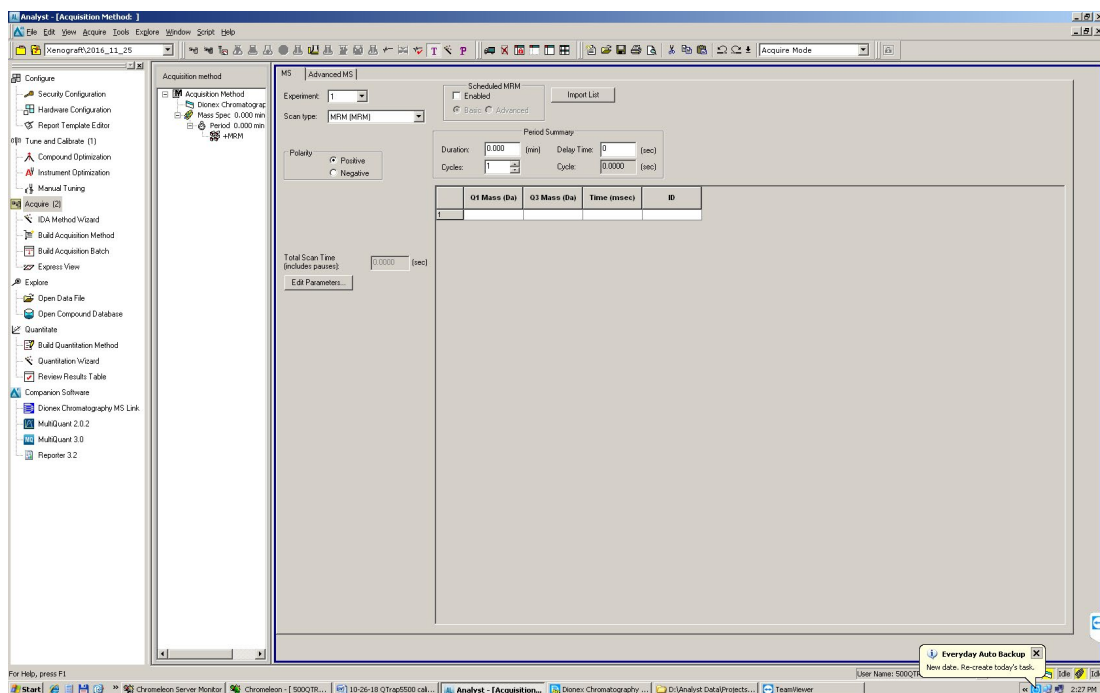


Figure 36.1. Screenshot of Analyst 1.6.2 software. At the left part of the screen, there is a command column with Hardware Configuration or Build acquisition method discussed in the text. At the main part of the screen, the MS method configuring dialogue is shown.

are the maximum volumes. With the use of pre-column, the maximum sample volume is limited to loop size only (usually 20 µl).

9. In the next step, sample injection properties, such as draw and wash volumes and speed, puncture depth etc. are set. Those values need to be optimized on each instrument separately.
10. After such properties, there is an important step, which is the selection of injection mode. There are multiple choices: FullLoop, Partial, ulPickUp and UserProg. Their properties are:
 - a. **FullLoop** The sample will be filled to the full loop. This is the most accurate and most reproducible method. However, it does not allow to change sample volume between samples and consumes most of the sample (approximately three times of sample loop volume).
 - b. **Partial** This method is in between of FullLoop and ulPickUp. The sample will be drawn to the needle and only selected volume of the sample to the loop. The accuracy and reproducibility lie here on the accuracy of autosampler syringe. It is thus necessary to avoid any bubbles in a syringe.

- c. **ulPickUp** This method allows to pick only a desired amount of sample. Thus the amount of sample injected and sample drawn from vial will be equal. This is done using transport liquid in a separate vial. This liquid will be drawn to the loop, the exact volume of the sample will be drawn and needle and part of the loop will be filled again with transport liquid. Although this method reduces sample consumption, it increases the risk of sample carry-over. Thus it is necessary to change transport liquid often.
- d. **UserProg** This method allows the user to program injection method.
11. The main properties of the LC method were already set. In the following tables there remain to set which devices will be recorded. There is a choice to record column oven temperature and pressures on pumps. Each value offers separate options for recording.
12. The last page of the wizard allows setting when turn pumps or instrument on and off and time when to do that. This is an advantage in higher flow rates. It is recommended to maintain continuous flow in nano pumps.

TABLE 36.1. Gradient composition for different length of peptide separation. These methods are standard at the proteomic facility of Institute of Molecular and Translational Medicine in Olomouc

Gradient composition		Gradient elution program for nanoLC separation				Switch valve position
% A	% B	60 min	120 min	180 min	240 min	
98	2	0	0	0	0	1_2
98	2	5	5	5	5	10_1
65	35	65	125	185	245	
10	90	67	127	187	247	
10	90	73	133	193	253	
98	2	75	135	195	255	1_2
98	2	95	155	215	285	
Total time [min]	95	95	155	215	285	

13. The protocol is now complete. It will appear as a program of commands. However, dialogues for different modules can be opened any time again. It is recommended to review the protocol Check before saving. This checks if all the commands are filled properly.
14. After saving the method, open the method again in Analyst and check if the LC method has been added. If not, close the protocol in Analyst and save it in DCMS link again.
10. In Analyst, choose the proper hardware profile including LC separation.
11. Choose Acquire/Build Acquisition batch
12. A new dialogue will open. In this dialogue, first, write the name of the sample set and then push the Add Set button. A button Add Samples will become active.
13. In Acquisition field, choose the acquisition method used. If there will be multiple methods used in the sample set, click Use Multiple methods. It will be then possible to change the method in sample lines with the method selected as acquisition as a default value.

D. Analysis of test sample

1. The sample of interest has to be prepared prior proteomic analysis. There are various protocols of protein isolation, pre-fractionation, digestion and purification. Those protocols are discussed in detail in Chapter 34. Here we will describe just basic in solution digestion of liquid protein sample (e. g. plasma) for demonstration.
2. Put 10 μl of sample to test tube. The sample should have concentration of proteins in the range $0.5 \div 1 \mu\text{g}/\mu\text{l}$.
3. Add 20 μl of acetonitrile and 20 μl of 50 mM ammonium acetate in water.
4. Add 5 μl of 65 mM dithiothreitol and incubate for 30 minutes in 57 °C. This will reduce the disulfide bonds.
5. Add 5 μl of 135 mM iodoacetamide and incubate for 1 hour in room temperature and dark. Iodoacetamide will alkylate free sulfides and prevents their re-folding.
6. Add 20 μl of 50 mM ammonium acetate in water and 2 μl of trypsin in concentration 0.5 $\mu\text{g}/\mu\text{l}$. Incubate overnight in 37 °C.
7. The cleaved peptides should be desalted using C18 sorbent. Those devices may vary from tip with sorbent to small columns or 96-well plates with sorbent. Basically, it is necessary to activate the sorbent with methanol, flush subsequently with acetonitrile and water, load the sample, desalt the sample by water flushing and elute peptides using acetonitrile. The weak acid, eg. 0.1% formic acid or trifluoroacetic acid, may be added to increase the performance of desalting.
8. The test sample should be either a mixture of synthetic peptides or sample digested as described.
9. Insert the test sample into autosampler.
14. Push the button Add Samples. The opened dialogue will ask of sample name with the possibility to number the samples, the name of the file(s), where the samples will be saved and the number of samples.
15. In the table will occur the selected number of samples named as was entered in the previous dialogue. The sample name can be changed and, more critical, sample location in an autosampler is recorded. There is a need to take attention in one feature of Analyst. Analyst asks for Rack Code (Red, Green or Blue) or Plate Code (Fixed Vials, 40 Standard vials, 96 well plate etc.). Correspondingly to that, Analyst also asks for Rack or Plate number which corresponds to the code (1 for Red, 2 for Green, 3 for Blue for Rack and 1 for Fixed vials and 2 for Standard vials for Plate). Vial position is not determined as A1 or C5 as is written on the plate, but numerically as 1 or 21 respectively. For better orientation in this system, see the Locations folder which shows the locations of samples graphically. Another values in the line are straightforward without any issues – it is the name of storage file, injection volume (default is set in LC method and it cannot be changed in Full loop injection) and if multiple methods are allowed, the acquisition method.
16. When all sample parameters are filled, continue to Submit folder. Select the samples, which are going to be sent into the sample queue. If all parameters are filled correctly, the Submit button will be active. After pushing it, the dialogue will open. The dialogue will ask if you wish to submit all samples or selected only. Only those will be submitted into queue.

17. Sample Queue itself will be visualized after selecting View/Sample Queue or by Ctrl+Q. The queue window shows basic information about the instrument – if the instrument is standby, ready or acquiring in one field and if the instrument is in normal mode or tuning mode. If the instrument is in standby and tuning mode, it is necessary to prepare it for analysis. First, turn off tuning mode by pushing icon with big T in the upper toolbar (Fig. 36.2). In the same toolbar, there is an icon with a wave. This icon will start equilibration of the instrument. In the same

time with equilibration of MS, equilibration of LC will also start. It will be shown as a dialogue window of DCMS link showing current and planned LC values. When both instruments reach equilibrium (typically after 1 minute), instrument status will turn into ready state signaled as green color on stoplights. By pushing the icon with flask and start signal (Start Sample; Fig. 36.2) the queue will start. If done correctly, the instrument status will change into Pre-run during sample injection and Acquiring status when sample acquisition will start.

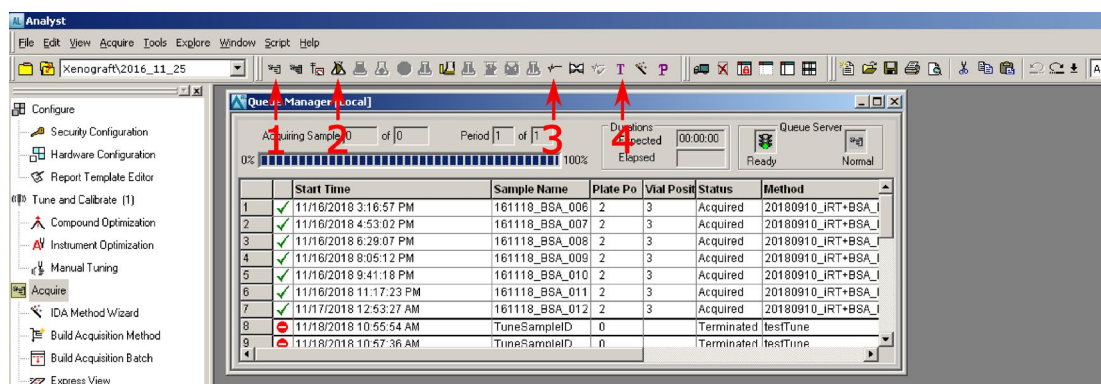


Figure 36.2. Snapshot of Analyst software. This screen will show in Acquire folder. The highlighted icons are: 1 – Show queue; this icon opens the queue window (on the main part of the screen), 2 – Start, 3 – Equilibrate icon, 4 – Tuning icon

E. Sample evaluation and set of scheduling

1. In the Skyline, open the document created for transition development. Then set up the mass spectra and peptide properties in Settings menu by choosing Transition / Peptide / Document settings... according to parameters of your experiment.
2. In File/Import/Results... open the new dialogue. Here can be selected, if the samples have been analyzed as single or multiple injections replicate. When selected, click OK and select the files with mass spectrometry data. Currently, Skyline can process mass spectra from Agilent, Bruker, SCIEX, Shimadzu, Thermo and Waters (9).
3. When the files are selected, Skyline will ask, if it should remove the common prefix and will start to upload the files. One special

feature of AB Sciex data is that multiple runs can be saved into one file. This is set in part D, point 7, where sample and dataset names are set. Dataset is saved as one file and samples are saved within this file. Skyline will thus ask even which samples extract from the file. If the import is done correctly, it will be visualised as the import of chromatograms of individual samples.

4. In the Skyline, individual transitions can be now overviewed and processed. Skyline uses its scoring mechanism when next to each peptide will appear a green dot if the peptide has been identified with high confidence. Such peptide is identified on more than one transition in the same retention time. Orange dot will appear if the confidence is not high, e.g. there is only one transition at the time, and a red dot means that peptide

has not been recognized.

5. The Skyline now offers multiple ways how to observe samples. One of those ways will be used in the next step of optimization. Go to View/Retention Times and select here either Replicate comparison or Peptide comparison. In both cases, a retention time across the test samples will show. In the Replicate comparison, there will be retention stability across samples better visible; meanwhile, in Peptide comparison, there will be visible retention times of all analyzed peptides.
6. With the knowledge of retention times, the scheduled run can be set. In Analyst, open the acquisition method in Acquire mode and File/Open... In MS part of the method, tick the Scheduled MRM enabled. The table of transitions will change and instead of dwell time in milliseconds, and retention time in minutes will appear. Fill the retention time recorded in Skyline.
7. Run test sample again to check if scheduling works.

F. Regular analysis

1. The samples for regular analysis are run in the same manner as test samples. Procedures

of inputting of samples into instruments software have been described in chapter D.

2. After analysis, the samples will be evaluated as described in chapter E.
3. Statistical analysis of samples is always very dependent on original experimental design and its description is beyond the scope of this book. Depending on experimental design and number of samples, there could be used multiple tools. Skyline offers basic statistic evaluation, which is sufficient for less complicated studies. For more complicated tasks, we recommend collaboration with a statistician.

IV. Comments

- The workflow described in this chapter expects at least basic training with the instrumentation. The workflow described here applies to Thermo Ultimate 3000 nano HPLC coupled with AB Sciex QTrap 5500. However, it should not be a problem to transfer the described method to another instrument with basic knowledge of their software environment.

V. Pitfalls

Problem	Cause and cure
Signal is missing	• The Skyline did not import the files. Check Skyline settings. Check Analyst menu Explore/Open data file if the analyzed data are recorded correctly. • MS recorded no data. Check if the sample has been injected. Check if LC has no leaks. Check if electrospray device works correctly.
Retention time is not stable	• Check LC for gas bubbles and leaks. Check pump wellness if flow and gradient are stable.
Peak intensity is not stable	• Check electrospray stability. Check autosampler whether injects the same amount of sample. Clean MS.
Noise intensity increases, peak intensity decreases	• Clean MS.

- Methods of targeted proteomics could be used as verification tools after the discovery phase with a regular shotgun MS proteomic approach. They can also be used as an alternative of array studies in cases when missing values coming from the shotgun approach could be a significant problem.
- Due to the very complex nature of the described method, there can many different errors occur. The vast majority of them

will occur during method optimization and after solving, they will not interfere with the regular analysis. Another kind of errors may arise from technical issues with instrumentation. It is thus recommended to keep the instrumentation clean, calibrated and in good technical condition. The following table introduces just a few most important pitfalls and their solution.

VI. Acknowledgment

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Methods of targeted mass spectrometry in the analysis of small molecules

Tomáš Oždian

I. Introduction

In a previous chapter, methods used for targeted mass spectrometry in proteomics has been described. However, targeted analysis is not beneficial only in proteomics, but the proteomics is only a small and very specialized subgroup of analytes which have benefited from targeted mass spectrometry.

It is beneficial to introduce targeted mass spectrometry in proteomics first – relatively established methods of proteome separation and homogeneity across peptides reduces variables and allows to better explain the principle of targeted mass spectrometry and its basic settings. In small molecules world, the situation becomes more complicated. With a practically endless count of different organic compounds, it is impossible to use only one separation method for all of them.

The selection of instrumentation is strongly dependent on the expected matrices and expertise of the operator. Today the most versatile system is liquid chromatography (LC) using a reverse phase column. This system allows separating most of the compounds with sufficient effectivity.

The other modes of liquid chromatography can be used in specific cases, usually when the reverse phase is not suitable for sample separation. The proper mode of separation and exact conditions has to be optimized during the setup of the whole LC-MS system. For volatile matrices, gas chromatography may be used with better effectivity. For an illustration of the workflow of targeted MS analysis, a reverse phase LC separation with triple quadrupole mass detection will be described. This model is the most widely used in practice. Targeted mass spectrometry in this setup is a very valuable tool for quantification of small molecules. It achieves high separation power on the one side and produces very clean spectra using mass filtration on the other side.

II. Materials and instrumentation

Instrumentation

- **Liquid chromatograph**

For connection with MS, the best options are high-performance LCs with flow up to approximately 500 $\mu\text{L}\cdot\text{min}^{-1}$. This limitation lays in the

effectivity of electrospray to ionize larger flows effectively. For LC-MS connection, there can be used even smaller scales of chromatography like a capillary or nanoflow chromatography. Those variants offer a lower flow of solvents and higher sensitivity. On the other side, those techniques require specific pumps and columns and are more challenging to optimize. In this chapter will be used Thermo Ultimate 3000 LCs as they are standard instrumentation in the Institute of Molecular and Translational Medicine in Olomouc. However, the described steps apply to different LCs as well.

• **Mass spectrometer**

There are multiple mass spectrometers suitable for targeted mass analysis. They are recognizable by a hybrid architecture with a quadrupole mass filter in front of ion path. The golden standard of such instruments is triple quadrupole, eventually with linear ion trap instead of the last quadrupole. Those instruments are available from plenty of vendors (AB Sciex, Agilent, Shimadzu, Thermo etc.). The higher level of instruments consists of a high-resolution detector like time-of-flight or Orbitrap at the end of the ion path. This allows more precise analysis with very narrow fragment windows further increasing specificity without compromising sensitivity. The instrument used for this chapter is quadrupole – linear ion trap AB Sciex Qtrap 5500.

Chemicals

All chemical needed in this experiment has to be the highest purity. This purity is usually marked as HPLC or LC-MS grade. The lack of impurities in those chemicals will provide a clear signal without elevated noise.

The basic solvents for reversed phase LC are water and organic solvent. The organic solvent may vary depending on sample polarity. The solvent of choice for the majority of small molecules is acetonitrile because it does not interfere with the UV spectrum as methanol and has good ionization properties for MS (1).

The solvents for LC has to be often modified to enhance separation and ionization efficiency. pH is the most often modified property is pH by a variety of buffers. The specific of LC-MS is that those buffers have to be volatile. Thus, during the process of evaporation, the buffers have

to decompose to inert gases like ammonia or carbon dioxide. Thus the most favorite modifiers are formic and acetic acid for acidification and ammonium acetate and ammonium formate buffers for higher pH values.

Software

Software used in this experiment is Analyst v. 1.6.2 for mass spectrometer control and Chromeleon v. 6.8 (DCMSlink v. 2.1 respectively) for LC control. That software is instrument-specific and will differ across the vendors. MultiQuant v. 2.1 is used for sample evaluation.

III. Procedures

The basic flow chart is the same as in Chapter 36. The main differences are in transition optimization, which has to be done manually for each compound, the evaluation software differs and more attention is taken about method validation parameters. On the other side, the scheduling is not usually an issue. The methods for small molecule analysis, except large scale metabolomics and lipidomics studies, are usually faster and with fewer transitions than proteomic studies.

A. Transition development

1. Dissolve the standard of the compound. The recommended solution is 1 - 10 μM in acetonitrile. This concentration is based on the author's experience to see sufficient signal for the broadest variety of compounds. Load the diluted standard into Hamilton syringe.
2. The workflow described here applies to AB Sciex QTrap 5500 and its software environment. For different instruments, the workflow may differ.
3. Connect the Hamilton syringe to the ion source tubing and the pump. In the case of AB Sciex instruments, the syringe pump is integrated into the instrument. In Analyst, select the hardware profile containing the mass spectrometer and syringe pump in Configure/Hardware Configuration.
4. The transition can be set in two ways. The first one, automated, is accessible through Tune

and Calibrate/Compound Optimization. This option will open a dialogue, where are set the details of injection and the masses of optimized compounds. After the finish of this dialogue, the automatic optimization of the instrument will begin. In the end, there will be a table of parent compound masses, the masses of fragments and specific settings, such as collision energy, optimized for a maximal yield of particular fragment ion.

5. In some cases, e.g. when fragment ion is known from the literature but is not selected by automatic optimization, it may be beneficial to set up the transition manually. This is done by Tune and Calibrate/Manual Tuning. In this window, manual measurement will open. This measurement allows testing all instrument possibilities and tune all-important parameters. For transition settings, select Product Ion (MS2). Set the Parent ion mass and mass range and click Start. Some of the parameters can be changed during the run, and the direct impact can be observed. Alternatively, there can be several parameters such as declustering potential or collision energy ramped. To do this, click Edit Ramp, the button next to the Start button. Select the ramped parameter, starting and ending values and click OK. Then click Start and ramp experiment will start. Those experiments help to find optimal values for a single transition.

B. Creation of MS method

1. The MS method creation protocol is almost the same as in Chapter 36. The main difference is in the way how transitions are uploaded.
2. If the compound transitions have been developed by automated protocol or are being transferred from the different instrument (of the same type), they can be imported.
3. If the transitions have been developed manually, they have to be manually inserted into the method as well.

C. Creation of LC method

The LC method creation protocol is the same as in Chapter 36. There is only one main difference

– as was written in Introduction, the small molecules are a very heterogeneous group, and thus only one LC method will not be universally valid. Because of its versatility, we recommend to start with reversed phase chromatography for all compounds with at least limited solubility in water and switch to different modes of separation such as HILIC, normal phase, phenyl columns, chiral separation etc. mainly in case that reverse phase does not provide satisfactory results. It is beneficial to consult the LC method of the particular compound with published literature. Due to the complexity of HPLC optimization, we will recommend referring specialized literature available both from scientific authors and companies in the HPLC industry (2,3).

D. Analysis of test sample

The test sample should be a mixture of compound standards. The procedure of inputting samples into the LC-MS system is described in Chapter 36. The care has to be taken for all compounds to prevent their co-elution.

E. Evaluation of results

1. Usually, the MS vendor provides its software for evaluation of SRM analyses. In the case of AB Sciex, this program is MultiQuant (v. 2.1 in our case). The following lines apply to this software.
2. Create a new result table. In MultiQuant, it is marked as a magic wand in the upper left corner. A new dialogue will open. In this dialogue, select the result file data first. Then you can use the already existing evaluation method or create a new one. If a new method is created, it should be saved with a new name first. In the next table, the MultiQuant will automatically select a representative file. All settings will be optimized on this file. In step after, MultiQuant will show the list of transitions. It is the same list as was imported into MS method. In the following window, integration parameters for each transition will be set. The impact on different settings will be directly seen on peak overviews from the representative file. This is the last step in the creation of the import method.
3. The results will be now uploaded in the form of a table. In this table, there are all transitions in all uploaded runs. The main

parameters shown here are peak area and intensity. There is a possibility to filter single transitions and set the calibration standards. When the calibration standards and their respective concentrations are set, MultiQuant will calculate concentrations and accuracy of unknown peaks. The peaks will be analyzed by possible structures. In some cases, depending on the solvent minor shifts can occur.

4. The MultiQuant allows for creating a complete evaluation of quantification results. It allows to visualize calibration curves, overview single transition peaks or plot specified values.
5. When all calculations are finished, the results can be exported via File/Export/Results table. The following dialogue will allow to set-up details of the resulting file. The output table will be saved as "*.txt".

F. Method validation

Contrary to proteomics studies, where the stability of retention time and peptide intensity in time

are usually the main validated parameters, the method validation in small molecule analysis is a very complex procedure. It is often connected with the requirements of the pharmaceutical, environmental, food industry or health authorities. The validation procedure thus must follow the rules of particular authority. The requirements of the European Medicinal Agency or the United States Food and Drug Administration (4,5) can serve as an illustration.

IV. Comments

The workflow described here is common workflow typical for most compounds. However, it is necessary to develop an assay directly for compound or compounds of interest with separation and ionization which will work best. Such method development often brings a lot of specific issues. Therefore, we will direct the reader to specialized literature and his/her LC and MS experience.

V. Pitfalls

Problem	Cause and cure
No signal observed	Check standard solution to control instrument performance. Check the sample injection. Check MS wellness. Check LC for leaks and pump wellness. If possible, check UV as an alternative to MS.
Signal of the standard is not uniform	Check ionization wellness. Check the sample injection. Check LC for leaks and pump wellness
The noise and signal variability is high	Clean the MS.
The peak retention is shifting and peaks are broadening	Check the column wellness. The simplest way is to reverse the column and repeat run. If necessary, Exchange column for a new one.
Peaks are fronting or tailing	Continue with LC optimization. Consult specific literature. Try a different column.

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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***In vivo* methods for evaluation of radiolabelled compounds**

Miloš Petřík and Zbyněk Nový

I. Introduction

The initial *in vivo* studies with a novel radiotracer are generally performed using rodents (mice or rats). Preclinical evaluation of radiolabelled compounds usually comprises *ex vivo* biodistribution, *in vivo* stability and *in vivo* imaging studies using healthy animals or appropriate disease animal models. Together, these studies can provide an overall information about pharmacokinetic and pharmacodynamic parameters of tested compound (1, 2).

For *ex vivo* biodistribution study, radiotracer is administered in desired manner, most often intravenously, into the animal. At different time points after application, depending on the half-life of the radioisotope used, the animals are sacrificed and tissues and organs of interest are removed and weighed. The amount of radioactivity in the tissue and organ samples is measured in a gamma counter. Results are expressed as percentage of injected dose per organ (%ID) or per gram organ (%ID/g) (Fig. 38.1) (3, 4).

In vivo stability studies of radiolabelled compounds are carried out by studying of body fluid samples (blood, urine and bile) and/or excretion

organs (kidneys and liver) homogenates. After the radiotracer administration, blood, urine, bile and organs are sampled at corresponding time points. The collected samples are subsequently processed (e.g. blood is precipitated and centrifuged; organs are homogenized and suspended in buffer at neutral pH) and analyzed by means of high performance liquid chromatography with radio-detection (rHPLC) or thin layer chromatography (TLC) methods (Fig. 38.2) (5).

In vivo animal imaging studies of radiotracers are performed with dedicated animal imaging systems. These are usually complex devices that can combine several imaging methods such as positron emission tomography (PET), single photon emission tomography (SPECT), computed tomography (CT) and/or magnetic resonance imaging (MRI). Before the imaging, experimental animals are applied with the studied tracer. At selected time points after the application, animals are anesthetized and placed into the imaging system. The imaging time is highly dependent on the type of the study and quality of the imaging system. It usually takes several tens of minutes. After the imaging, acquired data have to be reconstructed and subsequently can be viewed and analyzed by appropriate software (Fig. 38.3) (4, 6).

II. Materials and instrumentation

***Ex vivo* biodistribution study**

- Materials

scintillation vials, syringes, needles, microtubes, saline solution, and studied radiotracer.

- Instrumentation

analytical balance, personal computer, ionization chamber, and gamma counter.

***In vivo* stability study**

- Materials

syringes, needles, microtubes, centrifuge tubes, beakers, saline solution, acetonitrile, trifluoroacetic acid, deionized water, phosphate buffered saline pH = 7.4, and studied radiotracer.

- Instrumentation

personal computer, ionization chamber, high performance liquid chromatography with radio-detection, phosphor imager, centrifuge, and homogenizer.

***In vivo* imaging**

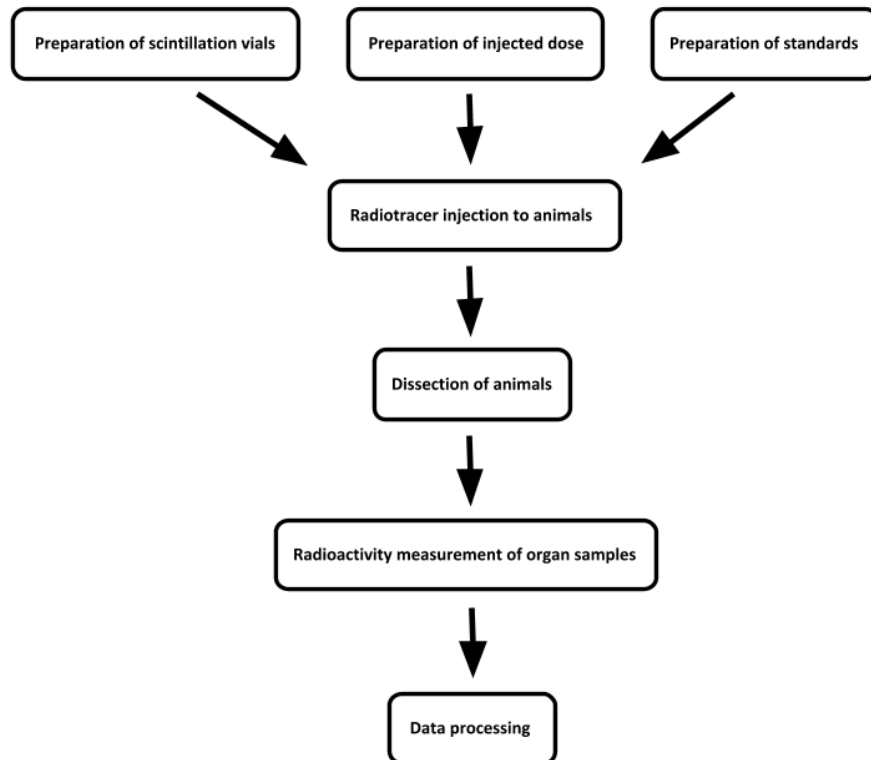
- Materials

syringes, needles, microtubes, saline solution, isoflurane, medical oxygen, and studied radiotracer.

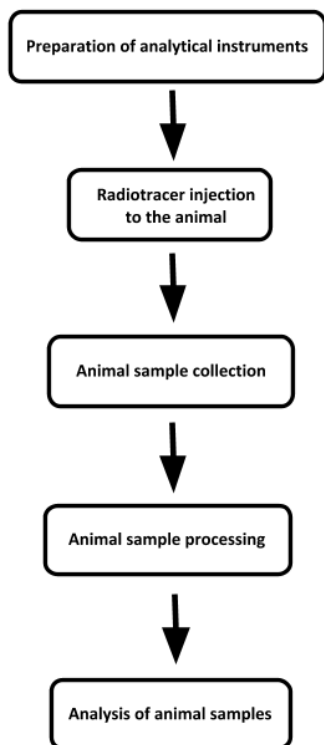
- Instrumentation

personal computer, ionization chamber, animal imaging system, anesthetic system, and dedicated software (e.g. PMOD or Volview).

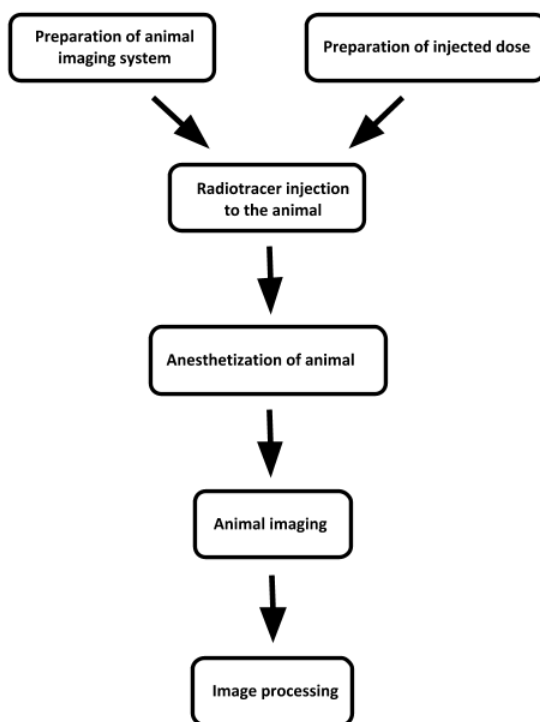
III. Procedures



Scheme 38.1. Ex vivo biodistribution study



Scheme 38.2. *In vivo* stability study



Scheme 38.3. *In vivo* imaging

A. Ex vivo biodistribution

Steps

1. Weigh and mark empty scintillation vials for organ and tissue collection and for standards.
2. Store the weights of scintillation vials electronically (e.g. MS Excel file).
3. Prepare the standards (1:1, 1:10, and 1:50) of the radiotracer in scintillation vials and weigh them.
4. Prepare the dose of studied radiotracer for administration to animals.
5. Weigh the syringes with prepared dose of the radiotracer.
6. Inject the radiotracer to animals and weigh the empty syringes.
7. Sacrifice the animals in selected time points.
8. Dissect the animals and collect the organs and tissues of interest in scintillation vials in selected time points.
9. Weigh scintillation vials filled with animal organs and tissues.

10. Measure the radioactivity in individual organs and tissues in a gamma counter.
11. Store the data obtained by gamma counter electronically (e.g. MS Excel file).
12. Process the results and express them as percentage of injected dose per organ (%ID) or per gram organ (%ID/g).

B. In vivo stability study

Steps

1. Prepare the analytical instruments for rHPLC and/or TLC testing
2. Inject the radiotracer to the animal.
3. Collect the body fluids and/or dissect the animals and collect the organs of interest in selected time points.
4. Process the collected body fluids and excised organs.
5. Analyze the obtained samples with rHPLC and/or ITLC methods.
6. Process the results.

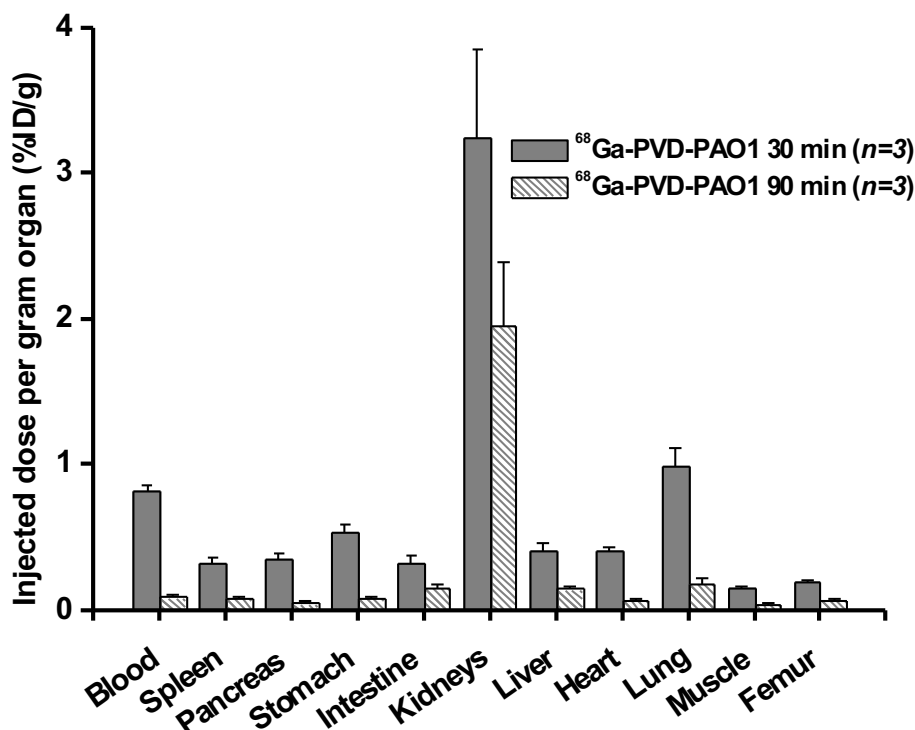


Figure 38.1. Example of *ex vivo* biodistribution data of radiolabelled compound (^{68}Ga labelled Pyoverdine PAO1 (^{68}Ga -PVD-PAO1)) injected to the experimental mice sacrificed 30 and 90 min post injection (three animals per time point).

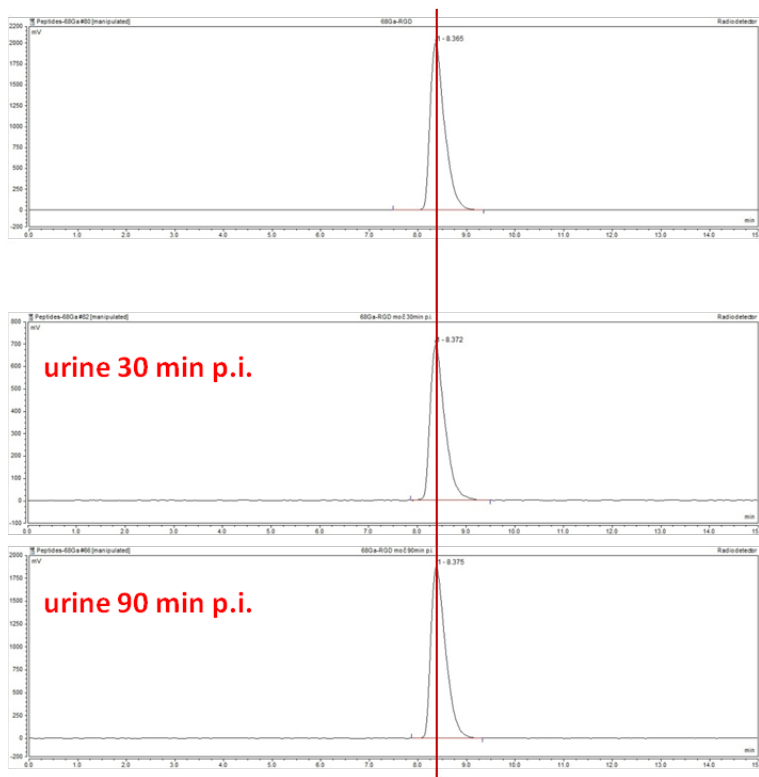


Figure 38.2. Example of *in vivo* stability testing of radiolabelled compound injected to the experimental mouse by *r*HPLC analysis of urine collected 30 and 90 min post injection.

C. *In vivo* imaging

Steps

1. Turn on the animal imaging system.
2. Prepare and measure the dose of studied radiotracer for administration to the animal.
3. Inject the radiotracer to the animal.
4. Set the imaging requirements in the imaging software.
5. Anesthetize the animal 5 min before the start of imaging.
6. Place the animal into the animal imaging system and keep it under the anesthesia.
7. Start the animal imaging.
8. Remove the animal from the animals imaging system after the end of the imaging.
9. Reconstruct the obtained data with the imaging software.
10. Process the animal images in relevant software.

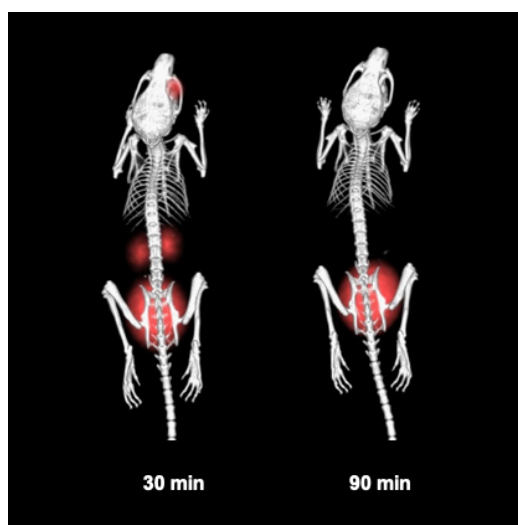


Figure 38.3. Example of three-dimensional PET/CT images 30 and 90 min after the injection of radiolabelled compound to the experimental mouse.

IV. Comments

Safety is an important issue in working with radioactive compounds. Exposure to ionizing radiation should be avoided as much as possible by following the operating rules of the laboratory. Whereas these methods include handling with living organisms, increased attention should

be also paid to the compliance with the ethical framework of working with experimental animals. Project of experiments on laboratory animals has to be approved by responsible authorities before the start of the investigation. The experimental procedures described in the project of experiments must be strictly followed.

V. Pitfalls

Problem	Cause and cure
Radioactive contamination	• Immediate decontamination as described in the operating rules of laboratory
Jam of measuring devices	• Restart the measuring device and repeat the measurement
Measurement of incorrect radionuclide	• Stop the acquisition and restart the measurement with correct radionuclide
Incorrect (paravenous) application of radiotracer	• Use another animal and repeat the injection
High deviation (>5%) in standards for ex vivo biodistribution	• Prepare new standards
Lack of mobile phase for rHPLC analysis	• Check the volume of mobile phase before the analysis; prepare and add the mobile phase if necessary

VI. Acknowledgement

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Basic *in vitro* methods for evaluation of radiolabelled compounds

Zbyněk Nový and Miloš Petřík

I. Introduction

Chemical compounds (drugs, new potential diagnostics, etc.) can be radiolabelled for various purposes like biodistribution studies, *in vivo* imaging, or internal radiotherapy. The process of radiolabelling consists of multiple steps, where all have to be set up correctly. At first, an appropriate radioisotope should be chosen with respect to intended purpose of the labelling. Beta plus or gamma emitters are suitable for *in vivo* biodistribution studies or imaging, while alpha or beta minus emitters are the isotopes of choice for radiotherapeutic applications. Also, physical half-life of the radioisotope has to correspond to the following analytical procedures and to biological half-life of the labelled compound in case of *in vivo* studies (1).

Briefly, we can distinguish two basic types of radiolabelling from radiochemical point of view – radiohalogenation and chelation of radiometals (2). In case of the radiohalogenation, we can directly attach the isotope to labelled structure, typically with various isotopes of iodine (e.g. ^{123}I , ^{125}I , ^{131}I) or fluorine-18. A compound, which is going to be labelled with radiometal, should be at first modified by conjugation with appropriate

metal chelator (e.g. DTPA, DOTA, NOTA, HYNIC, DFO, etc.)¹. The choice of correct chelator is crucial question and must respect the properties of selected radiometal (3).

Inevitable step in radiolabelling workflow is the quality control (QC), where the radiochemical purity is checked. Basically, there are two main approaches how to verify the purity of labelled compounds – rHPLC or TLC. The rHPLC (radio-detection-high-performance liquid chromatography) provides better analytical opportunities, how to optimize the analytical method, but requires costly HPLC system coupled with radiometric detector (4). On the other hand, TLC (thin layer chromatography) or especially iTLC-SG (instant TLC on silica gel) is not so challenging to perform, but its analytical options are limited compared to HPLC, especially when it comes to exact quantification of impurities. The required limit for radiochemical purity has to be stated on the beginning. Typically, such limit is 95%, so 95% of radioisotope is present

¹ DTPA=diethylenetriaminepentaacetic acid, DOTA=tetraazacyclododecanetetraacetic acid, NOTA=triazacyclononanetriacetic acid, HYNIC=hydrazinonicotinic acid, and DFO=desferrioxamine

in required chemical form, i.e. coupled to the drug or tracer, resting 5% are impurities, it means other chemical forms of used isotope, e.g. free isotope (5).

After successful radiolabelling of the compound, there are many subsequent *in vitro* tests, in which it has to be verified, whether the prepared tracer has appropriate properties and could be moved forward to *in vivo* assays. These *in vitro* tests are focused mainly into verification of radiochemical stability and establishing basic pharmacokinetic or pharmacodynamic parameters (6).

The basic primary stability test is performed in the same buffer as labelling itself and its goal is to analyze radioisotope detachment from the labelled compound under these mild conditions (e.g. in water buffer, room temperature). In case of satisfactory results, in this first test labelled compound undergo series of more sophisticated stability tests. The most common one is the plasma stability test, which should reveal whether plasmatic enzymes could decrease the radiochemical purity of tested compound in reasonable period of time. Similar assay could be done using liver microsomes to establish hepatic clearance of the tracer. These two tests are common for all types of radiolabelled tracers, i.e. both radiohalogenated and radiometal labelled (7).

For the tracers containing radiometals there is a series of additional stability test in order to prove the durability of compound-radioisotope bond under various conditions. The labelling with metals is usually performed in acidic pH buffer, so the first test is verifying the stability in pH elevated close to physiological conditions i.e. pH 7.4. The other test is done with excess of Fe^{3+} cations and has to reveal if the iron cations (most abundant metal in biological materials) are able to replace the radiometal chelated by the labelled compound. And finally, there is the stability test using buffer containing appropriate rival chelator. This assay should demonstrate ability of labelled compound to maintain its stability in presence of competing chelator (8).

The basic pharmacokinetic tests are i) determination of partition coefficient (log P value) and ii) plasma protein binding. The partition coefficient determination is performed in water/octanol system and gained log P value indicates the rate of lipophilicity of tested

compound. Usually more hydrophilic properties are welcomed. The incubation with human plasma for defined time period is serving to reveal plasma protein binding, where commonly the lower binding values are more favorable.

For specifically targeted radiotracers, we have various tests investigating their ability to bind to the target structure. These tests could be performed with isolated targets *in vitro*, e.g. immobilized receptors or on living organisms expressing appropriate targets like primary cells, continuous cell cultures or microbial/fungal/tissue samples, etc. The most simple affinity study is **binding assay**, where we incubate the ligand with the chosen target system (with and without competitor), but the results provides just the information, if the tracer is binding to the target (percentage of bound activity) and if the interaction is specific. The tracer accumulation is blocked by competitor of the similar structure in case of specific interaction. But the binding assay do not describe the strength of the target-ligand interaction, in other words the rate of affinity (7).

The affinity rate of the tracer could be estimated in the **competition assay**, where we incubate the tested compound in non-labelled form in increasing concentrations with target structure (e.g. isolated receptor) and constant concentration of labelled competitor. The gained results of decreasing accumulation of the competitor could be fitted to sigmoidal curve and then the half maximal inhibitory concentration - **IC₅₀** (concentration of tested ligand which decreases the accumulation of labelled competitor to 50 % of its maximum) is calculated in appropriate software. The **saturation assay** uses increasing concentrations of tested incubated with target structure. The analysis of the gained data gives us **K_D** value (dissociation constant), i.e. the concentration where the half of the targets is occupied, the value reflects affinity of the ligand to target. Such study can also determine **B_{max}**, which is the number of the binding sites on the target (9, 10).

In specific cases, the **internalization assay** could be used to determine the percentage of radioligand uptaken into the cells by washing the cells with appropriate buffer. Nonspecific uptake is revealed by incubation in 4 °C or in excess of cold (non-labelled) ligand. The **washout study** is describing percentage of released radioligand after its internalization into the cell (11).

II. Materials and instrumentation

A. *In vitro* plasma stability study

- **Materials**
labelled compound, human plasma, microtubes, and acetonitrile (ACN).
- **Instrumentation**
automatic pipette, vortex, hot air incubator, centrifuge, and HPLC with radiodetector.

Determination of partition coefficient

- **Materials**
labelled compound, phosphate-buffered saline (PBS), octanol, and microtubes.
- **Instrumentation**
vortex, automatic shaker, centrifuge, automatic pipette, and automatic gamma counter.

Plasma protein binding assay

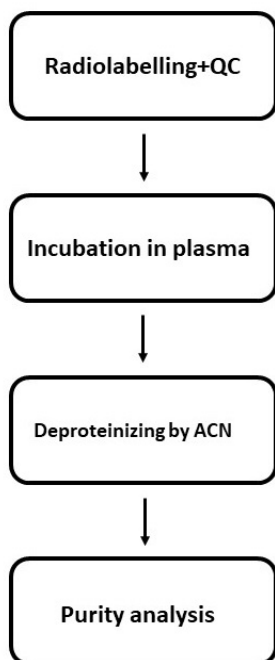
- **Materials**
labelled compound, human plasma, phosphate-

buffered saline (PBS), illustra MicroSpin G-50 columns (GE HealthCare), and microtubes.

- **Instrumentation**
automatic pipette, centrifuge, hot-air incubator, and automatic gamma counter.

Competition assay

- **Materials**
appropriate cell line in culture, tested compound, radiolabelled competitor, Tris/HCl, KCl, CaCl_2 , MgSO_4 , glucose, NaCl, 24-well cell culture plates, phosphate-buffered saline (PBS), NaOH, BCA protein kit, and scintillation vials.
- **Instrumentation**
automatic pipette, hot-air incubator, automatic gamma counter or liquid scintillation counter.



Scheme 39.1

III. Procedures

A. *In vitro* plasma stability study

Steps:

1. The studied compound is labelled in a standard way, passed through the quality control with sufficient radiochemical purity.
2. Process all the steps in triplicate.
3. Mix 100 μL of labelled compound with 400 μL of human plasma in Eppendorf tube. Vortex the mixture well.
4. Incubate the mixture at 37 °C and remove 100 μL of solution in defined time points, e.g. 0, 30, 60, and 120 minutes.
5. Immediately after the sample is removed, mix it with 200 μL of acetonitrile. Vortex this mixture intensively for 60 seconds.
6. Centrifuge the sample for 3 minutes with 20 000 g.
7. Remove the sample of the supernatant for analysis of radiochemical purity.
8. Analyze the sample using HPLC system with radiodetector or TLC. Example of such results is listed in Table 39.1.

B. Determination of partition coefficient

Steps:

1. The selected volume of labelled compound (e.g. 400 µL) is diluted with 600 µL of PBS (phosphate-buffered saline). Vortex well.
2. Prepare six samples as follows.
3. Take out 50 µL from the mixture prepared in step 1.
4. Mix these 50 µL with 450 µL of PBS and 500 µL of octanol.
5. Shake these six samples extensively for 20 minutes using automatic shaker.
6. Centrifuge the samples for 1 minute (15 000 RPM) to allow the separation of octanol and water phases.
7. Take out carefully the samples of 50 µL from octanol (upper) and water (lower) phases for further analysis to prepared Eppendorf tubes.
8. Count the activity of collected octanol and water fractions on automatic gamma counter.
9. Calculate the partition coefficient expressed as log P as follows:

$\log P = \log (A_{oct} / A_{water})$

Where A_{oct} is the activity of the sample taken from octanol phase and A_{water} the activity of the sample from water phase. The final log P value is shown as mean of the six analyzed samples $\pm$ standard deviation. Optimal log P value is usually under 5.

C. Plasma protein binding assay

Steps:

1. Process the samples in triplicate.
2. Take the sample of 10 µL from the solution of labelled compound and mix it with 190 µL of human plasma.
3. For verification of correct function of the gel column (control sample): mix 10 µL of labelled compound with 190 µL of PBS.
4. illustrate MicroSpin G-50 column preparation: open the column on its both ends, insert the column into the Eppendorf tube and spin it for 1 minute at 2 000 g. Number of prepared columns should correspond to number of samples prepared in step number 5.
5. Incubate both mixtures from step 2 and 3 at 37 °C and pipet 25 µL samples for analysis in defined time points, e.g. 0, 30, 60,

TABLE 39.1. Example of *in vitro* plasma stability study of radiolabelled ^{68}Ga -NODAGA-c(RGDyK) $_2$ followed for 120 minutes. The results are expressed as mean $\pm$ standard deviation (n=3).

NODAGA=triazacyclononaneglutaric acid-diacetic acid, RGDyK=Arginine-Glycine-Aspartic acid-D-Tyrosine-Lysine

Incubation time (min)	Radiochemical purity (%)
0	99.78±0.22
30	99.51±0.17
60	99.31±0.11
120	98.02±0.33

- and 120 minutes. Apply these samples on the columns prepared in step 4.
6. Spin the column with the sample in Eppendorf tube (without the tube cap) for 2 minutes at 2 000 g immediately after application of the sample onto the column.
 7. Separate the column and the eluate. Measure the radioactivity of these two parts in automatic gamma counter.
 8. Calculate the percentage of plasma protein bound fraction of radioactivity (P_b) as follows:

$P_b = (A_e / A_t) \times 100$

Where A_e is activity of the eluate and A_t is activity of the eluate plus activity of the column. Example of the results is given in Table 39.2. The same procedure applies for the control sample from step 3, where the total applied dose of radioactivity should theoretically remain in the column and so the P_b should be 0%. But practically about 1-2% of the activity always goes through the column into the eluate. Thus if P_b is under 2% for the control column, we consider this results as suitable and so the function of the column is correct.

D. Competition assay

Steps:

1. The experimental system containing target structure has to be prepared, e.g. immobilized receptors, adherent/suspension cell culture or microbial culture. For purposes of this protocol, we will describe the procedure for the adherent cell line culture.
2. In this study, we use tested compound in unlabelled (cold) form as competitor and selected radiolabelled ("hot") ligand targeting the same structure as tested compound.
3. Labelled ligand has to be diluted to appropriate concentration allowing reliable measurement of samples, e.g. the final activity should be within the dynamic range of automatic gamma counter or liquid scintillation counter. The concrete concentration depends on actual specific activity of the ligand and on binding rate of chosen target system.
4. Tested compound is diluted in appropriate concentrations using dedicated "uptake buffer" (25 mM Tris/HCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 5 mM glucose, 140 mM NaCl, pH adjusted to 7.4) to achieve final concentrations on the cells ranging e.g. from 0.1 nM up to e.g. 10 mM (in ten gradually increasing concentrations).
5. The cells cultured in 24-well plate are washed with PBS (500 µL each well). PBS is discarded and 300 µL of uptake buffer is added.
6. 100 µL aliquot of tested compound is added to achieve concentrations mentioned in step 4. Every concentration of the tested compound should be incubated in triplicate of wells at least.
7. The cells are incubated for 15 minutes at 37 °C.
8. 100 µL of selected is pipetted to the cells and incubated for 60 minutes at 37 °C.
9. After the incubation, the uptake buffer is removed from the cells and discarded as radioactive waste.
10. The cells are washed with lukewarm PBS (500 µL per well) and PBS is discarded again as radioactive waste.
11. 200 µL of NaOH (0.1 mol/l) per well is added to the cells and the plate is incubated

TABLE 39.2. Example of *in vitro* plasma protein binding of radiolabelled ⁶⁸Ga-NODAGA-c(RGDyK)₂ followed up to 120 minutes expressed (n=3).

Incubation time (min)	Plasma protein binding (%)
0	1.20
30	1.74
60	1.89
120	3.40

for 30 minutes at 37 °C to allow cell lysis and release of the radiolabelled ligand.

12. The cells are well homogenized using a pipette.
13. The sample of 10 µL is taken from every well for protein content determination in bicinchoninic acid (BCA) assay (12).
14. The lysed cells are moved to scintillation vials.
15. Three vials are used for standards, i.e. 100 µL of the radioactive ligand solution prepared in step 3 is added there instead of the cells.
16. All vials are counted in automatic gamma counter or liquid scintillation counter depending on what type of radioisotope is used.
17. Gained data are processed as follows: Percentage of accumulation = (As/Ast)*100, where As is activity of given sample and Ast is activity of standard (prepared in step 15). The percentage of accumulation is plotted to the graph as in Figure 39.1. Then sigmoidal inhibition curve is fitted to the data employing appropriate software e.g. GraphPad Prism or SigmaPlot. IC₅₀ value is calculated from this curve in the software.

IV. Comments

The radiation safety is very important issue when working even with low amounts of radioisotopes. The laboratory where the work is done must possess with appropriate authorization required by national authorities. In all cases, the elemental principles of work with radioactive sources should be followed, i.e. always use the lowest radioactive dose possible, reduce the time of personnel exposure to minimum and maintain the longest distance from the source of radiation as possible. Work with short-living isotopes requires well-planned schedule of the experiment, the key issue is proper synchronization of isotope delivery with preparation of the cell culture or tumor-bearing animals. Also, the radioactive waste produced within the experiments has to be handled and stored according the specific regulations, which usually requires dedicated shielded room reserved exclusively for storing the radioactive waste.

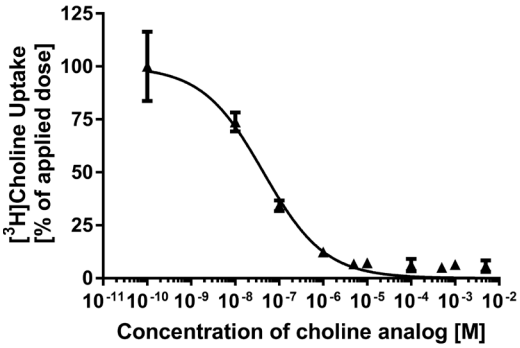


Figure 39.1. Example of the inhibition curve for choline analog. X-axis is in logarithmic scale. IC_{50} for the compound is $4.39 \times 10^{-8} \pm 9.69 \times 10^{-9}$ mol/L.

V. Pitfalls

Problem	Cause and cure
Radioactive contamination	• Immediate decontamination as described in the operating rules of the facility.
Inhibition curve does not yield a reasonable top plateau	• Use more data points in the region where you expect the top plateau.
Plasma protein binding: more than 2% of radioactivity in control sample is found in the eluate	• The column used for sample separation has probably expired. Use the fresh batch.
High standard deviation for log P value	• Probably inadequate sampling from the octanol-water system. Repeat the centrifugation and pipet the samples more carefully.
Adherent cell culture detachment during the incubation with radioligand	• Use collagen-coated cell culture flask or switch to more adherent cell line

VI. Acknowledgment

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GPCR high-throughput screening for ligands of adenosine receptors

Jana Kotulová

I. Introduction

Adenosine receptors (ARs) belong to the G protein-coupled receptor (GPCR) family and have four subtypes (A1, A2A, A2B, and A3) (1). Each receptor type is characterized by different abundance, expression level, downstream pathway preference, and the induction of the expression of different genes (2, 3). ARs are involved in the regulation of a plethora of biological processes, including pathophysiological conditions. Biological response includes for example modulation of brain and heart ischemia (4), pain (5), neurodegenerative diseases (6, 7), rheumatoid arthritis (8), cancer (9), inflammation, and immunomodulation (10, 11). In general, GPCRs belong to the most studied pharmacological targets for approximately 34 % of all Food and Drug Administration (FDA)-approved drugs. Currently, five AR ligands are used as analgesics, bronchodilators, CNS stimulants, and anti-inflammatory agents. Twenty-seven AR ligands are in clinical trials for a variety of conditions, including cancer (12).

Nonetheless, AR ligands are often reproached for their non-selectivity. Hence, emerging new

avenues in GPCR pharmacology, such as allosteric modulation or biased agonism, targeting only a single transduction pathway could substantially contribute to the elimination of any potential off-targets. High-throughput screening (HTS) is a great tool to facilitate GPCR drug discovery in its early phase. Moreover, luminescence detection offers a valuable replacement for fluorescence assays without interference from autofluorescent compounds that could result in high rate of false positive or false negative hits in agonist and antagonist screenings, respectively (13).

The protocol presented in this chapter describes an HTS assay for the identification of novel ligands (so-called primary screen, PS) of ARs that is also applicable to other GPCRs utilizing the same technology. For establishing HTS assay as well as for verification of identified hits after completing PS, several validation steps are crucial. Relevant details on pre-study validation and cross-validation of compounds are mentioned in part IV. Comments.

II. Materials and instrumentation

Instruments

- Echo555 liquid handler (LabCyte)
- FLIPR^{TETRA} multimodal reader (Molecular Devices)
- Heracell 150i cell culture CO₂ incubator (Thermo Scientific)
- MSC Advantage biological safety cabinet (Thermo Scientific)
- MultidropTM Combi Reagent Dispenser (Thermo Scientific)
- Vi-CELL Cell Counter & Cell Viability Analyzer (Beckman Coulter)
- Rotating wheel shaker SB2 (Stuart)

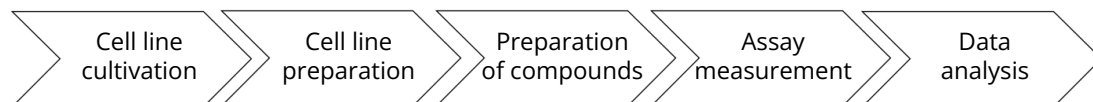
Materials

Ham's F12 (SH30026.02, HyClone), Dulbecco's modified Eagle's medium/F-12 1:1 (D-MEM/F-12; SH30272.02, HyClone), Eagle's minimum essential medium (EMEM; BE06-174G, Lonza), GlutaMAX (35050-061, Gibco), Geneticin (G418 sulfate, 11811-031, Gibco), Zeocin Selection Reagent (R25001, Gibco), TrypLETM Express Enzyme (12604054, Gibco), dimethyl sulfoxide, cell culture grade (DMSO, A3672, AppliChem), digitonin reagent, USP (A1905, AppliChem), coelenterazine h

(10111-1, Biotium), bovine serum albumin, protease free (BSA; P6155, Biowest), ethylene glycol bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA; 11290, Serva), potassium chloride (KCl; 30076-AP0, Lachner), di-sodium hydrogen phosphate dihydrate (Na₂HPO₄·2H₂O; 30388-AP0, Lachner), potassium dihydrogen phosphate (KH₂PO₄; 30016-AP0, Lachner), ethylenediaminetetraacetic acid tetrasodium salt (EDTA; E6511, Sigma-Aldrich), sodium chloride (NaCl; S7653, Sigma-Aldrich), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; C101, Sigma-Aldrich), adenosine (A9251, Sigma-Aldrich), HEPES (54457, Sigma-Aldrich). AquaMax sterilant (R8156, Molecular Devices), ethanol (EtOH; 85824.360, VWR international), heat inactivated foetal calf serum (FCSi; FBS-12A, Capricorn), double transfected reporter cell line CHO-K1 expressing mitochondrially targeted apoaequorin and A1 adenosine receptor (A1AR) (A1AR AequeoScreen cell line; ES-010-A, Perkin Elmer).

Culture flasks 150 cm² (90151, TPP), centrifuge tubes 50 ml (91050, TPP), LDV microplate (compound plate; LP-0200, LabCyte) and ViewPlate-384 (read plate; 6007480, Perkin Elmer).

III. Procedures



Scheme 40.1. General work flow of GPCR HTS for ligands utilizing AequeoScreen platform

Solutions

• Complete growth medium

Ham's F-12 medium

10% FCSi

2 mM GlutaMAX

Prepare the complete growth medium under the sterile conditions in the flow biological safety cabinet. Use the sterile labware for the medium preparation. Add appropriate volume of FCSi and GlutaMAX to the liquid culture medium. Filter the prepared complete growth medium using e.g. TOP filter Filtermax 0.22 µm. Close the bottle with the sterile cap. Store the prepared medium at 4 °C in the dark. Complete growth medium is stable for about 3÷4 weeks at 4 °C.

Selection antibiotics (working solution)

geneticin (G418, 0.4 mg/ml)

zeocine (0.25 mg/ml)

10× PBS buffer (phosphate buffered saline; stock solution)

- 1.37 M NaCl
- 27 mM KCl
- 100 mM Na₂HPO₄
- 18 mM KH₂PO₄

Dissolve the above mentioned chemicals in sterile water (tissue culture quality; eight tenth of the final volume), adjust pH to 7.4 with 1 M NaOH or HCl and add water to the final volume. Sterilize in an autoclave. Store at 4 °C.

1× PBS (working solution)

Dilute ten times the stock solution of the sterile 10× PBS in the sterile water (tissue culture quality). Use the sterile glass cylinder, bottle and the sterile bottle cap. Store at 4 °C.

Assay medium for HTS

DMEM/F-12 1:1 medium

0.1% BSA protease free

15 mM HEPES

Mix the above-mentioned substances. Prepare fresh prior the experiment under sterile conditions.

1× PBS/EDTA

Dissolve 0.2 g of EDTA (final concentration is 0.48 mM) in 1l of 1× PBS, adjust pH to 7.4.

- 10mM adenosine (stock solution)

Dissolve adenosine in DMSO. Aliquot and store at -20 °C. Avoid freeze-thaw cycles.

50mM digitonin (stock solution)

Dissolve digitonin in DMSO. Aliquot and store at -20 °C. Avoid freeze-thaw cycles.

10mM DPCPX (stock solution)

Dissolve DPCPX in DMSO. Aliquot and store at -20 °C. Avoid freeze-thaw cycles.

10mM tested compounds (stock solution)

Dissolve tested compounds in DMSO. Aliquot and store at -20 °C. Avoid freeze-thaw cycles.

Reference agonist solution

Prepare adenosine solution in assay medium from frozen stock solution, final concentration is EC80.

Wash fluid 1

Tissue culture quality H₂O.

Wash fluid 2

50 % ethanol diluted in tissue culture quality H₂O.

Cleaning solution

AquaMax sterilant solution prepared according to manufacturer's instructions, prepare fresh solution prior the post assay cleaning procedure.

A. Assay principle and work flow

For HTS purposes, AequoScreen platform is used. In addition to stably expressing apoaequorin and A1AR, this cell line is co-expressing promiscuous Gα16 subunit that can direct intracellular signalling to a calcium flux. The assay is based on the luminescent detection of transient increase/decrease in intracellular calcium level caused by GPCR-ligand interaction. Upon ligand binding, intracellular calcium released from endoplasmic reticulum is stimulated or blocked depending on agonistic or antagonistic response induced, respectively. Calcium ions together with photoprotein aequorin reconstituted by coelenterazine (lipophilic prosthetic group) are necessary for the reaction. Aequorin then oxidizes coelenterazine into coelenteramide accompanied by the production of CO₂

and emission of light (Fig. 1). The following measurement of emitted light is proportionally equal to GPCR activation (14, 15). There are different derivatives of coelenterazine on the market. However, coelenterazine h is a native

coelenterazine derivative that displays excellent bioluminescence properties while keeping its chemical stability at a reasonable level.

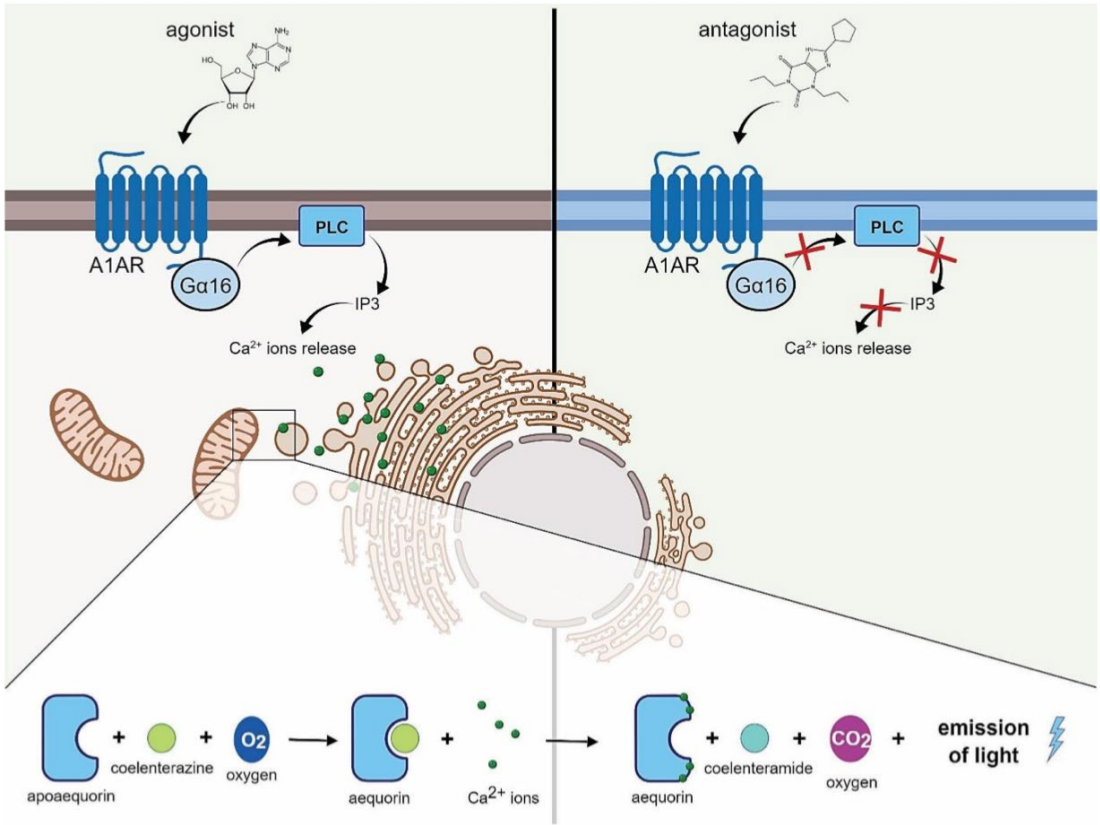


Figure 40.1. Aequorin assay principle. In presence of an agonist, A1AR activation is coupled to Gα16 subunit dissociation and execution of phospholipase C (PLC) function. PLC further stimulates inositol triphosphate induction of calcium release from endoplasmic reticulum. In addition, active aequorin is reconstituted by coelenterazine which upon calcium binding oxidizes coelenterazine into coelenteramide accompanied by production of CO₂ and emission of light. Subsequent measurement of emitted light is proportionally equivalent to measurement of intracellular Ca²⁺ flux and G protein-coupled receptor activity.

B. Cell line cultivation

1. Cultivate cells (A1AR AequeoScreen cell line) in complete growth medium with selection antibiotics according to manufacturer's instructions. Ensure that cells are in log phase of growth when subcultured, for further details on cell culture maintenance see Chapter 1. Volumes are given for a 150 cm² cell culture flask.
2. Remove and discard culture medium.
3. Briefly rinse the cells with 30 ml of 1× PBS.
4. Add 3 ml of TrypLE Express Enzyme and incubate cells for 5 minutes at 37 °C.
5. Add 10 ml of complete growth medium and resuspend cells by gently pipetting.
6. Add appropriate volume of cell suspension to a new culture vessel.
7. Passage cells three times a week at a split ratio of 1:8.

C. Cell line preparation

1. Cultivate adequate number of cells for an experiment. For aequorin assay of A1AR AequeoScreen cell line, 10,000 cells in suspension per well are used. In this respect, analysis of one read plate requires approximately 4×10^6 cells.
2. Cultivate cells at least 18 hours without antibiotics prior to the experiment.
3. Remove and discard culture medium.
4. Briefly rinse the cells with 30 ml of 1× PBS.
5. Add 5 ml of 1× PBS/EDTA solution to non-enzymatically harvested cells and incubate for 5 – 10 minutes.
6. Add assay medium and resuspend cells by gently pipetting.
7. Transfer the cell suspension to a centrifuge tube(s) and count the cell number.
8. Spin down the tubes for 5 minutes at $180 \times g$.
9. Discard the supernatant and resuspend cell pellet thoroughly in 1 ml of assay medium at first (in order to obtain a single cell suspension). Then, adjust the cell concentration to 2×10^6 /ml in assay medium.
10. Add coelenterazine h at a final concentration of 5 μ M to the cell suspension, avoid direct light during the addition.
11. Incubate the cells in centrifuge tubes at room temperature with constant gentle agitation (e.g. on rotating wheel) for 5 hours in the dark.

D. Preparation of compounds

HTS is conveniently run with the suspension cells in order to increase throughput. Therefore, an instrument capable of injecting cell suspension to the read plate and reading the signal at the same time is desired, i.e. aequorin assay requires to analyze the treated cells with pre diluted compounds.

1. Prepare the compound plate by adding 10 mM stock solutions of adenosine, digitonin, DPCPX, DMSO, and the compound to be tested (10 μ l per well).
2. Prepare the read plate by adding 25 μ l of assay medium and required volume of compounds (adenosine, digitonin, DPCPX, DMSO and tested compounds) from the compound plate using ECHO555 liquid handler. The final concentration of the tested compounds in the read plate should be 10 μ M. The read plate contains control wells for (i) DMSO interference (the final concentration 0.1 % DMSO), (ii) receptor-dependent positive control (adenosine, the final concentration 10 μ M in assay medium), (iii) receptor-independent positive control (digitonin, the final concentration 100 μ M in assay medium), and (iv) receptor-dependent negative control (DPCPX, the final concentration 10 μ M in assay medium).
3. Prepare microplate with reference agonist solution of final concentration EC_{80} in assay medium (any 384-well microplate).

E. Assay measurement

FLIPR^{TETRA} instrument requires several steps for initialization. Use Yellow test plate to ensure the standard deviation to be less than 3 %. Prior to start of the assay, cell reservoir and lines to and from spinner flask should be rinsed with (i) 70 % ethanol, (ii) endotoxin-free deionized water, and (iii) assay medium. Each washing step should be performed five times. Tips should be washed several times with endotoxin-free deionized water (tissue culture quality) and ethanol, 70 %.

1. Place the prepared suspension cells into spinner flask protected from light with cell flask spinning control set to 5.
2. Rinse the cell reservoir and tubing with cell suspension.
3. Place the reference agonist microplate into

position 2.

4. The read plate is automatically located into read plate position by TETRAcycler plate handler (if present), otherwise place the read plate in the indicated position inside the instrument.
5. Set signal acquisition to 35 reads with the read time interval of 1 second in agonist mode, and after 15-minute pause set the signal acquisition to another 35 reads with the read time interval of 1 second for antagonist mode.
6. During optimization phase adjust aspirating and dispensing speed and height to obtain a consistent response.
7. Using the FLIPR^{TETRA} automatic injection system, add 25 μ l of cell suspension to the read plate with compounds and read the signal in agonist mode. After 15 minutes of incubation with compounds, using the FLIPR^{TETRA} automatic injection system again, inject 15 μ l of reference agonist at a final concentration of EC₈₀ and immediately record the light emission for antagonist response.
8. For further analysis, export the obtained raw data (in relative light units) from ScreenWorks software as area under curve (AUC) for luminescence measurements.
9. Include the post assay cleaning procedure using AquaMax sterilant solution, note that post assay cleaning procedure should always follow according to manufacturer's instructions.

F. Data analysis and decision making

In routine PS data analysis, luminescence response is generated as AUC in 384-well plate format. Obtained data are subsequently evaluated whether they possess any activity against given GPCR. The compound signal is assessed as a percentage of activation for the agonistic response (cut-off value is set to 50 % of reference agonist) or percentage of inhibition for the antagonistic response (cut-off value is set to 50 % of reference antagonist), respectively.

Results from PS are further studied in secondary screen (SS) as 8-point dose-response analysis. Sigmoidal dose-response curves are generated, EC₅₀/IC₅₀ values are calculated and compared with the reference compounds.

Typically, methods using different approaches are involved in cross-validation phase to reveal non-active or interfering compounds, so-called false positive or false negative¹³, see part IV. Comments.

In both, PS and SS, in-study validation based on controls in plates to assess data robustness is performed (16, 17). Only robust data are included in calculations.

IV. Comments

In our setup, we use FLIPR^{TETRA} multimodal reader. Other instruments, e.g. MicroBeta (Perkin Elmer) or FDSS6000 (Hamamatsu Photonics), could also be used as far as they enable direct dispensing of cell suspension to the read plate and immediate recording of the signal. In addition to that, a turning point in producing high-throughput data is represented by automation and robotics. In general, utilizing advantages of robotic platform ensures assay robustness and minimize the impact of human error.

The procedure described herein concerns PS of compounds with unknown GPCR activity within an already well-established HTS functional assay. Special attention should be also applied to steps leading to the assay establishment and to following confirmation of identified active compounds (hits). Pre-study validation is an indispensable component of HTS assay development. Prior to the PS, testing of AequeScreen platform overall suitability for HTS by analysing series of parameters, including signal intensity, stability and consistency over time, existence of potential assay artefacts (edge effect, some patterns, etc.), and assay robustness is recommended.

Another critical point is further confirmation of hits from PS, i.e. cross-validation of findings comprised of (i) a different method to distinguish between active compounds and interfering ones directly with the detection technology (counter-screen), and (ii) a different method to verified active molecule properties (orthogonal assay). For our purposes, we use calcium mobilization assay as (i) counter-screen for unspecific calcium release from parental cells in the presence of compounds, and e.g. 3',5'-cyclic adenosine

monophosphate (cAMP) production analysis as (ii), which meets our requirements as an entirely different method based on the detection of cAMP presence by a specific antibody.

This chapter is intended to serve as a brief manual only. For additional information on HTS

assay development, validation, and HTS data analysis, the reader is referred to other literature, e.g. comprehensive manuscript Assay guidance manual (18, 19) or articles focused on validation and analysis such as (13, 20).

V. Pitfalls

The major problems encountered in HTS AequeoScreen assay are associated with either cell culture pitfalls or instrumentation pitfalls. Both types of difficulties can result in signal inconsistency and low data reliability. HTS

is a complex of different procedures that are of course susceptible to errors. In order to easily identify or prevent such errors following suggestions could be helpful.

Problem	Cause and cure
Obtained luminescence signal is low or unstable	- The prerequisite is to have more than 90 % of viable cells in suspension prior to the coelenterazine loading, otherwise a low luminescence response of cells may be expected. It is also possible to use adherent cells but it would decrease the throughput. It could be possible to compare cells from culture vs. freshly thawed cells, that might be used instead. - Be careful to keep the cells during incubation with coelenterazine and throughout the HTS assay in the dark. Coelenterazine is light sensitive and is subjected to decomposition in the presence of light. Also, coelenterazine is prone to auto-oxidation, especially when stored in solution. Hence, it is recommended to keep coelenterazine in vials under an inert gas atmosphere (e.g. nitrogen or argon). - Assessment of cell concentration per well in pre-study validation phase is recommended. Suggested cell density is 2,500 - 10,000 cells per well for 384-well plate format. - Coelenterazine loading could be extended to overnight at 4 °C.
Signal intensity could decrease over time (its stability)	Check the time for which the assay is intended to use without showing a decline in luminescence response. It may not be possible to run the assay for longer time span.
Signal acquisition delay	It is also advisable to use proper instrumentation. In GPCR or ion channel screenings signal must be acquired immediately after mixing cell suspension with compounds.
Different compounds have different dynamics of response	There are two parameters of luminescence response – intensity and the profile of cellular response. Adjustment of the signal acquisition parameters according to the reference compounds response characteristics would be eligible.

VI. Acknowledgement

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A combination of kinetic fluorometric assay and microscopy for the screening of potential anti-tau aggregating agents

Narendran Annadurai and Viswanath Das

I. Introduction

Gene of human microtubule-associated protein tau comprises 16 exons on chromosome 17q21 and encodes six tau isoforms ranging from 352 to 441 amino acid residues in size (1). Inclusion or exclusion of exon 10 results in the production of tau isoforms containing 4 or 3 imperfect repeats (R1-R3, Fig. 41.1). These repeats along with adjoining parts constitute the microtubule-binding domain (MBD) (2) (Fig. 41.1). Physiological tau is highly soluble, disordered, and heat-stable protein sensitive to proteases (3). Tau modulates the stability of axonal microtubules, recruits signaling proteins and regulates the microtubule-mediated transport (4).

Tau aggregation is central to the neurodegenerative process in tauopathies, a class of neurodegenerative disorders. In tauopathies, tau dissociates from microtubules and self-assembles into fibrillar aggregates. Tau is a major constituent of neurofibrillary tangles (NFTs) in Alzheimer's disease. NFTs are highly ordered and protease-resistant β -sheet structures (5). In MBD, both R2 (275-305 residues) and R3 (306-336 residues) domains of the tau protein exhibit the

tendency to form β -sheet structures (6). Tau R3 domain carries one of the important nucleating hexapeptide sequences of tau aggregation called as PHF6 (VQIVYK) along with disulfide bond forming cysteine residue (7). Tau R3 domain has been reported to form aggregated fibrils under self-aggregation conditions in the absence of heparin (8). R3 domain is the minimal sequence and active conformational nucleus that defines aggregation of the full-length tau, and R3 domain hereafter referred as R3 peptide aggregates is also able to induce aggregation of endogenous tau in 4R-tau MBD expressing cell line models (9).

Tau aggregates, in the specific oligomeric fibrillar form, are the major element of the neuronal cell toxicity in tauopathies. Aggregated tau protein is associated with many neurological disorders, which include Alzheimer's disease, and therefore establishing the *in vitro* tau aggregation assay for the screening of the potential small molecules could be helpful in targeting the formation of tau aggregates to halt the progression of tauopathies (10).

Here, we describe an *in vitro* R3 aggregation

assay in the presence of heparin which enhances the rate of aggregation of R3. The formation of aggregated R3 fibrils is monitored by the Thioflavin T (ThT) which binds to the β -sheet fibrils. Upon binding to the growing R3 aggregates, ThT exhibits the red shift in fluorescence (excitation: 450 nm; emission: 485 nm). In screening assay, active compounds with the inhibitory potential results in the reduced ThT fluorescence compared to the untreated control due to the reduced R3 aggregate formation. This robust, highly reproducible assay is helpful in screening larger compound libraries for the potential tau aggregation inhibitors.

II. Materials and instrumentation

Instruments

- Enspire multimode plate reader (PerkinElmer)
- Operetta High content screening system (HH12000000) (PerkinElmer)
- Benchtop microcentrifuge (5452000018) (Eppendorf minispin)
- GraphPad Prism (GraphPad)

Materials

Tau R3 peptide (AS-65435-1, AnaSpec), Thioflavin T (T3516-5G, Sigma Aldrich), heparin sodium salt (H4784-1G, Sigma Aldrich), CellCarrier 384-well plate (black/clear bottom) (6007550, PerkinElmer). Platemax (Aluminium-sealing film) (PCR-AS-200, Axygen Inc), Agarose (low gelling temperature) (A9414-25G, Sigma Aldrich), paclitaxel (Ebewe Pharma).

III. Procedures

Solutions

Aggregation buffer

- 20 mM Tris, pH 7.4
- 100 mM NaCl
- 1 mM EDTA

Dissolve appropriate amounts of the above-mentioned chemicals in deionized water (eight tenth of the final volume, e.g. 800 ml), adjust pH to 7.4 with 1 M NaOH and add deionized water to the final volume (e.g. to 1,000 ml). Filter using 0.22 μ m sterile syringe filter. Store at 4 $^{\circ}$ C.

1 mM Thioflavin T (ThT; stock solution)

Dissolve appropriate amount of the ThT in deionized water in aggregation buffer and mix it using vortex and filter using 0.22 μ m sterile syringe filter. Aliquot and store at -80 $^{\circ}$ C in a micro-centrifuge tube wrapped in aluminum foil.

150 μ M Heparin (stock solution)

Dissolve the heparin in deionized water and store at -80 $^{\circ}$ C.

R3 peptide (stock solution)

Dissolve R3 peptide in deionized water and mix it by pipetting. Aliquot in a 1.5 ml microcentrifuge tube and store at -80 $^{\circ}$ C. For the assay, remove stock solution of R3 domain from -80 $^{\circ}$ C and thaw on ice. Spin the solution at the maximum speed (12,000 rpm) for 45 seconds in a benchtop microcentrifuge.

Aggregation reaction mixture

Prepare the aggregation reaction mixture (total volume) containing ice-cold 1 $\times$ PBS, 10% aggregation buffer (v/v), 5 μ M heparin, 15 μ M ThT, and 20 μ M Tau R3 in the stated order.

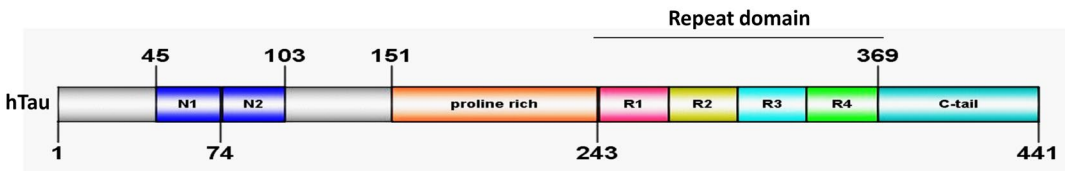
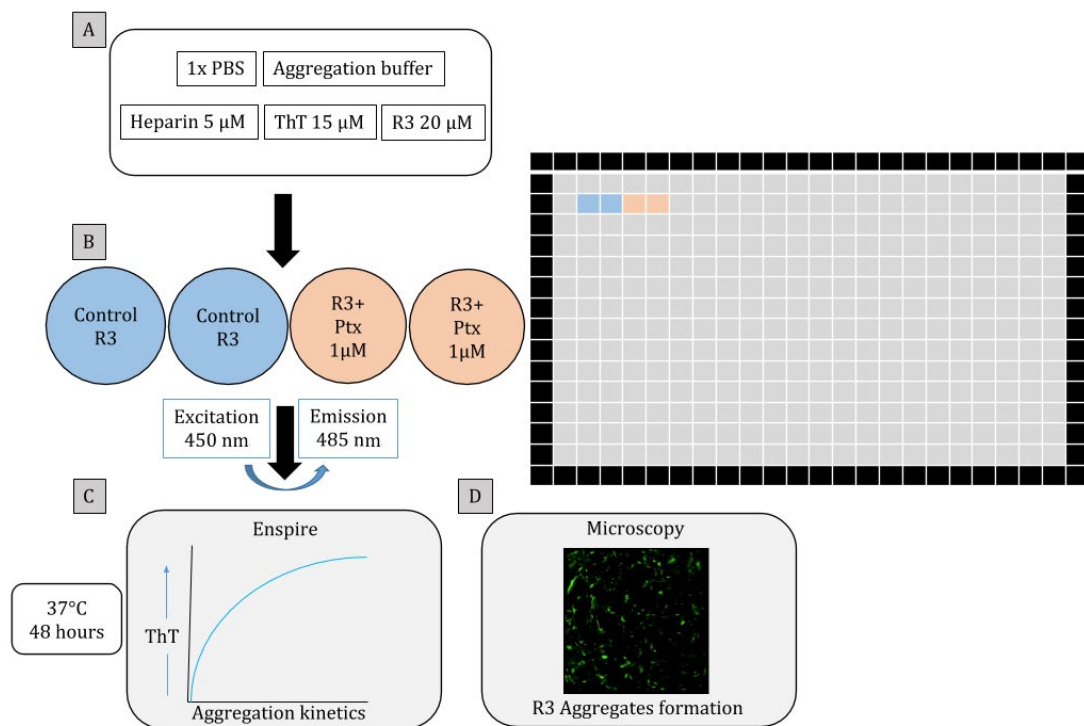


Figure 41.1. Structure of the longest human Tau isoform (hTau441).



Scheme 41.1. Simplified scheme of the assay for screening of R3 aggregation inhibitors. **(A)** Preparation of aggregation mixture, **(B)** Setting up assay in a 384 well plate, **(C)** Aggregation kinetics measurement in Enspire multi mode plate reader and, **(D)** Imaging formed R3 aggregates.

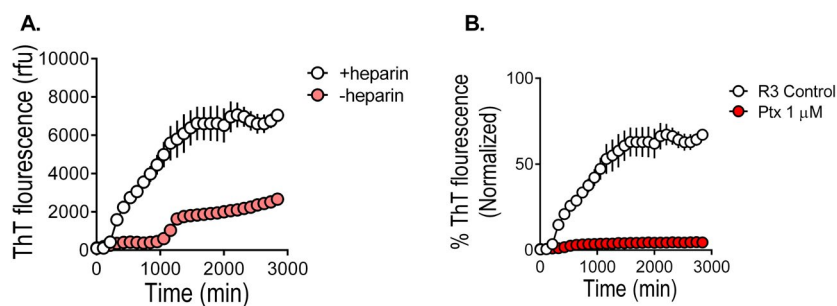


Figure 41.2. A. Graph showing the aggregation kinetics of Tau R3 domain in the presence and absence of heparin (48 hours). Mean $\pm$ SEM, $n = 3$. B. Graph showing the inhibition of R3 domain aggregation by Ptx. Mean $\pm$ SEM, $n = 3$.

Instrument setup

1. Turn on the computer and the Enspire multimode plate reader.
2. Start the Enspire software and create a protocol by following the steps below:
 - a. Select 384-general well plate type.
 - b. Set the kinetic measurement as 576 plate repeats at every 5 minutes.
 - c. Set the temperature to 37 °C, and set the upper heater temperature to 2 °C to prevent condensation in sealed plates.
 - d. Let the temperature of the instrument reaches 37 °C before starting the aggregation assay.
 - e. Set the shaking conditions as linear shake mode, 1,000 rpm with 5 minutes duration followed by measurement.
 - f. Set the excitation to 450 nm and emission to 485 nm.

A. Continuous-mode ThT aggregation assay (*in vitro*)

1. Prepare the aggregation reaction mixture and place it on ice.
2. Maintain 384-well plates on ice.
3. Add 40 µL of the aggregation reaction mixture to each well of the 384-well plate, seal the plate with Platemax and close the plate sides with the parafilm.
4. Place the plate into the Enspire multimode plate reader.
5. Maintain the plate under constant agitation (1,000 rpm) during the resting period.
6. Measure the changes in ThT fluorescence at 450 nm excitation and 485 nm emission in a continuous mode for 48 hours. Record the fluorescence intensity changes at a 5-minute intervals for 48 hours.
7. Normalize the raw data (ThT fluorescence) to the lower (0 %) and higher (100 %) relative fluorescence intensity values in each dataset. Plot the normalized data as the percentage of ThT fluorescence intensity relative to the time using e.g. GraphPad Prism software.

B. R3 peptide aggregation inhibition assay – drug screening

1. Prepare the aggregation reaction mixture

and dispense 36 µL of the mixture per well in 384-well plate.

2. In the untreated control wells, 4 µL of aggregation buffer add to make the final reaction volume as 40 µL per well in 384-well plate.
3. Add 4 µL of 10 µM paclitaxel (Ptx) to the aggregation reaction mixture in the drug treated wells of the 384-well plate. This will ensure that the final concentration of Ptx is 1 µM.
4. Perform all procedures on ice.
5. Seal the plate and measure the ThT fluorescence as described above.

C. Immobilization of formed R3 aggregates

1. Add 20 µL of 0.75 % low-melting agarose to each well of 384-well plate and allow the agarose to gel for a few minutes at room temperature.
2. Evaluate the plate using an Operetta high content screening system using 2x objective (numerical aperture: 0.08) and 488-laser line (Ex/Em: 488 nm/509 nm).

D. Representative results

ThT aggregation assay

Heparin-induced R3 peptide aggregation was confirmed by the time-dependent increase in the fluorescence intensity of ThT binding to R3 aggregates (Fig. 41.2A). The aggregation pattern is similar to *in vitro* aggregation of the full-length Tau.

Ptx inhibition of R3 aggregation

Ptx reduced ThT fluorescence and this confirms Ptx inhibited R3 aggregation (Fig. 41.2B).

ThT-stained R3 aggregates

The effect of Ptx on inhibition of R3 aggregation was characterized by reduced ThT-stained fibrillar aggregates compared to the untreated control sample. Ptx decreases the formation of R3 aggregates (Fig. 41.3).

IV. Comments

The *in vitro* aggregation process starts with a lag phase corresponding to the nuclei formation followed by fibril growing phase of R3. ThT, which binds to the β -sheet rich fibril structure allows the real-time automatic assessment of the growth of R3 aggregates as the aggregation rate can be monitored continuously. Thus compounds with an inhibitory potential of R3 aggregation will be identified with less ThT fluorescence compared to the untreated control R3 aggregation. The effect of compounds on

reducing the growth of aggregated R3 fibrils confirmed by fluorescence microscopy. Since it is a fluorescence-based assay, validating the inhibitory potential of colored compounds with self-fluorescence might be a problem which can be overcome by including compounds only controls. This reproducible, easy to handle and cost-effective method with the reduced assay time is a robust tool for the high-throughput screening for compounds that can inhibit the aggregation of R3.

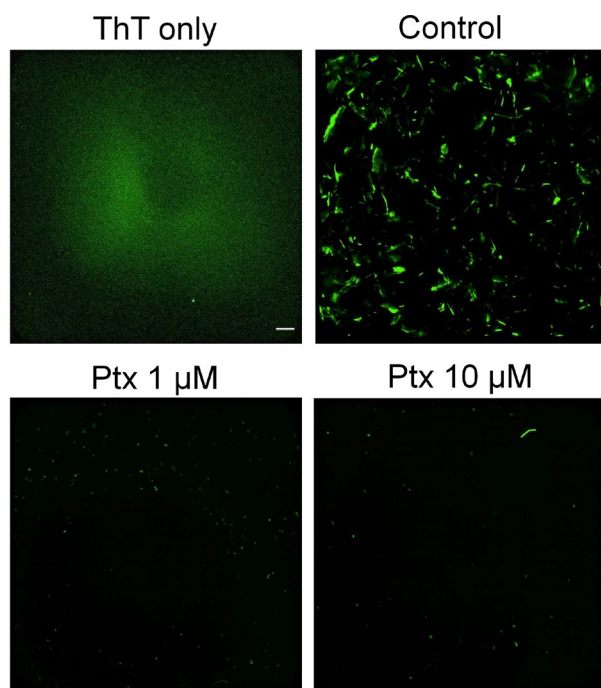


Figure 41.3. Images of ThT only, control and Ptx-treated samples embedded in low-melting agarose. Scale bar: 50 μ m, Objective: 2 $\times$.

V. Pitfalls

Problem	Cause and cure
Evaporation	
Evaporation of reaction mixture	As the assay is continuously running for 48 hours, there is a chance of evaporation of the reaction mixture. It results in the reduction of kinetics of R3 aggregation that is not related to the effect of compounds. Set the condensation temperature and seal the plate with parafilm to prevent the evaporation.
Self-fluorescence	
Compound self-fluorescence	Compounds that shows higher fluorescence than the ThT fluorescence in the absence of R3 peptide leads to the selection of false positives. Exclude those compounds further from testing.

VI. Acknowledgement

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Study of absorption, distribution, metabolism, and excretion (ADME) properties of a new drug candidates in pre-clinical development

Martina Medvedíková, Alejandro Carazo, and Barbora Lišková

I. Introduction

Pharmacokinetics is one of the subgroups of pharmacology, dealing with the time course of the fate of a drug, compound or new chemical entity (NCE) within the body after their administration. The four fundamental pharmacokinetic processes are absorption, distribution, metabolism and excretion. Their acronym gave the name of the scientific discipline of preclinical drug discovery called ADME.

The development of new drugs comprises prediction of the structure, determination of pharmacokinetic properties and the development of suitable models for predicting ADME properties at *in silico* and *in vitro* levels. Knowledge of the ADME properties of an NCE is critical to its selection as a leading candidate in a drug discovery program and/or its use as a functional research tool (1).

Absorption involves the entry of the drug into the bloodstream, which is referred to as a central compartment together with well-perfused organs. Most drugs are administered orally, therefore, the role of gastrointestinal tract (GIT) transporters and metabolic enzymes in drug absorption are

a subject of scientific interest. In addition, drugs can be administered by other routes: topical (skin, nasal mucosa, ocular topical), inhalational, intravenous, intramuscular, intraperitoneal and subcutaneous (2).

Distribution means a process where is studied the movement of xenobiotic into, through and out of body compartments. This process is highly related to the physicochemical properties of the drug: chemical structure, molecular weight, the ionization properties, lipophilicity, water solubility and capability of associations with physiological macromolecules (e.g. plasma or other tissue proteins). These properties determine the permeability of drugs across the epithelium of tissues and organs (3).

Metabolism is the chemical transformation of the drug into a metabolite that is eliminated or in specific situations a prodrug is activated to the drug. Metabolism, also called biotransformation, is subject to most drugs and xenobiotics that are absorbed into the body, through the activity of metabolizing enzymes. Biotransformation enzymes are grouped into two classes; Phase I and Phase II reactions. Phase I reactions include oxidation, reduction and hydrolysis reactions

primarily carried out by cytochrome P450 enzymes (P450; CYP) and flavin-containing monooxygenases (FMO). Phase II reactions refer to conjugations of xenobiotics with glucuronic acid, sulfuric acid, specific amino acids, etc. In general, biotransformation reactions that aid in the elimination of NCE increases the water solubility of the compound (decrease the lipophilicity) (4).

Excretion is the process of elimination of a drug from the body. A drug may be excreted in its original chemical state or as metabolites (after chemical modification). The majority of drugs are eliminated by pathways that involve the kidneys (renal excretion) or the liver (hepatic metabolism - phase I and phase II biotransformation reactions) in the form of urine or faeces (biliary elimination). Other minor routes of drug excretion from the body are through the lung, sweat, tears, skin, hair, or saliva (5).

Physicochemical properties (e.g. solubility), cell membrane permeability, transporter and drug metabolizing enzyme specificity are important factors in the characterization of NCE in the evaluation of leading candidate in pre-clinical drug discovery.

High-throughput Screening (HTS) is a method used in drug development research, which enables the testing of a large series of chemicals (identify “lead” compounds or “hits”), under established conditions in a short period of time. This method uses robots specially designed for these applications to test chemical properties or biological activity of newly developed compounds. Interestingly, several drugs used to date (sorafenib, maraviroc etc.) have been discovered thanks to HTS (6). The main advantages of this method are the effectiveness, relevance, speed, robustness and the accuracy of the provided data. Once hit compounds are identified in HTS via target engagement methods, *in vitro* ADME techniques play an important role in the further selection of leads based on optimal pharmacological properties.

II. Materials and instrumentation

Chemicals

All chemicals for preparing of buffers are purchased from Penta s.r.o., Czech Republic.

1x Phosphate Buffer Saline (PBS) (containing 137 mM NaCl, 2.7 mM KCl, 10mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 10 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4) and 100 mM K/PO_4 , pH 7.4. Isocitric dehydrogenase (I2002), isocitric acid (I1252), NADP+ (N0505), Lecithin (L- α -phosphatidylcholine, 61755) and dodecane (D221104) are obtained from Sigma-Aldrich, Czech Republic. Human plasma (Transfusion Department, University Hospital Olomouc, Czech Republic), human liver microsomes (HMMCPL, Thermo Fisher Scientific, USA), methanol (1.06035, Merck Millipore, Germany), formic acid (I2220, Fluka, Germany), acetonitrile (83640.320) and water (83645.320, VWR Chemicals, USA).

Culture media supplemented for each cell line according to requirements. DMEM (D5796), Fetal Bovine Serum (F0804), Penicillin-Streptomycin (P4333), Non-essential amino acids (M7145), L-glutamine (G7513), Lucifer Yellow (L0144) are from Sigma-Aldrich. HBSS (14025-050, ThermoFisher Scientific, USA).

All standards for each assay are purchased from Sigma-Aldrich: propranolol (P0884), atenolol (A7655), verapamil (V4629), rhodamine 123 (83702), antipyrine (A5882), and ranitidine (R101).

Instruments

pH meter (Chromservis, Czech Republic), Shaker Vortex IKA MS3 (Merci, Czech Republic), Thermo-Shaker TS-100C, Orbital Shaker PSU-10i and Orbital Shaker – Incubator ES-20 (BioSan, Latvia), Centrifuge Hanil 514R (Hanil, Republic of Korea), Freeze Dryer system – CoolSafe (ScanVac, Denmark), vacuum pump (Vacuubrand, Germany) and SpeedVac Concentrator (Thermo Scientific, USA), Biological safety cabinet HeraSafe KS12 and Incubator HeraCell 150i (Thermo Scientific, USA), Microplate reader Infinite M200 Pro (Tecan, Switzerland), TEER instrument (Merck, Czech Republic), Rapid Fire 300 (Agilent, MA), and Mass spectrometer QTrap 5500 (AB SCIEX, Canada).

Accessories for permeability assays

MultiScreen plates for PAMPA assay (donor – MATRNPS550 and acceptor – MAIPN4550,

Merck Millipore, USA). Inserts (pore size 1 μm , polyester membrane) of 24 well plate (PSRP010R1) for MDCK cell line assay are purchased from Merck Millipore, USA and all Transwell® plates and inserts are from Corning, USA. Receiver plates of 24 well and 96 well plates (3524 and 3382, respectively), Inserts (pore size 0,4 μm , polyester membrane, 3379) of 24 well plate and inserts (pore size 0,4 μm , polycarbonate membrane, 3391) are for Caco-2 cell line assay. Inserts (pore size 1 μm , polyester membrane, 3392) are for MDCK cell line assay.

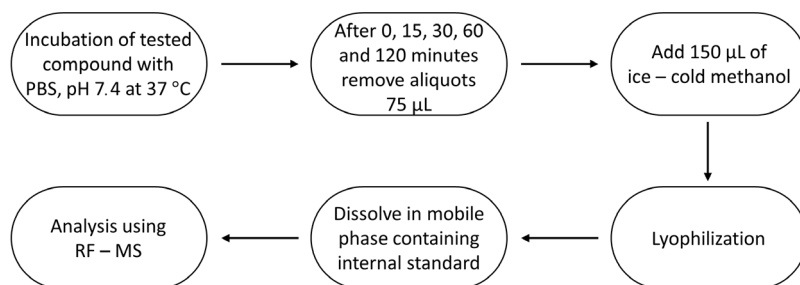
III. Procedures

Chemical stability

Stability in solution is a fundamental property of successful drug candidates. Only the dissolved fraction of a new chemical entities (NCE) can move through membranes by diffusion or via transporter mechanisms or interact with receptors/enzymes. Solubility is an important parameter in drug development as it is limiting factor for the free-to-interact fraction of the compound in the gastrointestinal fluids, plasma and other matrices in the body. Physicochemical parameters, such as solubility or chemical stability, are used therefore for the prediction of the compounds' behaviour *in vivo*. Lack of stability may be due to non-enzymatic processes, e.g. a variety of mechanisms include hydrolysis, oxidation, light-catalyzed degradation and others. These reactions are initiated by physicochemical factors such as pH, other components in the same solution (e.g. metals), enzymes, light, heat, humidity, and oxygen (7).

Chemical stability always involves dissolution of the compound in appropriate buffer. Care should be taken to ensure, that the compound is not unstable in the buffer. A prime consideration is the buffer pH. At early stages of drug discovery, screening for stability in buffer solutions at acidic, neutral and basic pH is sufficient. Many *in vitro* screens are performed at physiological pH to mimic the *in vivo* situation. If the compound is unstable at pH 7.4, samples generated in the *in vitro* screens may decay rapidly prior to analysis (8). Oral administration is the route of choice for new drugs. Compounds must be chemically stable at the low pH values observed in the stomach. Therefore, knowing if a compound is stable at pH 2 is useful information in predicting if oral administration is likely to pose a problem.

1. Add the studied compound to the PBS (pH 7.4) for final concentration 7.4 μM in solution.
2. Incubate at five time points (0, 15, 30, 60, and 120 minutes) at 37 °C.
3. After each incubation, remove aliquots of reaction solution (75 μL) in the triplicate into 96 well plate.
4. Terminate a reaction by the addition of 150 μL of ice-cold methanol.
5. Lyophilize.
6. Dissolve dry pellet in 200 μL of the mobile phase containing internal standard and analyze by the RapidFire 300 system coupled with QTRAP 5500 mass spectrometer (RF-MS).
7. Calculate the percentage of tested compound remaining at the individual time points versus time 0.



Scheme 42.1.

Plasma stability

Blood plasma is a yellowish colored liquid component of blood, containing a large number of biomarkers including dissolved proteins (albumins, globulins, fibrinogens), nucleic acids, enzymes (esterase, amidase or protease), and other metabolites. Determination of the stability of new chemical entities (NCE) in plasma is essential for maintaining acceptable drug concentration and half-life in order to achieve desirable pharmacological effects (9). Certain classes of drug molecules are potentially labile to the plasma enzymes. For instance, structures containing ester or amide-linked groups are prone to hydrolysis by plasma enzymes.

Instability in plasma can result in rapid clearance, short half-life and poor *in vivo* performance. Furthermore, they cause difficulties for pharmacokinetic (PK) studies, because the compounds will continue to degrade even after the blood samples were taken from the animals.

On the other hand, plasma instability is desirable for prodrugs. The prodrug contains a protecting group in its molecule which makes it pharmacologically inactive but exhibits better solubility or favourable intestinal absorption. Plasma hydrolysis will rapidly release the active form of the drug (10). Plasma stability is therefore also very useful for screening of prodrugs and antedugs, where rapid conversion in plasma is desirable.

1. Add the tested compound to the preheated human plasma for final concentration 3.3 μM in solution.
2. Add the control compounds (verapamil and propranolol) to the preheated human plasma for final concentration 3.3 μM in solution.
3. Five time points of incubation are 0, 15, 30,

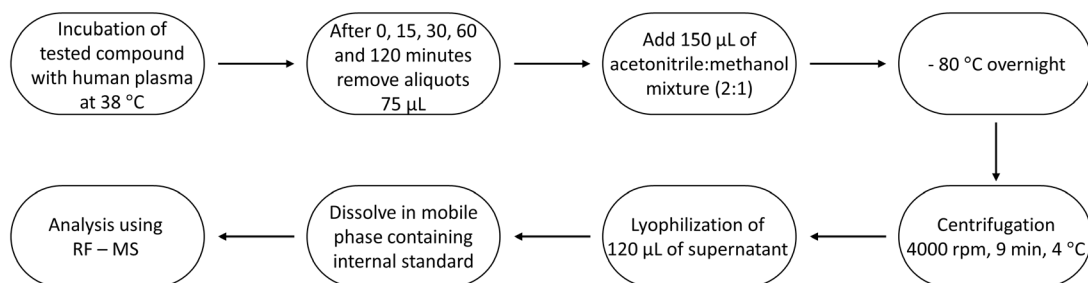
60 and 120 minutes at 38 °C.

4. After each incubation, remove aliquots of reaction solution (75 μL) in the triplicate into 96 well plate.
5. Terminate of a reaction by the addition 150 μL of acetonitrile:methanol mixture (2:1).
6. Store - 80 °C overnight.
7. Centrifuge (4000 rpm, 9 min, 4 °C).
8. Pipette 120 μL of supernatant into clear 96 well plate and lyophilize.
9. Dissolve lyophilizate in 200 μL of the mobile phase containing internal standard and analyze on the RF-MS.
10. Calculate the percentage of tested compound remaining at the individual time points versus time 0.

Microsomal stability assay

The *in vitro* microsomal stability assay is the model of choice for drug metabolism and for drug interaction studies. Liver microsomes, as vesicles of the hepatocyte endoplasmic reticulum, contain membrane phase I enzymes, namely cytochromes, flavine-containing monooxygenases, esterases, amidases, epoxide hydrolases, and phase II enzymes such as UGTs (4). Usually, the microsomal stability assay is based on a particular CYP450 metabolic pathway (apply NADPH generating system).

Liver microsomal preparations are the most common metabolic reagents used in metabolism studies because of convenience, low cost, ease of use, and reproducibility. Hepatocytes are expensive; therefore they are commonly used in late stages of drug discovery and in development of a small number of compounds. It is important to remember that in addition to Phase I microsomal metabolism, plasma



Scheme 42.2.

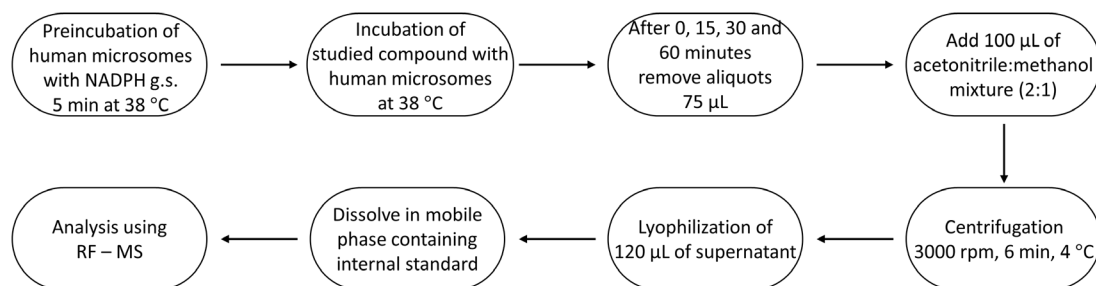
clearance can also be affected by other processes such as Phase II metabolism, extramicrosomal hepatocyte metabolism, renal excretion, biliary clearance, lung metabolism, plasma degradation, and erythrocytes binding. Every compound will be cleared to a different extent by the different mechanisms. Although microsomal stability is the main mechanism of plasma clearance, it is only one of several *in vivo* clearance mechanisms (11).

1. Use human liver microsomes (0.5 mg/mL).
2. Buffer reaction mixtures by 100 mM KH_2PO_4 (pH 7.4) and add NADPH generating system consisting of NADP⁺ (0.5 mM), isocitrate dehydrogenase (6 U/mL), isocitric acid (4 mM), and MgSO_4 (5 mM) according to (12).
3. Preincubate human liver microsomes within the NADPH generating system (in a 12-well plate) in a thermoblock at 38 °C with agitation at 250 rpm for 5 minutes.
4. Add compound of interest to the reaction mixture for a final concentration of 5.74 μM .
5. After incubation, transfer 75 μL of each reaction mixture to a 96-well plate in triplicate.
6. Terminate the reaction by adding of 100 μL of acetonitrile:methanol (2:1) to the reaction mixture.
7. Incubate the samples variable of time points as 0, 15, 30, and 60 minutes.
8. Centrifuge the sample (3000 rpm, 6 min, 4 °C).
9. Pipette 120 μL supernatant into 96-well plate and lyophilize it.

10. Dissolve residues in 200 μL of the mobile phase with internal standard and analyze it by the RF-MS.
11. Calculate the intrinsic clearance using the formula: $\text{CL}_{\text{int}} = V \cdot (0.693 / t_{1/2})$, where V is the volume of incubation in μL related to the weight of microsomal protein in mg per reaction. Half-life values ($t_{1/2}$) are calculated using the equation $t_{1/2} = 0.693 / k$, where k is the slope of the ln of the percent compound remaining versus time curve (13).

Plasma protein binding

The binding of studied molecules to plasma proteins can impact their distribution and efficacy as it is assumed that only the free fraction of the drug is available to interact with the disease target and exhibit pharmacological effects. The plasma proteins that are capable of binding a compound (and its metabolites) include albumin, α 1-acid glycoprotein (AAG), lipoproteins and α -, β -, and γ -globulins. Serum albumin and α 1-acid glycoprotein in particular are present in sufficient quantities and are capable of binding a broad variety of compounds. For example, albumin is a 66-kDa globular protein of 585 amino acids and is the major protein component of plasma (~4.5% w/v). Albumin has multiple hydrophobic binding sites and is capable of binding endogenous ligands (e.g. fatty acids and metal ions) as well as drugs (14).



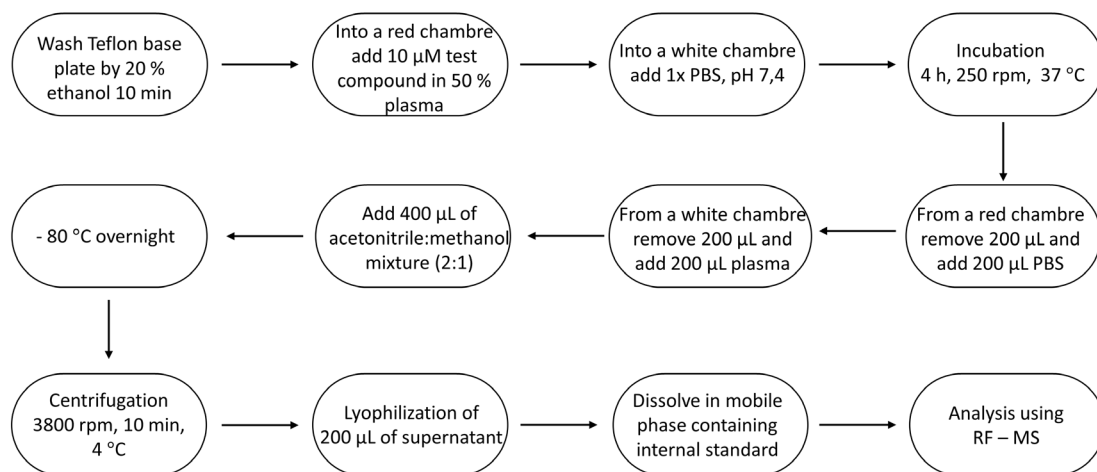
Scheme 42.3.

In recent years, several *in vitro* methods for measuring the unbound fraction in plasma have been developed, including equilibrium dialysis, ultrafiltration, ultracentrifugation, microdialysis, solid phase microextraction, and high-performance frontal analysis. Equilibrium dialysis is the most frequently used method. Rapid Equilibrium Dialysis (RED, Fig. 42.1) device is designed for efficiently performing protein binding assays and uses inserts that consists of two chambers (Red and White) separated by a semi-permeable membrane that has a high surface area-to-volume ratio allowing for rapid equilibrium (15). The semipermeable membrane between the two chambers of dialysis buffer and plasma, separates the free drug fraction from that bound to plasma proteins.

1. Use the RED (Rapid Equilibrium Dialysis, Thermo Scientific™, Rockford, USA) test.
2. Wash the teflon base plate with 20 % ethanol for 10 minutes.
3. Remove ethanol and wash twice with ultra-pure water.
4. Let the teflon base plate dry.
5. Place chambers on the teflon base plate - ensure that they do not touch the semipermeable membrane.
6. Prepare 50 % of the plasma in 1x PBS (pH 7.4).
7. Prepare 1 mL of the studied compound

and reference substances (atenolol and propranolol) at a concentration of 10 μM in 50 % plasma.

8. Pipette 300 μL of plasma with studied compound into the chamber R (red) and add 500 μL of PBS buffer into the chamber W (white).
9. Overlay teflon base plate with adhesive foil.
10. Incubate at 37 °C with shaking 250 rpm for 4 hours.
11. After incubation, transfer equal volumes (200 μL) of solution into 48 well plate, add the same quantity of 50 % plasma to the PBS buffer (from chamber W). Likewise, the PBS buffer to the solution of the test substance in plasma (from chamber R).
12. Terminate the reaction by the addition of 400 μL of acetonitrile:methanol (2:1).
13. Store at -80 °C overnight.
14. Centrifuge at 3800 rpm, 10 min, 4 °C.
15. Pipette 200 μL supernatant into 96 well plate and lyophilize.
16. Dissolve lyophilizate in 200 μL of the mobile phase with internal standard prior to the RF-MS analysis.
17. Calculate unbound fraction of studied compound in plasma as the ratio of the concentration of unbound compound in plasma (chamber W) to the total plasma concentration of the compound (chamber R).



Scheme 42.4.

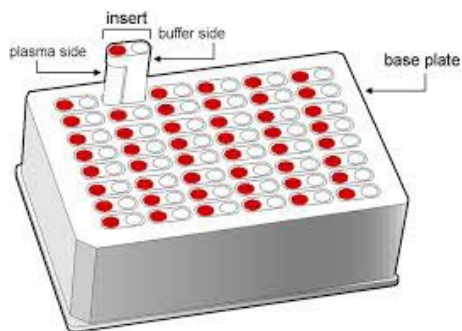


Figure 42.1. The rapid equilibrium dialysis device, base plate and inserts (16).

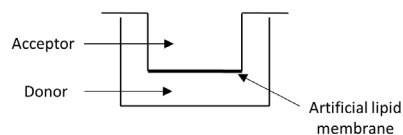


Figure 42.2. PAMPA provides information about passive diffusion of the tested compound.

PAMPA (Parallel Artificial Membrane Permeability Assay)

PAMPA (Parallel Artificial Membrane Permeability Assay) is a cell-free assay that simulates the passive permeability absorption of compounds through an artificial membrane. Commonly, drugs enter the circulation through several processes such as: passive diffusion, facilitated transport, active transport or by processes such as endocytosis, exocytosis, and pinocytosis. PAMPA assay studies the passive diffusion of compounds, which is the process by which most of the small and lipophilic drugs enter circulation. Through this process, a drug migrates from a high drug concentration environment to a lower drug concentrated environment, until the concentrations at both sides are equilibrated. Passive diffusion is the only transport process by which the presence of other membrane proteins (transporters, ion channels etc.) is not needed, since it happens without energy requirements. The membrane used in PAMPA assays is complemented with a lipid solution and separates two compartments: donor (lower compartment, initially containing the compound) and acceptor (upper compartment, initially free of compound) (Fig. 42.2). The plate is assembled with the donor and acceptor compartments and after the incubation period, the amount of compound that spontaneously diffused to the acceptor compartment is quantified.

PAMPA is used as a useful screening tool in the early steps of drug discovery. This assay provides predictive data regarding the absorption of

new chemical entities (NCE) and allows to classify tested compounds based on their passive diffusion properties. The obtained information is relevant since this kind of transport happens in GIT and brain among other parts of the human organism. Advantages of this method are the high throughput, reduced amount of time required for the experiment and the low cost of the experiments. In addition, PAMPA assays have shown good correlation with Caco-2 permeability experiments (17).

1. Prepare 10% lecithin solution with dodecane (work in hood cabinet due to dodecane toxicity), calculate the needed solution volume for each experiment in advance.
2. Prepare initial (1996 μL PBS with studied compounds at final concentration 20 μM) and equilibration (225 μL PBS + 450 μL initial solution) solutions, in 12-well plates – also for low log P_e (atenolol or ranitidine) or high log P_e (propranolol) standards.
3. Pipette 300 μL of the initial solution to the donor compartment.
4. Add 5 μL lecithin solution to each well, carefully dropping a bubble to the membrane and never touching it or the walls of the well - check that all the area of the membrane is successfully covered by the solution, as shown in figure 42.3.
5. Pipette 150 μL of PBS to the acceptor compartment, bring together both parts of the plate.
6. Seal the plate with tape and place in a box containing humidified paper, incubate for

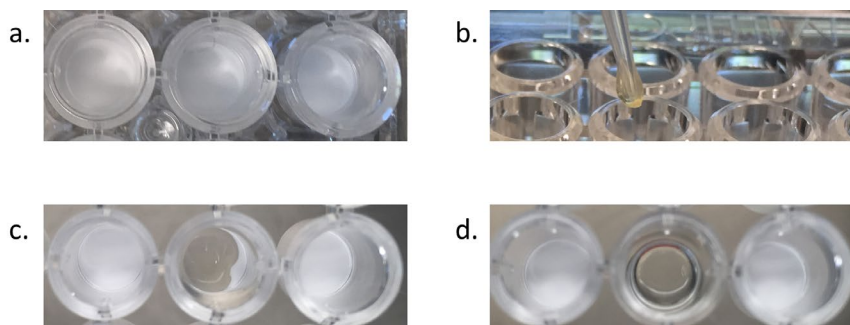
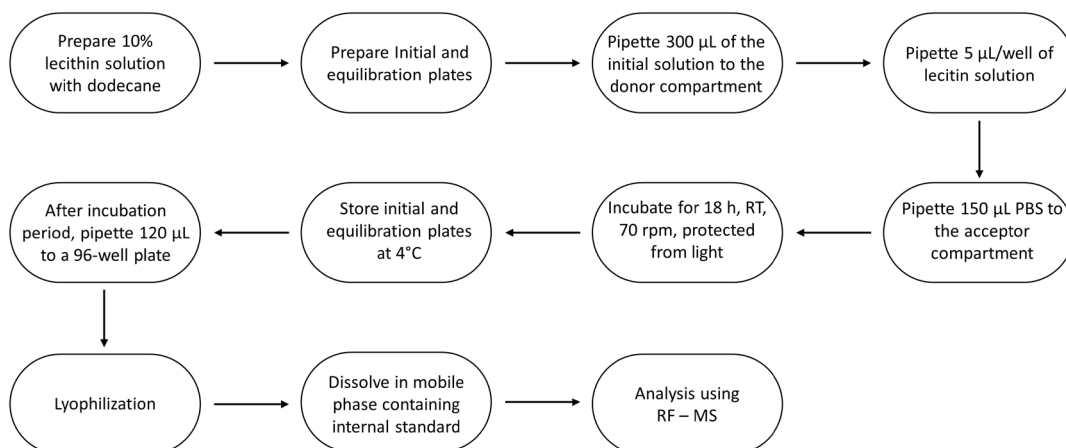


Figure 42.3. a. original appearance of the membrane; b. and c. 10% lecithin solution drop, which gradually covers the membrane; d. final appearance of the artificial lipid membrane.

- 18 hours at 70 rpm, room temperature.
7. Store initial and equilibration plates at 4 °C
8. After incubation is over, pipette in triplicates 120 μ L of each well to a new 96-well plate, according to figure 42.4.
9. Lyophilize the samples in 96-well plate.
10. Reconstitute lyophilized product in the corresponding mobile phase containing 10 μ M of internal standard.
11. Calculate in the corresponding volume of mobile phase the effective permeability logarithm ($\log P_e$) and recovery from data acquired by this assay in RF-MS system:

Acceptor	○ ○ ○
Equilibration	○ ○ ○
Donor	○ ○ ○
Initial	○ ○ ○

Figure 42.4. Well distribution of PAMPA assay after incubation.



Scheme 42.5.

$$\text{Log } P_c = C \times \left(-\ln \left(1 - \frac{\text{Drug acceptor}}{\text{Drug equilibration}} \right) \right)$$

$$\text{where } C = \frac{V_D \times V_A}{(V_D + V_A) \times A \times t}$$

$$\text{Recovery (\%)} = \frac{D + A}{I} \times 100$$

Drug acceptor: drug concentration in acceptor compartment
Drug equilibration: drug concentration in equilibration plate

V_D: volume in donor compartment
V_A: volume in acceptor compartment
A: area of the artificial membrane
t: time of the experiment (in seconds)

D: drug concentration in donor compartment by the end of the experiment

A: drug concentration in acceptor compartment by the end of the experiment

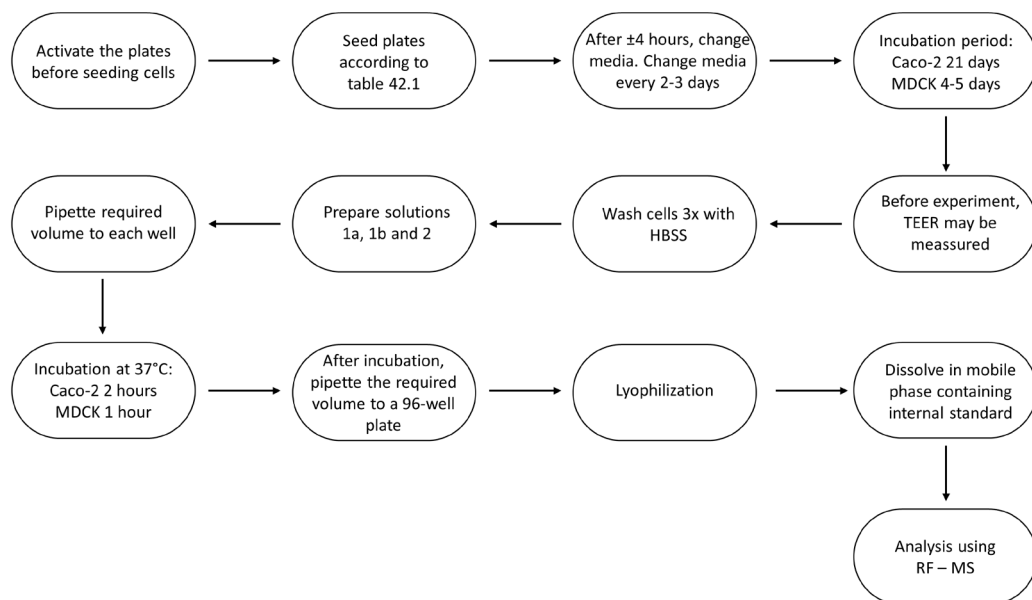
I: drug concentration at the start of the experiment

Cell permeability assays: Caco-2 and MDCK cell lines

Caco-2 and MDCK cell lines are well-established *in vitro* models for the study of drug permeability and absorption. These models provide significant information regarding permeability of the studied compounds and have proven to nicely correlate with the absorption processes in the human organism. Unlike PAMPA, these assays yield information regarding the active and passive transport of the studied compounds. Active transport is mediated through drug transporters (uptake and efflux) which are

membrane-bounded proteins. Some of the most relevant efflux transporters are P-glycoprotein (P-gp, MDR1) and breast cancer resistance protein (BCRP). Important uptake transporters are solute carrier transporter family (SLC). These transporters are widely distributed in the organism: liver, intestine, kidney, and brain. Therefore, these assays are useful tools in drug development.

Caco-2 cell line is originally derived from human colorectal adenocarcinoma and when cultured for 21 days, cells form a monolayer with tight junctions and differentiate into enterocytes,



Scheme 42.6.

expressing several enzymes and transporters. Caco-2 cells possess uptake and efflux systems and have been used for several years as the reference screening tool and for the study of the intestinal absorption process for NCE (18). Permeability experiments with these cells differentiate between high, moderate and low P_{app} drugs.

MDCK (Madin-Darby Canine Kidney) cells were isolated from canine renal tissue. Unlike Caco-2 cells, these cells are rapidly growing and thus, when cultured in semi-porous membranes, only 3 to 5 days are needed to develop a monolayer, differentiate and be ready for experimentation. In addition to the parent MDCK cells, a stably transfected cell line with MDR1 (P-gp, ABCB1) transporter has been developed (MDCK-MDR1) enabling to study the effect of MDR1 overexpression, serving as a model for the study of the blood-brain barrier (BBB) transport (19). MDCK permeability experiment differentiate between high and low P_{app} compounds. These cells represent another rapid tool for the screening of drug permeability and transport.

To perform permeability assays, cells are cultivated in semipermeable supports coupled to multiwell reservoir plates, typically 24 or 96-well plates. The data provided by these experiments are apparent permeability (P_{app} , units in cm/s), recovery and efflux ratio (ER). However, interlaboratory variations among permeability results have been observed over the years, and thus, well-established standards must be included in the experiments (see comments section) (20).

1. One hour before seeding, activate the plates by addition of the required volume of media (21) (for instance, for 96 well plates, 115 μ L to the insert wells and 250 μ L to the receiver wells) and incubate at 37 °C - the following steps of protocol are described for one drug, therefore, quantities should be adapted to the number of tested compounds.
2. Discard media from cultivation flask, detach cells and count them using a Bürker cell counting chamber, seed cells according to table 42.1.
3. After cell seeding change media when cells are attached to the membrane (during cultivation period, media should be changed every 2÷3 days).
4. Cells incubation period:
 - a. Caco-2 cells: 21 days
 - b. MDCK cells: 4-5 days
5. After incubation discard medium and wash the cells 3× with HBSS, leave the last wash for several minutes at 37 °C.
6. Prepare solutions for receiver (basolateral) plate (**solutions 1a** and **HBSS**) and for the insert (apical) plate (**solutions 1b** and **2**) – solutions must be prepared for the testing compounds and standards.
 - a. **Solution 1a:** prepare a 10 μ M solution of the tested compound in 2 mL of HBSS in a 12-well plate.
 - b. **Solution 1b:** transfer 750 μ L of solution 1a and prepare a 100 μ M solution of Luciferase Yellow, as a marker of cell monolayer integrity, in another 12-well plate.

TABLE 42.1. Volume, cell seeding number and surface of the insert plates used in cell permeability assays.

		insert well			receiver well
	plate type	volume [μ L]	cells seeded [cell number/mL]	surface [cm ²]	volume [μ L]
Caco - 2	24 well	150	112,000	0.33	600
	96 well	115	112,000	0.143	250
MDCK- MDR1	24 well	400	30,000	0.7	800
	96 well	115	30,000	0.143	250

- c. **Solution 2:** prepare 250 μL of HBSS with Luciferase Yellow at a final concentration of 100 μM .
7. Pipette the given volume to each well according to figures 42.5a and b.
8. Incubate the plates at 37 $^{\circ}\text{C}$ for 2 hours (Caco-2 cells) or 1 hour (MDCK cells).
9. After incubation period, pipette into a new 96-well plate: 80 μL (from 96-well plate) or 100 μL (from 24-well plate) according to the following scheme:
 - a. A0 1b solution
 - b. B0 1a solution
 - c. AB A₁₂₀ insert
 - d. AB B₁₂₀ receiver
 - e. BA A₁₂₀ insert
 - f. BA B₁₂₀ receiver
10. Lyophilize.
11. Reconstitute the lyophilizate in the corresponding mobile phase containing 10 μM of internal standard.
12. Calculate the permeability coefficient (P_{app}), recovery and efflux ratio (ER) from data acquired by this assay in RF-MS system:

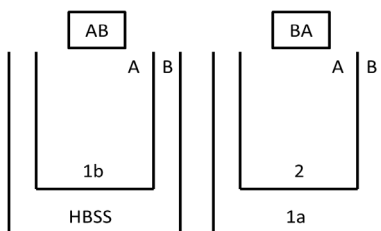


Figure 42.5a. Well distribution. *A* (apical) represents the insert with semi porous membrane and *B* (basolateral) represents the receiver well.

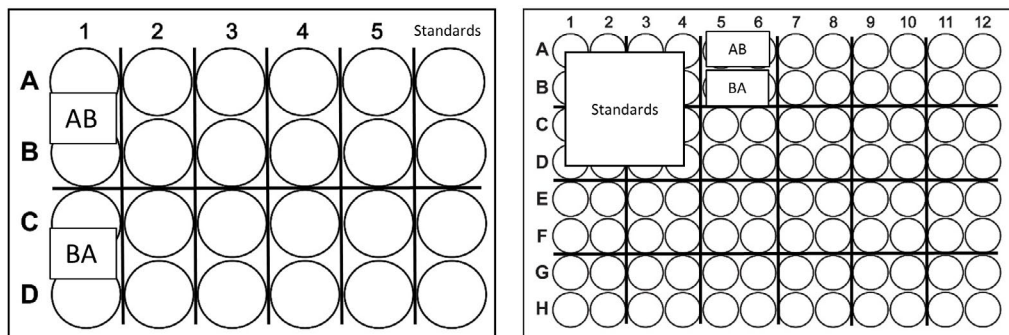


Figure 42.5b. Experiment distribution in 24-well (left) and 96-well (right) plates.

$$P_{app} = \left(\frac{dQ/dt}{A \times C_0} \right)$$

$$\text{Recovery (\%)} = \frac{D + A}{I}$$

$$\text{Efflux ratio} = \frac{P_{app} \text{ (BA)}}{P_{app} \text{ (AB)}} \times 100$$

dQ/dt: rate of drug permeation

C₀: drug concentration in acceptor compartment

A: area of the cell monolayer

D: drug concentration in donor compartment by the end of the experiment

A: drug concentration in acceptor compartment by the end of the experiment

I: drug concentration at the start of the experiment

ER > 2 *Efflux*

ER < 2 *No efflux*

Cell monolayer integrity measurement - quality assurance of the assay:

1. Luciferase assay: Lucifer yellow (LY) is a low permeability compound and is only able to pass through the cell monolayer by passive paracellular diffusion. LY is added to the insert wells, and if the cell monolayer has grown correctly and formed tight junctions, no LY should be found in the receiver well.

a. Transfer solutions 1a and HBSS from each well of the receiver plate into corresponding well of a black plate with transparent bottom: solutions for RFU_{basolateral} measure.

b. Transfer solutions 1b and 2 from 12-well plates into corresponding well of a black plate with transparent bottom: solutions for RFU_{apical} measure.

c. Read plates in fluorescent reader (Micro plate reader Infinite M200 PRO) measure fluorescence: Excitation wavelength: 485 nm, Emission wavelength: 530 nm, Plate: Corning 96 Flat Bottom black, clear bottom.

d. Calculate LY rejection: % LY rejection = 100 x (1 - RFU_{basolateral} / RFU_{apical}).

2. Transepithelial Electric Resistance (TEER): resistance measurement is another assay that can be performed to measure the cell monolayer integrity. This assay measures provide information about the cellular monolayer integrity by measurement of the resistance to pass current across a cell monolayer. TEER can be measured after plate activation, during the incubation period and prior to performing experiment.

- Sterilize the electrode.
- Prior to turning the power switch on, connect the required electrode and set mode to ohms (Ω) – cell resistance.
- Allow the cells to come to RT.
- Turn power on - meter must be disconnected from the charger.
- Place the electrode so the shorter electrode (wider base in the case of 96-wells electrode) is in the middle of the well, whereas the longer electrode outside of the insert.
- Allow the electrode (96-wells plate) to sit on the insert when taking the TEER read – the electrode should be placed 90° from the plate and should never touch the cell monolayer.
- Subtract the blank reads from the final reading.
- Values should be corrected to the area covered by the cell monolayer: Unit Area Resistance = Resistance (Ω) x Effective Membrane Area (cm²).

RapidFire RF300 system coupled QTRAP 5500 mass spectrometry of NCE

Concentration of studied compounds after performance ADME assays is determined by RapidFire RF300 system (Agilent Technologies) with QTRAP 5500 (AB Sciex) mass spectrometer (RF-MS). First step to prepare a method is the tuning of the studied molecules on QTRAP 5500 mass spectrometer. The QTRAP 5500 mass spectrometer is a sensitive triple quadrupole with the possibility to apply two different ionization techniques including electrospray ionization

(ESI) and atmospheric pressure chemical ionization (APCI) in two ion modes (positive and negative). ESI produces ions through ion evaporation. ESI can be used for highly polar, least volatile, or thermally unstable compounds. The generated ions are protonated molecules (positive ion mode) or deprotonated molecules (negative ion mode). The other technique is APCI ionization of solvent molecules to generate stable reaction ions. Protons are transferred between these reaction ions and sample molecules (ion-molecule reaction) to ionize sample molecules by either adding or removing a proton. APCI is used for analyzing in lipid soluble compounds or compounds that do not ionize in solution. These are usually non-polar compounds.

Studied compound is identified by parameters like tuned and optimized MS/MS transitions and de-clustering potential (DP), collision energy (CE) and collision cell exit potential (CXP). Daughter ion peaks (corresponding MS/MS transitions) are monitored using the multiple reaction-monitoring (MRM) mode.

The Agilent RapidFire 300 High-throughput instrument reduces the time needed for analysis by combining fast online solid phase extraction with feeding samples directly to the mass spectrometer (simple sample extraction). Each sample is processed over six to ten seconds, this ultra-high throughput mass spectrometry system consists of a proprietary sample purification and injection system which replaces the HPLC unit used in conventional LC-MS applications. Only 5 steps are needed for the analysis: Aspirate - injection the sample into the system, Load/Wash

- feed and extraction of sample to column, Elute and Re-equilibrate. Part of the RapidFire is 6-digit cartridge changer, the cartridges (columns) are available in a variety of designs - C4, cyano, C18, graphitic carbon, C8, Phenyl and HILIC (hydrophilic interaction liquid chromatography) for a wide range of screening, typical applications include: small molecules, peptides, and oligos, hydrophobic compounds, proteins, hydrophilic compounds and aromatic compounds. For example, a pyrrolo-fused nucleoside (Fig. 42.6) has been studied for cytostatic, antiviral activity and ADME properties (22).

It was necessary to find RF-MS method for determining the concentration of the studied nucleoside 22d after performance ADME assays. First, the MS parameters (Tab. 42.2) were identified by electrospray ionization (ESI) in positive ion modes.

The second step was to specify suitable conditions of solid phase extraction on RapidFire. Lyophilized samples of nucleoside 22d were dissolved in 200 μ L of the mobile phase (95 % water, 5 % ACN, 0,1 % formic acid). The samples were aspirated directly from 96-well plates into a 10- μ L sample loop and passed through a C4 cartridge (Agilent Technologies) with solvent A (95% 0.01% formic acid/5% acetonitrile) at a flow rate of 1.5 mL/minute for 3 seconds. After the desalting step, the analyte retained on the cartridge was eluted with solvent B (95% acetonitrile/5% 0.01% formic acid) to the mass spectrometer at a flow rate of 0.4 mL/minute for 7 seconds.

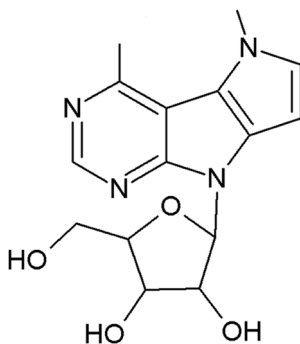
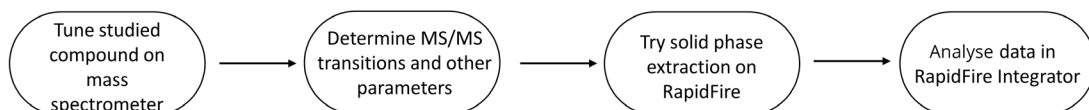


Figure 42.6. Structure of pyrrolo-fused nucleoside 22d.

TABLE 42.2. MS parameters of pyrrolo-fused nucleoside 22d, mass of protonated molecule (MS mass), mass of daughter ions (corresponding MS/MS transitions), de-clustering potential (DP), collision energy (CE) and collision cell exit potential (CXP).

MS mass [Da]	MS/MS mass [Da]	DP [volts]	CE [volts]	CXP [volts]
319.23	186.9	101	35	10
	170.8	101	97	8
	142.8	101	107	24



Scheme 42.7.

1. Follow the instructions of the instrument manuals of QTRAP 5500 (AB Sciex) mass spectrometer and RapidFire RF300 system (Agilent Technologies).
2. Tune studied compound on mass spectrometer by application of ESI or APCI ionization in two ion modes (positive and negative).
3. Determine MS/MS transitions and declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP).
4. Try solid phase extraction on RapidFire, establish – duration of 5 steps of analysis: Aspirate, Load/Wash, Elute and Re-equilibrate; suitable mobile phase and cartridge.
5. Check the extraction using program RapidFire Integrator.

IV. Comments

Chemical stability

If the tested compound shows PBS instability, make sure that the compound does not bind to plastics. Then try to repeat the assay in glass vials.

Plasma stability

Controls – stable versus nonstable drugs (verapamil and propranolol, respectively) were used as reference in plasma stability assay.

If the tested compound is unstable in the plasma, make sure it is not prodrug for which instability is desirable.

Microsomal stability assay

It is important to prepare exact and reproducible NADPH generating system and abide time of preincubation of microsomes (5 min) so that reaction system functions. It is not advisable to thaw microsomes more than once. Classification of microsomal intrinsic clearance for studied NCE is done according to (4).

Plasma protein binding

First, plasma protein binding of studied molecules is done in the 50 % plasma but in the case that 90 % or more of the studied substance binds to plasma proteins, it is necessary to repeat the experiment in 10 % plasma. In the case of weakly binding compounds (less than 10 %), they should be tested in 100 % plasma. Atenolol and

propranolol are used as standards for low and high plasma protein binding, respectively. The concentration of standards is the same as the studied NCE, namely 10 μ M in 50 % plasma.

PAMPA (Parallel Artificial Membrane Permeability Assay)

In order to obtain information regarding whether experiments worked as they are supposed to, some standards are included together with the tested compounds. These standards are drugs with well-established characteristics.

In PAMPA experiments, low log P_e drugs of reference are atenolol or ranitidine and high log P_e drug is propranolol (23). Some of the problems that can be found in this assay are the incorrect application of lipid solution, or errors from the incubation process.

Cell permeability assays: Caco-2 and MDCK cell lines

For cell permeability assays, reference drugs with established Papp and efflux values are used. In Caco-2 cell experiments, three categories are differentiated according to Papp values: low, moderate and high. Low Papp drugs are: atenolol and rhodamine 123, while high Papp reference drugs are: propranolol and verapamil (23). In

MDCK cell assays use the following standards for low Papp: atenolol (efflux) and ranitidine (efflux); and for high Papp drugs: propranolol (no efflux) and antipyrine (no efflux) (18). In these experiments, it should be taken into account that data can be influenced by several factors such as cell passage number, seeding density of cells, pH, filter size or experimental conditions. Attention should be paid to assess cellular monolayer integrity during incubation and experiment. This is particularly important for highly potent cytotoxic compounds.

RapidFire RF300 system coupled QTRAP 5500 mass spectrometry

It is important to choose an internal standard (IS) for the analysis of studied compounds using RF-MS. The internal standard should be similar (analogous chemical structure), but not the same as our studied compound (distinct molecular weight and/or MS/MS transitions), to have a similar height of signal intensity and the same conditions for solid phase extraction on RapidFire. Properly chosen and used IS can compensate for several types of both random and systematic errors which can happen during experiment and analysis. The significant problem for analysis can be potential interference when multiple metabolites of the same mass transition are formed. The used chemicals (especially solvents) must be declared as LC-MS grade.

V. Pitfalls

Problem	Cause and cure
Poor solubility of studied compounds in DMSO	Depending on the chemical characteristics of each compound, a suitable solvent should be found (water, methanol etc.).
Ignorance of physicochemical properties of tested compounds	Some compounds are sensitive to light or other factors that can alter their integrity. In such cases, measures should be taken to preserve the chemical integrity of the studied compounds (e.g. work in the dark).
False data due to the binding of compounds to plastics used in the experiments (plates and pipettes)	Experiments should be performed using glass labware.

VI. Acknowledgement

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HER2 gene copy number detection by quantitative polymerase chain reaction (qPCR)

Vladimíra Koudeláková

I. Introduction

The human epidermal growth factor receptor 2 gene (HER2, *ERBB2*) is localized on chromosome 17q and amplified in 15 to 20% of breast cancer patients. HER2 is a biomarker associated with poor prognosis, early recurrence, and reduced progression-free survival. HER2 expression is also a predictor of responses to drugs targeting HER2 (including trastuzumab, lapatinib, and pertuzumab) that are currently approved by regulatory agencies for treating HER2-positive breast cancer patients (1,2).

Various methods can be used to determine the HER2 copy number or abundance of the corresponding protein in a tissue sample, including *in situ* hybridization with detection by fluorescence, chromogenic, or silver (FISH, CISH, or SISH), immunohistochemistry (IHC), Southern/Western blotting, slot blotting, the polymerase chain reaction (PCR), reverse-transcription (RT)-PCR, and enzyme-linked immunosorbent assays (ELISA) (2–4). IHC and ISH are widely accepted as the gold standards for evaluating HER2 status. Immunohistochemistry is the primary recommended screening method,

with ISH being used to confirm IHC results. Patients with an IHC (HercepTest) score of 3+ are eligible for HER2-targeted therapy, whereas patients with 0 or 1+ scores do not overexpress HER2 and are therefore unsuitable for the treatment. Samples from patients with equivocal result (IHC 2+) must be retested for HER2 gene copy number using the ISH assay (1).

However, in approximately 5% of breast cancer samples, HER2 copy numbers cannot be evaluated using FISH due to poor tissue quality, which may be due to incorrect handling, especially in cases where inappropriate fixation methods are used or degradation occurs. Similarly, the choice of fixation methods can adversely affect IHC, but not gene amplification and FISH results, necessitating use of a DNA amplification based method to determine the HER2 gene copy number (2,5).

The method based on quantitative real-time PCR (qPCR) for determining HER2 gene copy numbers was therefore designed. The method involves three duplex qPCR amplifications to compare copy numbers of the HER2 gene to those of three reference genes: *GCSI* (glucosidase I),

DCK (deoxycytidine kinase) and *EPN2* (epsin 2). These genes were selected because their copy numbers rarely change in breast cancers (2).

II. Materials and instrumentation

Laboratory equipment

- Safety cabinet, Mars Safety Class 2 (ScanLaf)
- Real-time PCR thermal cycler, Rotor-Gene 3000 (Corbett Research, Generi Biotech)
- Microfuge for 1.5 mL tubes, MiniSpin (Eppendorf)
- Vortexer (Labnet International)
- Set of single-channel pipettes on stand (Eppendorf)
- Cold blocks (Sarstedt)
- Freezer, -20 °C (Gorenje)
- Refrigerator, 2 °C ÷ 8 °C (Gorenje)

Plastics and glass

- Microcentrifuge nuclease-free tubes, 1.5 mL (Cat. No. 1210-00), SSIbio, P-lab
- Strip tubes and caps, 0.1 ml (Cat. No. 981103), Qiagen
- Pipette tips (compatible with pipettes), Eppendorf

Chemicals

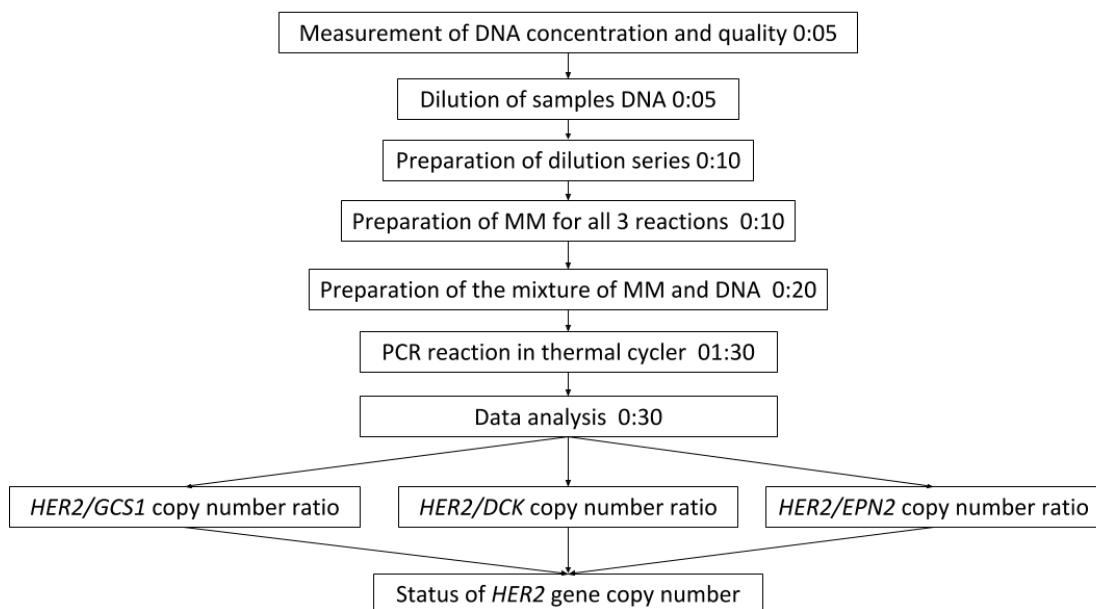
- DNeasy Blood & Tissue Kit (Cat. No. 69504), Qiagen
- Thermo-start PCR buffer, 10x, (Cat. No. AB-1217), ThermoFisher Scientific
- Magnesium chloride solution, 25 mM, ThermoFisher Scientific
- dNTP mix, 10 mM (Cat. No. 18427013), ThermoFisher Scientific
- ThermoStar polymerase, 5 U/µl (Cat. No. AB-0908), ThermoFisher Scientific
- DEPC-treated water (Cat. No. AM9906), ThermoFisher Scientific
- Standard DNA - DNA isolated from FFPE normal (non-cancerous) breast tissue samples that had physiological *HER2* gene copy number and showed no evidence of *HER2* amplification and expression (as determined by FISH and the HercepTest).
- *HER2*, *GCS1*, *DCK*, and *EPN2* forward (FW) primers, Tab. 43.1, Generi-Biotech
- *HER2*, *GCS1*, *DCK*, and *EPN2* reverse (RW) primers, Tab. 43.1, Generi-Biotech
- *HER2*, *GCS1*, *DCK*, and *EPN2* probes, Tab. 43.1, Generi-Biotech

TABLE 43.1. Characteristics of primers/probes (2)

Gene	Primer/probe	DNA sequence	Product size (bp)
<i>HER2</i>	Forward	5'-AGAGTCACCAGCCTCTG-CAT-3'	138
NM_001289 936	Reverse	5'-GCAACTCCCAGCTT-CACTTT-3'	chr17:39699791
	Probe	BHQ1-CTCCTTTTCACACTCTT-GCCGACGTC-FAM	+39699928
<i>GCS1</i>	Forward	5'-CAGGTGACCCTGAAAATT-CC-3'	133
NM_006302.3	Reverse	5'-CTTCAGCATGGCTCTCCAG-3'	chr2:74462670-74462822
	Probe	BHQ1-AAATCAAGCCCTGCCA-AGACTGGC-HEX	

DCK	Forward	5'-CCGCCACAAGACTAAGGA-AT-3'	109
NM_000788.2	Reverse	5'-CGATGTTCCCTTCGAT-GGAG-3'	chr4:70993798 +70993926
	Probe	BHQ1-AGAAGCTGCCC-GTCTTTCTCAGCC-HEX	
EPN2	Forward	5'-TGGTCATTTCTCCTTT-GGA-3'	130
NM_01496	Reverse	5'-AGTACCTGCTGCTGGA-CCAC-3'	chr17:19282871 +19283000
	Probe	BHQ1-AAACCCATTCCACTGC-GACGTAAGC-H	

III. Procedures



Scheme 43.1.

TABLE 43.2. *HER2* and reference gene copy numbers and dilution of standard DNA.

	Standard DNA	DEPC water	Concentration after dilution	DNA per reaction	<i>HER2</i> and reference gene copy number per reaction
Standard 1	4 µl	16 µl	4 ng/µl	20 ng	3330
Standard 2	6 µl	14 µl	6 ng/µl	30 ng	6660
Standard 3	8 µl	12 µl	8 ng/µl	40 ng	9990
Standard 4	10 µl	10 µl	10 ng/µl	50 ng	16650
Standard 5	12 µl	8 µl	12 ng/µl	60 ng	19980
Standard 6	14 µl	6 µl	14 ng/µl	70 ng	23310
Standard 7	20 µl		20 ng/µl	100 ng	33300

PCR reaction

1. After sample DNA extraction using DNeasy Blood & Tissue Kit, check that the A260/A280 ratio of the sample DNA is between 1.7 and 1.9 and measure the DNA concentration. Dilute sample DNA to concentration 10 ng/µL. Use only barrier pipette tips and change tips between tubes.
2. Dilute the standard DNA to concentration 20 ng/µL and prepare a dilution series of 20, 30, 40, 50, 70, and 100 ng/reaction mixture. Prepare 20 µL of each standard according to the Tab. 43.2.
3. Calculate the final volumes of individual components of master mix according to the Tab. 43.3 where the volumes of individual components are shown per PCR reaction. The final volume is depending on the number of tested samples. One no template control (NTC, negative control) and 7 standards are included in each PCR run. All tested samples have to be analyzed in duplicate at least. Prepare one master mix for *HER2*/GCS1 reaction, one master mix for *HER2*/DCK reaction and one master mix for *HER2*/EPN2 reaction.

Example: When you want to evaluate 10 clinical samples, you have to prepare 3 master mixes (for GCS1, DCK, and EPN2) for 31 reactions (10 samples in duplicate, 1 no template control, 7 standards, and 10% surplus).

4. Gently pipette the master mix up and down for thorough mixing and dispense 20 µL of the master mix into 100 µL.
5. Add 5 µl standard or sample DNA to each of 100 µL PCR tubes. Add 5 µl of DEPC water in the case of no template control (negative control). Rinse the mixture by pipetting up and down in each step and close the tubes.
6. Load the tubes to the real-time thermal cycler. Set the appropriate experiment setting and start the run according to conditions in Tab. 43.4. Reactions were optimized for RotorGene 3000. Validation is necessary in the case of using different thermal cycler.
7. The fluorescence is measured in channel FAM a JOE/HEX in the temperature of 60 °C.

TABLE 43.3. Composition of the 3 different master mixes for the analysis of one sample.

		Reaction 1		Reaction 2		Reaction 3	
Component	Final concentration	<i>HER2</i>	<i>GCS1</i>	<i>HER2</i>	<i>DCK</i>	<i>HER2</i>	<i>EPN2</i>
10 x PCR buffer	1 x	2.5 µl		2.5 µl		2.5 µl	
25 mM MgCl ₂	5 mM	5 µl		5 µl		5 µl	
10 mM dNTP	200 µM	0.5 µl		0.5 µl		0.5 µl	
25 µM FW primer	0.2 µM	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl
25 µM RW primer	0.2 µM	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl
25 µM probe	0.15 µM	0.15 µl	0.15 µl	0.15 µl	0.15 µl	0.15 µl	0.15 µl
DEPC water		10.7 µl		10.7 µl		10.7 µl	
ThermoStar polymerase (5 U/1 µl)	1 U	0.2 µl		0.2 µl		0.2 µl	
DNA	(5 µl)						
Total volume		25.0 µl		25.0 µl		25.0 µl	

TABLE 43.4. PCR thermal cycling conditions for RotorGene 3000

Step	Time	Temperature	Cycle
Enzyme activation	5 min	95 °C	1
Denaturation	15 s	95 °C	40
Annealing/Extension	60 s	60 °C	

Data analysis

1. Perform the data analysis after the end of the PCR. Data analysis is depending on the individual thermal cycler; nevertheless, the signal in channel FAM (filter comb 465-510 nm) show the copy number of *HER2* gene and signal in channel HEX/JOE (filter comb 533 – 580 nm) show the copy number of each individual reference gene (*GCSI*, *DCK*, or *EPN2*).
2. Prepare calibration (standard) curve using seven standards with known *HER2* and reference genes copy number by plotting copy numbers on the X axis, and the assay measurement on the Y axis (Ct value). Since it is duplex reaction, it is necessary to prepare calibration curve for each channel/ gene separately. To analyze the experimental data, locate the measurement (Ct value) on the Y-axis that corresponds to the assay measurement of the tested sample and follows a line to intersect the standard curve. The corresponding value on the X-axis is the *HER2* or reference gene copy number in the tested sample. Calculate the *HER2*, *GCSI*, *DCK*, and *EPN2* copy number of all tested samples for all 3 reference genes.
3. Evaluate the variation between individual duplicates. When the deviation is less than 10%, calculate the average of the duplicate results. When the deviation is more than 10%, repeat the reaction (dilution series and tested sample) for the primer pair which failed.
4. Calculate *HER2/GCSI*, *HER2/DCK*, and *HER2/EPN2* ratio for each tested sample.
 - a. *HER2* gene is considered to be amplified in samples where the ratio of the *HER2* copy number to that of at least two of the three references gene exceed the threshold 2.2.
 - b. *HER2* gene is considered to be non-amplified (physiological) in samples where the ratio of the *HER2* copy number to that of at least two of the three references gene is lower than the threshold 2.2.

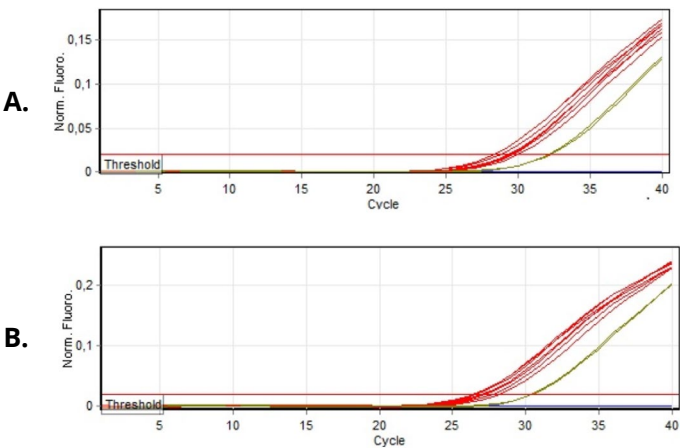


Figure 43.1. An example of the real-time PCR output for *HER2* (A) and *GCSI* (B) in dilution series (red) and one tested samples (yellow) (RotorGene 3000).

IV. Comments

- C_t (cycle threshold), also known as C_q (quantification cycle), value is defined as the number of PCR cycles required for the fluorescent signal to cross the threshold (background). C_t levels indirectly correspond to the amount of target DNA in the sample (the lower the C_t level the greater the amount of target DNA in the sample).
- If the only one or none reaction from the duplex PCR is evaluable, it is not possible to evaluate the *HER2* gene copy number and it is necessary to repeat the test for individual sample.
- If the C_t in channel FAM and/or HEX/JOE is lower than 35 in no template control (NTC), the run is contaminated and it is necessary to repeat all samples.
- If the C_t in channel FAM and/or HEX/JOE is higher than 35 for tested sample, the sample is not evaluable and it is necessary to repeat the test for individual sample.
- If the *HER2* and/or reference gene copy number is lower than 200 copies per reaction, the sample is not evaluable and it is necessary to repeat the test for individual sample.

V. Pitfalls

Problem	Cause and cure
Any signal ($C_t < 35$) in no template control	• Contamination during PCR reaction preparation. All samples results are invalid. • Decontaminate all surfaces and instruments. • Use only barrier pipette tips and change tips between tubes. • Try to perform PCR reaction with the same reagents with no template control only. If the no template control is still positive, repeat the PCR preparation with new set of reagents.
No or weak signal of dilution series samples (standard DNA)	• Degradation of the standard DNA. • Check the storage and manipulation conditions. Try to find out if the standard DNA is degraded (e.g. by electrophoresis) or directly use the new standard DNA. • The PCR conditions did not comply with instructions or thermocycler is broken. • Check the PCR conditions and thermocycler calibration.
No or weak signal of all tested and dilution series samples	• PCR inhibition. • Check that you use DNA extraction method recommended for your type of sample and follow the manufacturers instruction. • The reagent storage conditions did not comply with instruction. • Check the storage and manipulation conditions especially for DNA polymerase and primers/probes. • The PCR conditions did not comply with instructions. • Check the PCR conditions.

**Only one sample
replicate is evaluable**

- Inaccurate pipetting.
 - Repeat the test for the sample.
-

VI. Acknowledgment

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Site-Directed mutagenesis using PCR

Vaishali Uniyal

I. Introduction

Site-Directed Mutageneses (SDM) are specific, targeted changes in double stranded plasmid DNA (insertions, deletions, and substitutions) that allow to study genetically coded changes in protein activity or expression. It can be used to elucidate the role of nucleotides in regulatory region or in region that interacts with a drug. Also, they can introduce or remove sequences that facilitate further genetic manipulation, such as sites recognized by restriction endonucleases (1).

There are several methods of deliberate, targeted mutagenesis or genetic engineering, differing in precision of genetic modification. On one side of spectrum, there are error-prone PCR, multiplex automated genome engineering (MAGE), PFunkel, programmable nucleases (zinc-finger nucleases, ZFNs, transcription activator-like effector nucleases, TALENs, and CRISPR/Cas (2), orthogonal DNA polymerase-plasmid pairs, Targeting Glycosylase To Embedded Arrays for Mutagenesis (TaGTEAM), and retrotransposon-based targeted mutagenesis and their combinations (CRISPR-MAGE, CRISPR-error-prone PCR, and CRISPR-base

editing enzyme) (3). These methods possess a feature of randomness and have a potential to generate a number of mutations. On the other side, cassette mutagenesis, primer extension, and inverse PCR introduce a change of one or few nucleotides in a defined sequence position (4). Inverse PCR with mutations generated using a pair of oligonucleotide primers designed with mismatching nucleotides at their centers is the easiest and therefore widely used method (5).

The method described here is derived from Kunkel method (6) that uses the synthesis of template plasmid DNA in a dUTPase deficient *E.coli* strain. This strain has a high number of dUTPs that are incorporated into the plasmid in place of dTTPs. Such DNA template has a normal coding potential for the site-directed mutagenesis protocols but is not biologically active upon transfection into a wild-type (i.e., ung+) *E. coli* host cell. Expression of the desired change, present in the newly synthesized non-uracil-containing covalently closed circular complementary strand, is thus strongly favoured.

Our method creates a single nucleotide mutation in a double-stranded plasmid DNA. A linear double stranded DNA or gene can also be used as

a template but for protein expression it still needs to be cloned into a plasmid. If several mutations are needed in one gene, then it is better to clone the gene in a plasmid and use different mutated primers to create different mutations. A gene already inserted into a plasmid reduces the work of cloning each individual mutated cell line.

Our SDM method includes steps of denaturation, renaturation, and *DpnI* digestion of methylated parental plasmid DNA. Then, the products are directly transformed into host cells. The important components used in the SDM are described below:

Plasmid DNA

The template we want to mutate can be a linear DNA or plasmid with a selectable marker. Small-sized plasmids (3-5 Kb) are easily amplified while there are SDM Kits available in the market which can amplify plasmids up to 14 Kb. PCR extension time depends on the length of the plasmid. As this method includes the *DpnI* treatment (explained later), the plasmid has to be purified from a methylation competent bacterial strain (i.a. DH5 α dam⁺) which adds a methyl group to the adenine of GATC stretches of DNA. Later, the methylated DNA will be cleaved by *DpnI*, thus leaving only new template with mutations for further processing (<https://blog.addgene.org/plasmids-101-methylation-and-restriction-enzymes>).

Primers

Primers are designed to create a mutation in the amplicon. They are similar to normal PCR primers but with nucleotide with a deliberate mismatch in the middle of the primer. On approximately 22 bp long primer, a mismatch can

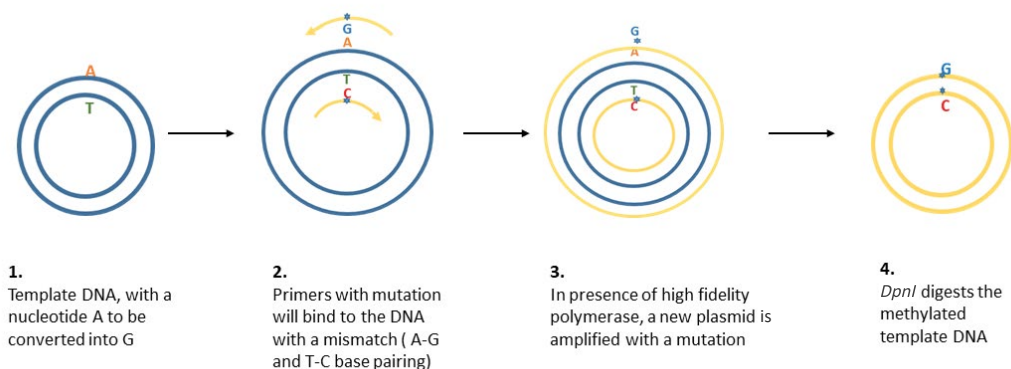
be incorporated after 11 bp of complementary binding. The placement of mismatch closer to 3' end of primer would hamper the amplification (would cause amplification drop-out). Forward and reverse primer should be complementary to each other - both the primers will carry the mutation at the same nucleotide position. Thus, mismatch closer to 5' end of primer would cause unwanted mismatch close to 3' end of complementary primer. The choice of primers follows the basic rules of primer design (7).

Polymerase

High fidelity DNA polymerase is used to reduce the risk of introduction of undesired mutation. The polymerase should have 5' to 3' polymerase activity for DNA synthesis and 3' to 5' exonuclease activity to proofread the wrongly incorporated nucleotide. However, it should not possess any 5' to 3' exonuclease activity as it might truncate the previously synthesized DNA. The polymerase should create blunt ends as the overhangs will interfere with the plasmid reconstitution. An example is *Pfu* polymerase that, moreover, synthesizes DNA with a 6 times higher fidelity than Taq polymerase (8).

Nuclease

As mentioned above, the template DNA has to be methylated. In our case it is methylated by DNA adenine methylase (Dam methylase). After DNA amplification, the template DNA strand is methylated while the new DNA strand which carries mutation has no methylation. *DpnI* specifically cleaves the template DNA at methylated adenosine, leaving the new mutated strand intact.



Scheme 43.1. Generation of point mutation in a plasmid DNA

Transformation & Screening

The PCR product treated with *DpnI* can be directly used for transformation. To achieve a high yield of plasmid, a bacterial strain which produces a high copy number of plasmid should be used. Based on selectable marker present on marker plasmid, bacterial colonies can be screened and used for plasmid purification.

Validation of mutation

Part of the purified plasmid encompassing the region of mutation is sequenced, using the primers with consensus sequence (without any mutation). Sequencing data can be compared with consensus to check whether the new DNA bears a change in the sequence at the desired position.

II. Materials and instrumentation

- Quick Change Lightning Multi Site-Directed Mutagenesis Kit (Agilent technologies, Catalog # 210514).
- Plasmid DNA purification kit (QIAprep Spin Miniprep Kit, Catalog # 27104)
- Plasmid DNA.
- Primers with the desired mutation (primer working solution should be prepared according to the manufacturer's instructions)
- Competent bacterial cells (DH5 α or any other suitable strain for amplification of plasmid).
- Thermocycler.
- Microcentrifuge tubes (0.2 μ l).
- Tips & pipettes (1 μ l-1000 μ l).
- MilliQ purified water .
- 70% ethanol.
- PB buffer (5 M Gu-HCl, 30% isopropanol)
- PE buffer (80% Ethanol, 100 mM NaCl, 10 mM Tris-HCl pH 7.5).
- Table top centrifuge with 17,900 g rotor speed.
- Dry bath incubators.

III. Procedures

A. SDM Template preparation

SDM template preparation is needed if the plasmid DNA will be purified using manual methods. Skip this step if plasmid DNA will be purified using commercial kits.

This purification steps involves the solution from commercially available kit and are given a code by the supplier kit (QIAprep Spin Miniprep Kit, Catalog # 27104).

1. To 1 volume of the stock plasmid DNA (any concentration) add 5 volumes of PB and mix the solution vigorously till it turns uniform. Incubate for 2 minutes at RT.
2. Transfer the solution to a silica gel column and incubate for 3 min at RT.
3. Centrifuge at $\sim 17,900 \times g$ for 2 min and discard the flow-through.
4. Add 750 μ l PE buffer to the column, incubate for 2 minutes and centrifuge at $\sim 17,900 \times g$ for 2 minutes. Discard the flow-through and repeat a spin on a new microcentrifuge tube. Discard a tube.
5. Put the column on a new microcentrifuge tube and allow it to dry at 65 $^{\circ}$ C on a dry bath incubator.
6. Add preheated MilliQ water to the center of the column, then centrifuge at $\sim 17,900 \times g$ for 5 min and collect the flow-through.

B. Preparation of PCR Mix

1. Thaw all the reagents on ice. Gently tap them with the finger and give a short spin to bring all the reagent volume to the bottom of the tube. Do not vortex.
2. Take a new PCR tube and keep it on ice.
3. Start adding all the reagent to a PCR tube according to the order in the Table 43.1 or start with the reagent having the highest volume in the PCR mix (usually water).
4. Add *Pfu* Polymerase (supplied with the kit) as the last component, just before PCR. The exact composition is defined in the Table 43.1.

C. *DpnI* digestion

1. Add 2 μ l of *DpnI* enzyme to each amplification reaction (50 μ l reaction volume).
2. Gently mix the solution with the help of pipette and briefly spin down all the volume.

TABLE 43.1. Reaction mixture composition for SDM reaction.

SDM REACTION CONDITION			
Component	Stock concentration	Final amount	Volume (in µl)
Buffer	10x	1x	5
dNTPs	10 mM	2 mM	1
Quicksolution	16.7x	1x	3
Forward primer**	125 ng/µl	125 ng	1
Reverse primer**	125 ng/µl	125 ng	1
DNA	61 ng/µl	20 ng	0.3
<i>Pfu</i> ultra	2.5 U/µl	2.5 U	1
MQ H ₂ O			37.7
Total Volume			50

****Primer sequences:**

Forward Primer (41 bp): 5' CACAGACCGATTGGTTGCCAGCCAGCCAAATGTCTTGGAGG 3'

Reverse Primer (41 bp): 5' CCTCCAAGACATTTGGCTGGCTGGCAACCAATCGGTCTGTG 3'

For SDM, primer concetration should be adjusted to 125 ng/µl to match the concentration of template. However, the primer concentration can be calculated from the molarity:

No. of pmoles of oligos = (ng of oligos/330 x no. of bases in oligos) x 1000

For 125 ng of 41 bp primers:

No. of pmoles of oligos = (125 / 333 x 41) x 1000 = 9.23 pmoles

TABLE 43.2. PCR conditions for setting up SDM reaction.

PCR condition for SDM						
	Initial Denaturation	Denaturat-ion	Annealing	Extension	Final extension	Hold
	18 cycles					
Temperature (°C)	95	95	60	68	68	4
Time (s)	60	50	50	30 s/Kb DNA*	420	∞

*Here, extension time would be 60 s for 2 kb DNA.

3. Keep it at 37 °C for 5 minutes to digest the methylated template DNA.
4. Transform this *DpnI* treated amplified product into DH5α bacteria to obtain more copy number of the plasmid.

D. Confirmation of Mutation

1. After transformation, randomly pick 3-4 colonies and mark them on the plates. Pick only half colony or streak the colonies on a fresh plate as a backup if it turns out to be a positive clone after sequencing.
2. Use these colonies for plasmid purification and validate the plasmid sequence by DNA sequencing.
3. If a mutation is confirmed, then amplify the plasmid from the same bacterial clone.

IV. Comments

- Template for SDM should be highly pure. Check the purity by electrophoresis of the plasmid on the agarose gel and spectrophotometrically.
- To evaluate DNA purity and detect possible contaminants, measure the ratio of the absorbance at 260 nm and at 280 nm. Good quality DNA will have an A_{260}/A_{280} ratio of 1.7–2.0. Other substances which also absorb light at 260 nm could interfere with DNA values, artificially increasing the result calculated from the absorption readings. To compensate for this, it is good to perform wavelength scan from 230 nm to 320 nm. A wide absorbance peak around 260 nm should be preceded by a ‘dip’ at 230 nm. If the readings at 230 nm are high (too similar to those at 260 nm), DNA is contaminated and cannot be measured accurately. There should also be a rapid tail-off from 260 nm down to 320 nm.
- All the reagents must be kept on ice during the reaction mix preparation.

V. Pitfalls

Problem	Cause and cure
No colony on the plate	• Check the transformation efficiency of the cells. • Check the plasmid DNA after PCR on an agarose gel. • Make sure the DNA polymerase has all the properties mentioned above.
No mutation after sequencing	• Check the primers and their sequence. • Check PCR conditions, to ensure that plasmid is getting amplified. • No or improper treatment with the DpnI may keep the parent DNA (non-mutated) and this gets amplified in the cells. • Use different colonies for sequencing. Try to pick up the colonies from different part of the plates.

VI. Acknowledgement

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Genetic risk calculation of cystic fibrosis in prenatal testing

Jiří Drábek

I. Introduction

Cystic fibrosis (CF) is a potentially fatal autosomal recessive disorder with distinctive mutational spectrum in the Czech Republic (1). Its incidence was originally 1/4023 (2) but this number is continually corrected by downsizing. For purpose of calculation with fractions in this chapter, we will use the number of 1/4489 that is close to 1/4500 published in online screening guidelines (<https://www.novorozeneckyscreening.cz/cysticka-fibroza>).

CFTR (cystic fibrosis transmembrane regulator) gene mutation encoding a defective chloride channel in epithelial cells responsible for salt, fluid, and pH balance in secretory and absorptive epithelial tissues was discovered as a causative agent of CF in 1989. Today (January 2020), more than 2000 variants are listed in Cystic Fibrosis Mutation Database (<http://www.genet.sickkids.on.ca/StatisticsPage.html>) but just 432 of them are annotated (https://www.cftr2.org/mutations_history). CFTR mutations can be grouped into five classes, where CFTR protein: 1) is not synthesised, 2) is inadequately processed, 3) is not regulated, 4) shows abnormal

conductance, and 5) has partially defective processing or production.

The most common mutation worldwide (66%) is a class 2 in-frame deletion of phenylalanine in position 508 (c.1521_1523delCTT, p.Phe508del, rs113993960). Cystic fibrosis pathophysiology proceeds from absent/abnormal CFTR protein through airway surface liquid depletion, defective mucociliary transport, mucus accumulation, bacterial infection, and airway inflammation to airway and lung tissue damage. Symptoms of CF are chronic airway disease, gastrointestinal disease, salt wasting with metabolic alkalosis, and obstructive azoospermia (3). Broad phenotypic CF spectrum is explained by contributing effects of complex alleles, modifier genes, genetic heterogeneity (mutation in non-CFTR genes), epigenetic factors, and environmental factors (4). For treatment options, see <https://www.cff.org/Life-With-CF/Treatments-and-Therapies/>.

CFTR screening in newborns in the Czech Republic is performed by immunoreactive trypsinogen (IRT) testing, followed by CFTR testing and sweat test when IRT $\geq$ 65 ng/ml. CFTR gene is genotyped in proband and their

parents in an ISO15189 accredited genetic laboratory. If CF diagnosis is confirmed, patient is treated in one of cystic fibrosis centres (e.g. University Hospital Motol Prague or University Hospital Olomouc). In some cases, CF risk for consultand needs to be calculated without their CFTR genotyping information (i.e. consultand is not proband). For such calculations and to incorporate all information available for reducing uncertainty about CF status, Bayes' theorem (5) should be used. Unfortunately, sometimes clinicians do not use Bayes' theorem properly what leads usually to overestimation of the CF risk of an individual (6). This chapter delineates the application of Bayes' theorem for calculation of genetic risk of CF, exemplified by one pedigree over four generations and two approaches – algebraic and Bayes net ones.

II. Materials and instrumentation

For Bayesian genetic risk assessment, the following is handy: agreed Notation, Parameters of relevant population, Hardy-Weinberg equilibrium, Mendel autosomal recessivity, Bayes' theorem, Parameters of genotyping method, Pedigree structure with definition of consultand(s), proband(s) and goal, CFTR genotyping results, and CF statuses of probands. The further reduction of uncertainty may be achieved by results of other tests (e.g. fetus ultrasound recogniton of echogenic bowel) (7,8).

Notation

P	probability
H	hypothesis
prior	before update using new information (e.g from genetic test)
posterior	after update
cf	hypothesis that CFTR mutation allele is present
WT	hypothesis that CFTR mutation allele is absent and wildtype allele present
cf_cf	hypothesis that CFTR mutation is present in genotype in ho mozygous form, CF is present

WT_cf	hypothes is that CFTR mutation is present in genotype in heterozygous form, CF is absent, client is healthy
WT_WT	hypothesis that CFTR mutation is absent in homozygous form, CF is absent, client is healthy
T+	positivity of test (mutation detected)
T-	negativity of test (mutation not detected)
 	on condition that, given that

Parameters of our population, Hardy-Weinberg equilibrium, and Mendel autosomal recessivity

Let incidence of CF = $P_{\text{prior}} = P(\text{cf}) * P(\text{cf}) = 1/4489 = 0.000222767$, from which

$$P(\text{cf}) = \sqrt{\frac{1}{4489}} = \frac{1}{67} = 0.014925$$

Bayes' theorem

Derivation: The probability of two events H and T happening, $P(H \cap T)$ is the probability of event H, $P(H)$, times the probability of T given that H has occurred, $P(T | H)$.

$$P(H \cap T) = P(H) \times P(T | H)$$

On the other hand, the probability of H and T is also equal to the probability of T times the probability of H given T.

$$P(H \cap T) = P(T) \times P(H | T)$$

Equating the two right sides of equations yields:

$$P(T) \times P(H | T) = P(H) \times P(T | H)$$

and thus

$$P(H | T) = \frac{P(T | H) \times P(H)}{P(T)}$$

Note: $P(T) > 0$

This equation, known as Bayes Theorem is the basis of logical (statistical) inference. If we want

to contrast two hypotheses, H^+ and H^- , when we obtain negative result of the test, T^-

$$P(H^+ | T^-) = \frac{P(T^- | H^+) \times P(H^+)}{P(T^-)}$$

vs

$$P(H^- | T^-) = \frac{P(T^- | H^-) \times P(H^-)}{P(T^-)}$$

then the odds form of Bayes theorem reads:

$$\frac{P(H^+ | T^-)}{P(H^- | T^-)} = \frac{P(H^+)}{P(H^-)} \times \frac{P(T^- | H^+)}{P(T^- | H^-)}$$

The three fractions (from left to right) are called **posterior odds**, **prior odds**, and **likelihood ratio**.

Prior odds can be repeatedly updated by multiplying with likelihood ratio using new information from different independent tests. When tests are not independent, situation becomes more complicated and should be handled by Bayes nets.

In the following text our hypotheses are compound. They consist of presence of two alleles, when one (WT_{cf}) or none (WT_{WT}) can be cystic fibrosis allele, while test for cystic fibrosis alleles is negative. Therefore, odds form of Bayes theorem reads:

$$\frac{P(WT_{cf} | T^-)}{P(WT_{WT} | T^-)} = \frac{P(WT_{cf})}{P(WT_{WT})} \times \frac{P(T^- | WT_{cf})}{P(T^- | WT_{WT})}$$

Parameters of genotyping method

Diagnostic kit CF StripAssays® from ViennaLab (cat. No. 4-410). The following 34 mutations are tested: CFTRdel2,3 (21kb), G85E, 394delTT, R117H, Y122X, 621+1G>T, 711+1G>T, 1078delT, R334W, R347H, R347P, A455E, I507del (-ATC), F508del (-CTT), 1717-1G>A, G542X, G551D, R553X, R560T, 1898+1G>A, 2143delT, 2183AA>G, 2184insA, 2184delA,

2789+5G>A, 3120+1G>A, 3272-26A>G, Y1092X (C>A), R1162X, 3659delC, 3849+10kb C>T, 3905insT, W1282X, and N1303K.

$P(T^+ | cf) = 0.9$ (Method sensitivity, ability of a test to correctly identify allele with the *CFTR* mutation)

$$P(T^- | cf) = 1 - 0.9 = 0.1$$

$P(T^+ | WT) = 0$ (Method specificity, let us optimistically assume that false positivity errors do not happen)

$$P(T^- | WT) = 1$$

Reference cDNA sequence for *CFTR* gene used is NM_000492.3.

Note: CF StripAssays® detects also *CFTR* phenotype modifier, T(n) polymorphism in intron 8 of *CFTR* gene (IVS8 T5/T7/T9) what we neglect for purpose of simplicity.

The last two needed parameters (Pedigree structure with definition of consultands and probands and *CFTR* genotyping results and CF statuses of probands) are case-specific.

In our case, Parents in expectation III.1 and III.2 want to know the risk of their so far unborn child to have CF, when genotyping results are available for persons III.1, III.2, and III.3, see Fig. 45.1.

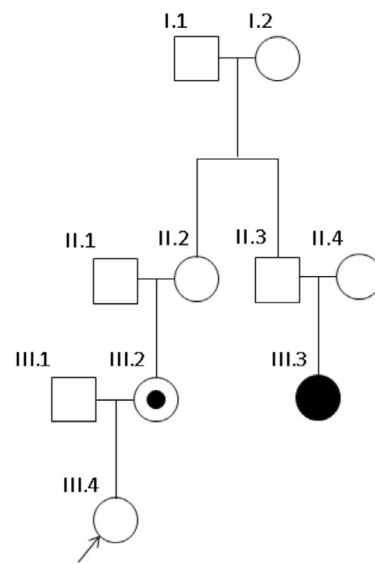


Figure 45.1. Pedigree with definition of consultand IV.1 and probands III.1, III.2, and III.3

CFTR genotyping results and CF statuses of probands

I.1, I.2, II.1, II.2, II.3, and II.4 are healthy, not genotyped

III.1 is healthy, wildtype homozygote WT_WT; c.[=]; [=]

III.2 is healthy, heterozygote WT_cf; c.[1624G>T]; [=], p.Gly542X

III.3 has CF, genotyped to have c.1521_1523delCTT, p.Phe508del in homozygous status (cf_cf).

IV.1 8th weeks old embryo, not genotyped

Bayes net software

GeNIe (<https://download.bayesfusion.com/files.html?category=Academia>) or other free software, e.g. HUGIN Lite (<https://www.hugin.com/index.php/hugin-lite/>)

III. Procedures

Steps of algebraic method of calculation

1. First, we have to calculate odds that man III.1 is hidden heterozygote. Though III.1 is healthy without any mutation detected, it can be either true finding (WT_WT) or case of false negativity (WT_cf). Thus, we calculate posterior probability of him being heterozygote (WT_cf) when (on condition that) genotyping is negative, T-. To calculate posterior odds (WT_cf) vs (WT_WT),

$$\frac{P(WT\_cf | T-)}{P(WT\_WT | T-)}$$

we need to multiply prior odds with likelihood ratio.

2. Hardy-Weinberg rule states that $P(cf)+P(WT) = 1$ and $P(cf\_cf)+2*P(cf)*P(WT)+P(WT\_WT) = 1$. So, $P(WT\_cf) = 2*P(cf)*P(WT) = 2*P(cf)*(1-P(cf)) = 2*1/67*66/67 = 2*0.014925373*0.985074627 = 0.029405212$ and $P(WT\_WT) = (1-P(cf))^2 = (66/67)^2 = 4356/4489 = 0.97037202$.

Therefore prior odds

$$\frac{P(WT\_cf)}{P(WT\_WT)} = \frac{0.029405212}{0.97037202} = 0.03030303.$$

3. CF StripAssay does not distinguish whether mutation is present in homozygous or heterozygous form. Therefore, likelihood ratio obtained from genotyping test is:

$$\frac{P(T- | WT\_cf)}{P(T- | WT\_WT)} = \frac{P(T- | cf)}{P(T- | WT)} = \frac{0.1}{1} = 0.1$$

4. Prior odds multiplied by likelihood ratio (giving posterior odds) are: $0.03030303*0.1=0.003030303$. Thus, using the formula for odds to probability transposition $P=odds/(1+odds)$, posterior probability of III.1 being "hidden" heterozygote is $0.003030303/1.003030303 = 0.003021148 \sim 0.3\%$.

More concise, tabular form of calculation is in Tab. 45.1.

5. The following question is, what is the probability that our man III.1 (hidden heterozygote with probability of 0.3%) together with our woman III.2 (proven heterozygote) will father a child with cystic fibrosis.
6. For further calculation, we will use Mendel law of segregation and probabilistic law of multiplication. Three independent phenomena must occur together: woman passing cf, man having cf, and man passing cf. **Therefore probability of their child having cystic fibrosis is $0.5*0.003021148*0.5 = 0.000755287 \sim 0.08\%$. Probability of child in this pedigree having CF is more than three times higher than Czech population incidence.**

Steps of Bayes net method of calculation

1. The same result should be achieved also by using Bayesian network approach. Bayes net is encapsulation of Bayes theorem by directed acyclic graph from graph theory

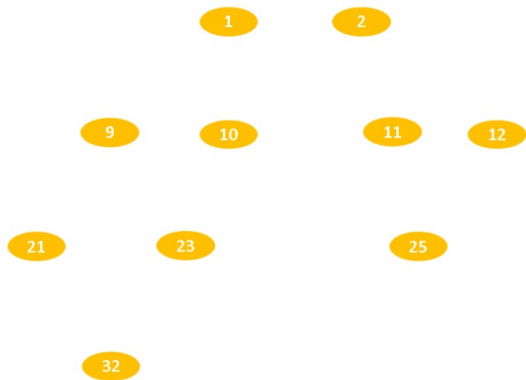
(9). Bayes nets are probabilistic expert systems suitable for elucidating probabilistic relations among the large number of variables and for inference. Bayes net can answer question of "what if" type just by clicking the execute button. For purpose of cystic fibrosis risk assessment, the causal application of Bayes net is presented as three steps: definition of nodes and edges, filling probabilistic tables, and instantiation (fixing

the known variables).

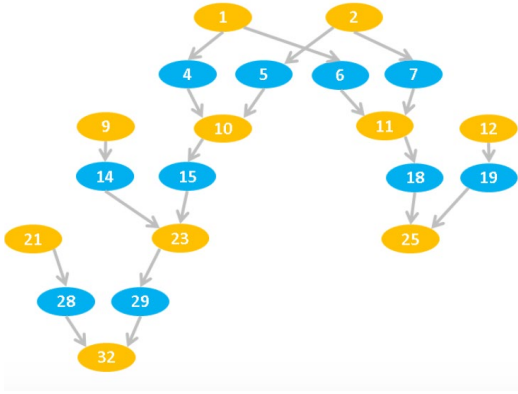
2. We define the genotype (orange) nodes of Byes net by transposing the genetic pedigree representation of relationship (Scheme 45.1). Let I.1 = 1, I.2 = 2, II.1=9, II.2 = 10, II.3 = 11, II.4 = 12, III.1 = 21, III.2 = 23, III.23 = 25, IV.1 = 32.

TABLE 45.1. Concise form of probability update

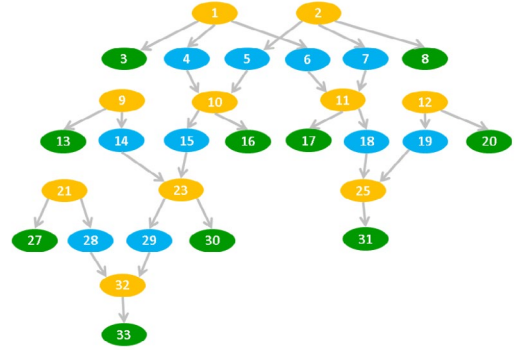
	Hypothesis H	Person III.1 is carrier, WT_cf: main hypothesis H1	Person III.1 is not carrier, WT_WT: alternative hypothesis H2
A	Prior probability of Hx from Hardy-Weinberg equilibrium	$2*WT*cf = 2*1/67*66/67 =$ $132/4489 = 0.029405212$	$WT*WT = (66/67)^2 =$ $4356/4489 = 0.97037202$
B	Conditional probability of H (condition of test T being negative) $P(Hx T-)$	1/10	1
C	Joint probability A*B	132/44890	4356/4489
D	Relative probability (joint probability with the same Hx denomi- nator)	132	43560
E	Posterior probability $D_{Hx}/(D_{Hx} + D_{Hnonx})$	$132/(132+43560) =$ 0.003021148	$43560/(132+43560) =$ 0.996978852



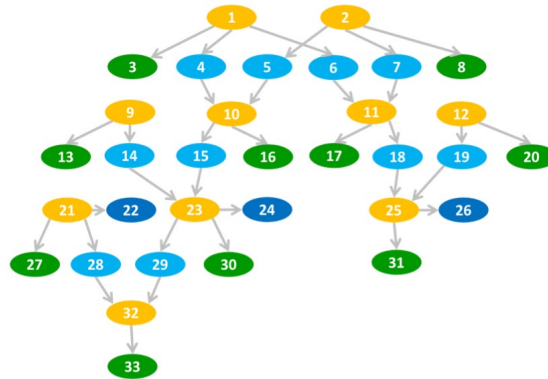
Scheme 45.1



Scheme 45.2



Scheme 45.3



Scheme 45.4

3. We add Mendelian segregation characteristics by adding (light blue) nodes and edges from parents to children (Scheme 45.2).
4. We add green node of health status of each person (Scheme 45.3):
5. We add dark blue node for available genotyping information (Scheme 45.4):

6. By clicking at each node, table reveals that must be filled by conditional probability values (row dependent on column). Though there are 33 tables to be filled, many of them are identical, with repeated values. Thus, only five conditional probability tables (Tables 45.2 to 45.6) are needed (table colour code matches the graph node colours):

TABLE 45.2. Conditional probability table for nodes 1, 2, 9, 12, and 21

WT_WT	0.970372
WT_cf	0.029404489
cf_cf	0.000222767

TABLE 45.3. Conditional probability table for nodes 3, 8, 13, 16, 17, 20, 27, 30, 31, and 33

	WT_WT	WT_cf	cf_cf
healthy	1	1	0
sick	0	0	1

7. Once nodes, edges, and conditional probabilities are ready, we can proceed by "asking" the Bayes net our question. By "Setting the evidence" we input the known values for health status nodes 3, 8, 13, 16, 17, 20, 27, 30, and 31 and genotype nodes 22, 24, and 26.
8. We click "update" button (flash sign) and click the node 33, Value sheet. We shall get **the same result as by algebraic method: 0.000755287 ~ 0.08 %**.

TABLE 45.4. Conditional probability table for nodes 4, 5, 6, 7, 14, 15, 18, 19, 28, and 29

	WT_WT	WT_cf	cf_cf
WT	1	0.5	0
cf	0	0.5	1

TABLE 45.6. Conditional probability table for nodes 22, 24, and 26

	WT_WT	WT_cf	cf_cf
test positive	0	0.9	0.9
test negative	1	0.1	0.1

TABLE 45.5. Conditional probability table for nodes 10, 11, 23, 25, and 32

	WT		cf	
	WT	cf	WT	cf
WT_WT	1	0	0	0
WT_cf	0	1	1	0
cf_cf	0	0	0	1

Node properties: health IV.1		
General	Definition	Observation Cost
Marginal probability distribution:		
► healthy	0.99924471	
sick	0.00075528741	

Figure 45.2. Result obtained through Bayes net

IV. Comments

Case may proceed further by suggestion of more detailed *CFTR* examination of father III.1. When mutation is confirmed, amniocentesis or chorionic villus sampling (CVS) will be offered to parents because of 25 % risk of CF child. When amniocentesis and CVS are not possible, ultrasound controls of echogenic bowel aimed at 18th to 20th week of pregnancy is offered instead. If child is born, *CFTR* genotyping is performed. Also, cascade testing of relatives of III.2 for carrier status is offered.

While Bayes theorem was used in genetics since 1930s (10), by now it seems like a lost art. I found it unfortunate. While massively parallel sequencing is finding its way into routine *CFTR* testing (11,12), the reason for omitting Bayesian interpretation cannot be the wet lab methodological change. The reason to stay with

Bayes is not only that most of CF laboratories still use low throughput molecular genetics techniques (allele specific PCR, chip array, Invader chemistry, oligonucleotide ligation assay, mass spectrometry, minisequencing, and Sanger sequencing) but the interpretation logic is missing. Though shift from genetic to genomic counseling would bring some changes in scope, approach, and process (13), the rationale of genetic risk assessment will not change with an update of testing technology. Therefore, the general principles described in this book chapter hold even for future (or more complicated) genetic risk cases (14) where other strands of evidence are to be combined (7,8,15). For such cases, using Bayesian net or disease-dedicated software (e.g. BOADICEA <https://ccge.medschl.cam.ac.uk/boadicea/forbreastandovariancancers>) is advisable.

V. Pitfalls

Problem	Cause and cure
Wrong result obtained in exemplar case	Probably clerical mistake. To obtain Bayes net for cystic fibrosis in *.xdsl, *.net, *.dne, or *.dsc format, contact author by e-mail drabekjiri@gmail.com.
Suspicious result obtained in other case	Care must be taken to relate the conditional information to the correct person. In Bayes net development, it is advisable to start with person nodes and then add segregation, genotyping, and health status nodes. Direction of edges should be causal (though it is not a general condition for Bayes nets). When unexpected probabilistic result is obtained after checking the net and probabilistic tables, one should bear in mind that it can be just expression of inadequacy of common sense.

VI. Acknowledgements

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Analysis of RNA by Northern blot

Zuzana Macečková

I. Introduction

Northern blot is one of the oldest method for mRNA expression pattern detection. Although nowadays, golden standard in expression is RT-PCR, microarrays, and transcriptome sequencing, Northern blots are still irreplaceable in cases of detection of rRNA processing or studying of splicing variants.

Northern blot method consist of several steps including RNA separation, RNA transfer, hybridization, and probe detection (Fig. 46.1). Generally, it is recommended to carry all the steps in instrument washed by DEPC-treated (RNA-ready) water and prepare all solutions in RNA-ready water to prevent RNA degradation.

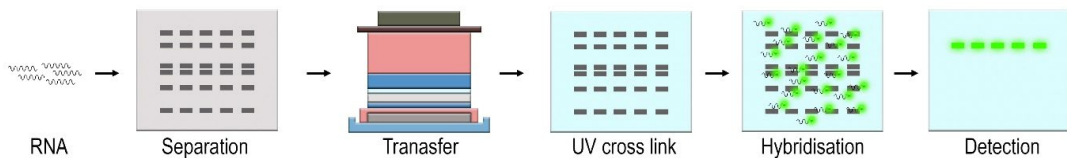


Figure 46.1: Northern blot method overview

II. Solutions

Apart from buffers enlisted below, we recommend to obtain probe dilution and antibody dilution buffer from one of many suppliers.

2× RNA Loading Buffer (450 µl)	
Formamide	200 µl
Formaldehyde	83 µl
10x MOPS	50 µl
2.5% bromophenol blue	10 µl
Glycerol	50 µl
Water	57 µl

20× SSC (1 l pH 7.5)	
NaCl	175 g
Sodium citrate	88g

10× MOPS buffer (1 l)	
MOPS	41.9 g
Sodium acetate	3.8 g
EDTA	7.4 g

High Stringency buffer (HSB, 50 ml)	
20X SSC	1.25 ml
10xSDS	500 µl

Low Stringency buffer (LSB, 50 ml)	
20X SSC	5 ml
10 % SDS	500 µl

Washing buffer (1 l, pH 7.5, 0.3% Tween)	
Maleic acid	11.6 g
10 % SDS	

III. RNA separation

For purpose of Northern blot, RNA is separated on denaturing agarose gel. Dependent on targeted RNA size, 6% formaldehyde and 0.5% ÷ 2.5% agarose gel can be used. Prior to sample loading, it is necessary to perform RNA denaturation. Depending on RNA abundancy in sample, 5÷15 µg of RNA sample is mixed in volume ratio 1:1 with RNA loading buffer and denatured in 75 °C for 2 minutes and then quickly cooled down on ice. It is desirable to

prevent RNA degradation and gel overheating, so gel should run in 4 °C using low voltage (15 V÷30 V), preferably overnight in 1X MOPS, 6% formaldehyde buffer.

IV. RNA transfer

Probes are not able to enter gel matrix. Therefore, RNA is transferred to positively

charged, usually nylon, membrane. Wash out of residual formaldehyde from gel prior to transfer is essential. Gel can be washed 2× 30 min in 20× SSC buffer on shaker. Furthermore, soaking membrane in 20× SSC buffer prior to transfer can significantly increase transfer efficiency. Membrane can be washed 5 min by distilled water, followed by 10 min wash in 20× SSC. When both gel and membrane are ready, capillary transfer can be set as described on Fig. 46.2. Transfer is done in 8 hours (it can be carried out overnight). After transfer, membrane is washed in 2× SSC buffer and RNA is cross-linked on membrane by UV for 5 min and air dried. After crosslinking, membrane could be stored in 4 °C for two weeks or in -80 °C up to half a year.

V. Hybridization

Purpose of hybridization is to mark targeted RNA with labeled probe. Oldest type of probe labeling is based on incorporation of radioactive phosphor isotope to 5' end of oligonucleotide probe. Currently, this type of labeling is overcome and is preferred to use digoxigenine labeled probes, which manifest same detection sensibility together with higher safety and stability (1).

These are several commercially available products for digoxigenine labeled probe generation. The biggest disadvantage of in house probe generation is different yield of

every set up and need to measure efficiency of every probe. From experience in our laboratory, we recommend usage of commercially labeled oligonucleotides. Almost all distributors offering oligonucleotides can provide digoxigenine labeled probes, which have stable yield and which are considerably cheaper than in house created probes. General recommendation is to use oligonucleotides longer than 25 nucleotides to achieve required specificity.

It is necessary to perform membrane-blocking step prior to hybridization. Blocking is performed in probe dilution buffer, usually for one hour in hybridization temperature, ideally in rotating hybridization chamber. Again, best result is achieved with commercially available digoxigenine blocking solutions.

After blocking, blocking buffer is wasted and replaced with probe diluted in probe dilution buffer. Concentration of probe we are using is 10 pM. Hybridization step is carried over night in rotating hybridization chamber in 37 °C ÷ 50 °C depending on melting temperature of oligonucleotide probe.

After hybridization probe wash out by LSB and HSB follows. Washing by LSB is performed 2× 5 min in RT and washing by HSB is performed 2× 15 min in hybridization temperature (it is recommended to preheat HSB to required temperature).

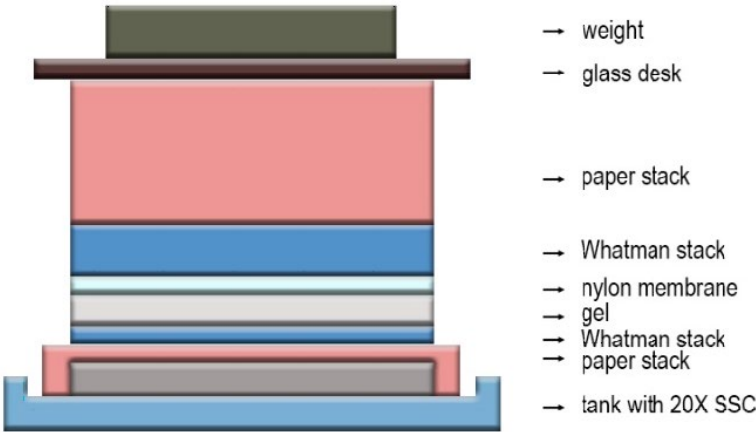


Figure 46.2. Capillary transfer set up

VI. Detection

After hybridization, membrane is washed 2× 15 min in washing buffer and then blocked in blocking buffer for 1 h at RT. Blocking buffers are commercially available. After blocking, membrane is incubated 1 h with digoxigenine

AP antibody in blocking buffer (dilution ratio should be set according to manufacturer’s recommendation) following by 3× 15 min wash in washing buffer. After washing, membrane is covered by alkaline phosphatase substrate and evoked in chemiluminescence detector.

V. Pitfalls

Problem	Cause and cure
Low sensitivity	• Low probe concentration: increase concentration of probe.
High background	• High probe concentration: decrease concentration of probe. • Inefficient washes: increase time of washes, and check wash temperature.
Result is blurred	• Increase RNA load, increase voltage.

VIII. Acknowledgement

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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MiRNA expression microarray

Hanuš Slavík and Josef Srovnal

I. Introduction

MiRNAs are short regulatory oligonucleotides showing promising analytical properties for *in vitro* diagnostics, that has been used particularly in oncology in recent years. In addition to NGS (next generation sequencing), microarray is a standard miRNA profiling technique, which is capable to capture a large number of different miRNAs even at low concentrations. The method is ideal tool for hit-mining approach for setting up some differentially expressed miRNAs for further investigations or global transcriptomic characterization. In comparison to NGS, microarray allows only relative quantification, but it offers much more standardized, and reproducible workflow and data acquisition. However, it is inappropriate method for the quantification and measurement of the expression of individual miRNAs. The reason is low specificity for homologous miRNAs, poor optimization of melting temperature for a large number of different sequences and high cost. Thus, the obtained data should be verified or validated using RT-qPCR (reverse transcription quantitative polymerase chain reaction) or another single-target technique. In principle, each

transcriptomic microarray method is based on hybridization of target molecules on pre-defined immobilized probe sets. Hybridized molecules are fluorescently labeled and the fluorescence is measured for each point on immobilized and washed array (1). Relative expression of investigated RNAs is represented by comparison of fluorescence between the group of samples. Specific microarrays for targeted hybridization were developed by several vendors, such as Illumina, Agilent or Affymetrix. For instance, GeneChip miRNA 4.0 Array from Affymetrix (now part of ThermoFisher Scientific) contains 30,434 probe sets for total mature miRNAs. The array is suitable for 203 organisms and there are pre-designed 2,578 mature miRNAs, 2,025 precursor miRNAs and 1,996 other small RNA species for human samples (2).

II. Materials and instrumentation

The majority of the material (otherwise the supplier is mentioned) is supplied by Applied Biosystems (Foster City, CA, USA), which is a part of ThermoFisher Scientific (Waltham, MA, USA).

Laminar box, appropriate centrifuge, thermocycler, Nanodrop ND 1000 (ThermoFisher Scientific) or another instrument for measurement of nucleic acid concentration, Hybridization oven 645, Fluidic station 450, GeneChip Scanner 3000 7G (ThermoFisher Scientific) and pipettes.

The 0.2 ml plastic tubes or strips and pipette tips in PCR quality, sodium hypochlorite (MERCK, Germany), 1mM Tris-Cl buffer, FlashTag™ Biotin HSR RNA Labeling kit, GeneChip Hybridization Wash and Stain kit, GeneChip Hybridization Control Kit Stain and miRNA 4.0 Arrays.

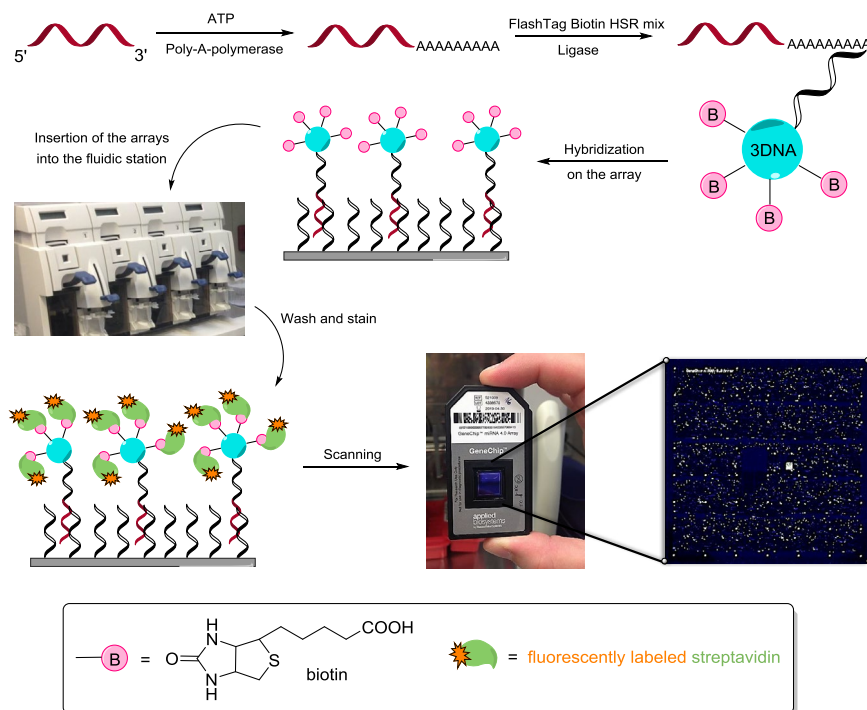
III. Procedures

At the beginning, the miRNA molecules are poly-adenylated at the 3' end, then a biotin branched nucleic acid (3DNA in this case) is ligated to the poly-adenylated terminus by ligase. 3DNA dendrimer technology serves here as "signal amplification connector" between analyzed molecules and biotin-based detection system for higher sensitivity and accuracy in fluorescence measurements. After hybridization

of the RNA to the microarray, biotin is detected by labeled streptavidin. Phycoerythrin is used as fluorescence probe connected to streptavidin. Detailed protocol is supplied by the manufacturer (3), thus here is only a summary guideline.

Steps

1. Preheat the Hybridization oven to 48 °C. Thaw the isolated samples on ice.
2. Modify the concentration of RNA samples with nuclease-free water to 130 ng/8 µl in 0.2 ml micro-tubes in PCR quality.
3. 2 µl Spike Control Oligos to each sample.
4. Dilution of supplied ATP mix with 1 mM Tris buffer in proportion ATP:Tris = 1:38.5.
5. Preparation of polyadenylation reaction according to manufacturer's instructions. Incubation of samples with the mixture for 15 minutes at 37 °C.
6. Preparation of the ligation reaction according to the manufacturer's instructions. Incubation for 30 minutes at room temperature.
7. Preparation of the hybridization reaction and incubation after adding to the samples



Scheme 47.1. Overview of the principle and the workflow of microarray method for miRNA profiling

for 5 minutes at 99 °C and next 5 minutes at 45 °C

8. Immediate loading of prepared mixes into the Affymetrix GeneChip miRNA 4.0 Array in 130 µl volume and incubation of the arrays in the Hybridization oven 645 for 16÷18 hours at 48 °C and 60 rpm.
9. Removing of the arrays from the oven and replacement of the hybridization mixture from the array with Array Holding buffer. Preservation of the hybridization mixture in the fridge.
10. Loading of the stain cocktails and the arrays into Fluidic station 450 after preparing program PRIME 450 and running of appropriate program for the arrays.
11. Washing and IDs scanning into the Affymetrix GeneChip Command Console software according to the manufacturer's instructions.
12. Replacement of the arrays from the station

and insertion (without visible bubbles on the array) into GeneChip Scanner 3000 7G. Scanning of one array takes only few minutes.

13. Modification of array scans using the Affymetrix GeneChip CommandConsole software and exporting of CEL files for data analysis.
14. Reading, quality evaluation and data analysis of CEL files using R-software or TAC supplied by ThermoFisher Scientific. Both of them can be obtained as full-version freeware.

V. Pitfalls

Problem	Cause and cure
Low concentration of RNA / low fluorescence of the array	Low abundant material: Sample concentration or re-extraction with higher amount of starting material.
Presence of bubble on the array after washing on fluidic station	Discard the liquid content from the array and refill it with new array holding buffer.
Scanning failure	Try to rescan the array and visually check every scan. If it is not possible to generate appropriate images after scanning, it will be the technical problem with array and the process should be repeated with the new one.
Sample do not pass QC	Do not use it for the analysis, it can be caused by contamination or too different sample type or low sample quality. Try to rescan the array or repeat the process from the start for this sample.

IV. Comments

Pre-analytical preparation of the samples is crucial for this method because microarray technologies itself are standardized and functionally optimized in these days. RNA extraction procedure should provide maximum possible yield of small RNA species because content of miRNAs in most biological materials is below 10% among all RNA species. For this reason, miRNeasy Mini kit (Qiagen, Hilden, Germany) is one of the most suitable universal kits for RNA isolation from various biological materials including cell-free body fluids or FFPE (formalin fixed paraffin embedded) samples according to our experiences. Validated spectrophotometric method is recommended for measurement of concentration of RNA samples for calculation of needed input for microarray. Whole pre-analytical process can take up to 3 h depending on number of samples and type of starting biological material. Microarray itself take about 3 h during the first day and after over-night incubation of the arrays in the oven, each four arrays can be processed in 1.5 h during second day. Time needed for the data analysis depends on number of analyzed CEL files, complexity of studied problem and the computing capacity of the used equipment. Standard differential expression investigations between two groups of samples is possible to be calculated in one day for reasonable amount of CEL files. Usually, it is needed to start with a robust multiple array normalization to stabilize the fluorescence among the whole data set. Afterwards, quality control analysis is recommended for each array (principal components, MA-plots, and others) which are part of the software intended for that purpose. Obtained logarithmic values of fluorescence for every probe sets can be used as an input for statistical calculations of significant differences between groups of samples (Wilcoxon regression analysis, Student t-test, etc.)

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Circulating tumor cells detection

Alona Řehulková and Josef Srovnal

I. Introduction

Detection of circulating tumor cells (CTCs) in cancer patients represents novel strategy to assess accurate prognosis and administer adequate therapy. There is a variety of methods that allow detection or separation of rare tumor circulating cells from peripheral blood. The approach described in this protocol is based on detection of mRNA transcripts that are not naturally present in peripheral blood, but occur in CTCs or disseminated tumor cells (DTCs) released from primary tumor or metastasis to the bloodstream. For detection of many solid tumors, specific mRNA of epithelial markers such as MUC-1 (mucin 1), cytokeratin 19 and 20, hTERT (human telomerase reverse transcriptase), CEA (carcinoembryonic antigen), EGFR (epidermal growth factor receptor), LunX (lung specific X-protein), c-met (hepatocyte growth factor receptor) and EpCAM (epithelial cell adhesion molecule) can be used. The identification of CTCs is performed using reverse transcription and quantitative real-time polymerase chain reaction (RT-qPCR). The disadvantage of RNA detection is a high rate of false-positive results (1). In this chapter, cut-off values were determined

using healthy control cohort for the CEA, EGFR, LunX, c-met and EpCAM mRNA copies that showed either CTC/DTC presence or absence.

II. Materials and instrumentation

Hypotonic lysis buffer: 1.55 M NH_4Cl (Cat. No. 30070-AP0, Lach-Ner), 0.1 M NH_4HCO_3 (Cat. No. 30065-AP0, Lach-Ner), 1 mM EDTA (Cat. No. 39760, Serva).

RNA isolation and reverse transcription

TRI Reagent (Cat. No. TR118, Molecular Research Centre, USA), RNeasy[®] (Cat. No. 76106, Qiagen), Nanodrop ND 1000 (Thermo Fisher Scientific, DE, USA), Random Primers (Cat. No. C118A, Promega), RevertAid H Minus Reverse Transcriptase (200 U/ μl) with 5X Reaction Buffer, (Cat. No. EP0451, Thermo Fisher Scientific), dATP, dGTP, dCTP, dTTP (Cat. No. U120C, U121C, U122C, U123C, Promega), RNasin ribonuclease inhibitor (Cat. No. N251B, Promega), DEPC (diethyl

pyrocarbonate) - treated water (AM9916, Ambion, Thermo Fisher Scientific), chloroform (Cat. No. 02194002 MP Biomedicals), 2-propanol (I9516, Sigma-Aldrich), ethanol (437433T, VWR Chemicals).

Quantitative real-time polymerase chain reaction

LightCycler® 480 System (Roche), LightCycler® 480 Probes Master (Cat. No. 04 887 301 001, Roche), DEPC treated water (AM9916, Ambion, Thermo Fisher Scientific), an optimized amount of primers (primer-Fw – 300nM for CEA and 400 for EGFR, LunX, c-met, EpCAM; primer-Rev – 600 nM for CEA and 400 nM for EGFR, LunX, c-met, EpCAM); probes (200 nM for each), standards with number of copies 30, 10², 10³, 10⁴, 10⁵, 10⁶, and 96-well plate for LightCycler® 480.

Primers and probes

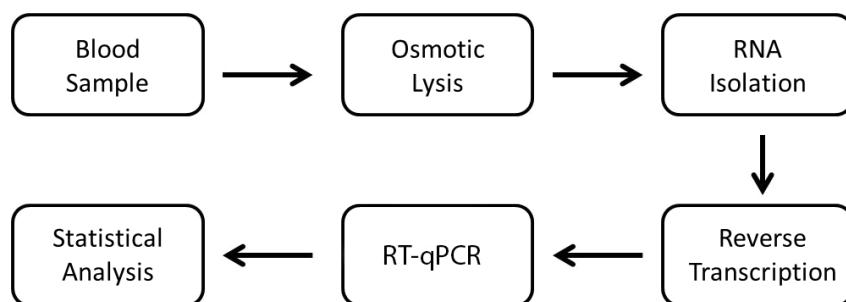
CEA-fw 5'-taagtgttgaccacagcgaccc-3', CEA-rev 5'-gttcccatcaatcagccaagaa-3' and CEA-probe 5'-atgtctctatggccagacgaccc-3' BHQ1-HEX (167 bp amplicon); EGFR-fw 5'-acttcaaaaactgcacctccat-3', EGFR-rev 5'-aatcagcaaaaacctgtgatt-3' and EGFR-probe 5'-acatcctgcgggtggcatttagg-3' BHQ1-HEX (149 bp amplicon); LunX-fw 5'-gatggccaccgtctctatgt-3', LunX-rev 5'-acagccagcctcaacagact-3' and LunX-probe 5'-ccatcctctcggcataaagctcc-3' BHQ1-HEX (93 bp amplicon); c-met-fw 5'-tggacaatgatggcaagaaa-3', c-met-rev 5'-gatgattccctcggtcagaa-3' and c-met-probe

5'-tcactgtgctgtgaaatccttgaaca-3' BHQ1-HEX (99 bp amplicon); EpCAM-fw 5'-aaacacaaagcaagagaaaaacct-3', EpCAM-rev 5'-aattttggatccagttgataacg-3' and EpCAM-probe 5'-ttgcggactgcacttcagaagga-3' BHQ1-HEX (95 bp amplicon) (Generi-Biotech, Hradec Králové, Czech Republic).

III. Procedures

Osmotic lysis

1. Keep the samples on ice throughout the protocol. All centrifugation steps are performed at 4 °C.
2. The total RNA is purified from 10 ml EDTA samples of blood or bone marrow.
3. Spin down the cells at 2,000 rpm for 10 min.
4. Aspirate supernatant (plasma).
5. Add 40 ml of cold hypotonic lysis buffer and incubate for 15 – 40 minutes on ice until the solution is clear.
6. Centrifuge at 4,500 rpm for 10 min.
7. Aspirate supernatant and resuspend cells in 20 ml of hypotonic lysis buffer.
8. Centrifuge at 4,500 rpm for 5 min.
9. Aspirate supernatant and resuspend cells in 20 ml of PBS.
10. Centrifuge at 4,500 rpm for 5 min.
11. Aspirate supernatant and resuspend the cells in 3-10 ml PBS, transfer 1 ml aliquots into 1.7 ml tubes.
12. Centrifuge at 2,000 rpm for 4 min.
13. Aspirate supernatant, add 1 ml TRI Reagent into each tube, mix and immediately process or store at -20 °C.



Scheme 48.1.

RNA purification

1. Extract total RNA from 1 ml of the sample in TRI Reagent (2).
2. Add 200 μ l of chloroform, vortex, and incubate 10 min at room temperature.
3. Centrifuge at 12,000 g for 15 min.
4. Collect the upper clear aqueous phase containing RNA into a new tube.
5. Add 500 μ l of 2-propanol to an aqueous phase, mix and incubate 5 min at room temperature.
6. Centrifuge at 12,000 g for 10 min.
7. Aspirate the supernatant and wash the pellet with 1.5 ml of 75% ethanol.
8. Centrifuge at 12,000 g for 5 min.
9. Aspirate the supernatant completely and let the pellet dry in the flow box for 3-10 min.
10. According to the size of the pellet, add 20-60 μ l of DEPC-treated water.
11. Incubate the tubes with RNA for 10 min at 60 °C.
12. Determine concentration and purity of the RNA using Nanodrop ND 1000.

Reverse transcription

1. Use 3 μ g of total RNA for reverse transcription.
2. Add DEPC-treated water to total volume of 19.5 μ l, vortex and spin down.
3. Pre-incubate 3 μ g of RNA with 0.3 μ g of Random Primers for 5 min at 70 °C.
4. Place the mixture immediately on ice.
5. Prepare MasterMix: Add 6 μ l of RevertAid 5x RT buffer (Fermentas, ThermoScientific), 3 μ l of 10 mM deoxyribonucleotide triphosphates (dNTPs) and 0.75 μ l of 40 U/ μ l RNasin ribonuclease inhibitor per one sample.
6. Add 9.75 μ l of MasterMix to the sample and mix using a pipette.
7. Incubate for 5 min at RT.
8. Add 150 U reverse transcriptase and incubate 10 min at RT.
9. Incubate at 42 °C for 60 min and then at 70 °C for 10 min.
10. Keep the samples with cDNA on ice or store at -20 °C.

Quantitative real-time polymerase chain reaction

1. Carry out the RT-qPCR with 100 ng of cDNA in a final volume of 15 μ l.
2. Prepare MasterMix: One sample contains 3.5 μ l DEPC water, 7.5 μ l LightCycler® 480 Probes Master, 1 μ l of forward primer, 1 μ l of reverse primer, 1 μ l of probe. Vortex and spin down.
3. Add 14 μ l of MasterMix into wells of 96-well plate for LightCycler® 480. Prepare wells with MasterMix for duplicate samples, six standards, positive control, and negative control.
4. Add 1 μ l of sample to a defined well. Typically, the PCR is performed with one sample in duplicates.
5. As a positive control, add to a separate well 1 μ l cDNA with expressed marker.
6. As a negative control, use 1 μ l of DEPC water.
7. Pipette 1 μ l of standards with number of copies 30, 10^2 , 10^3 , 10^4 , 10^5 , 10^6 in duplicates. Cover the plate with the PCR foil.
8. Set the following parameters of the reaction using LightCycler® 480 System.
9. Initial step 96 °C for 15 minutes.
10. 50 cycles of: 95 °C for 15 seconds and then either 65 °C for 15 seconds (CEA), 62 °C for 15 seconds (EGFR), 62 °C for 10 seconds (LunX), 58 °C for 15 seconds (c-met), or 59 °C for 15 seconds (EpCAM).

Standardization and normalization of the data

1. Construct standard curves to determine the absolute number of mRNA copies for a particular gene.
2. Calculate absolute mRNA copy numbers for each gene in standards using concentrations measured on NanoDrop ND 1000, the molecular weight of the amplicon, and Avogadro's constant ($6.023 \times 10^{23} \text{ mol}^{-1}$).

V. Pitfalls

Problem	Cause and cure
Abnormal amplification curve	• The sample contains degraded RNA. Repeat the isolation and reverse transcription (3).
Abnormal amplification curve	• The RNA is of a poor quality and contains proteins or other contaminants from the isolation steps. Purify the RNA.
Abnormal amplification curve or no amplification	• The constituents of the PCR reaction are degraded. Use new stock of reagents.
The amplification occurs in the negative control	• Contamination of reagents with the PCR products. Use new stock of reagents and clean the working space.

V. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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DNA copy number variants detection using microarray

Karolína Bartáková and Josef Srovnal

I. Introduction

Copy number variants (CNVs) or copy number polymorphisms (CNPs) are one form of the mammalian genomic variation (structural variation). The others include single nucleotide polymorphisms (SNPs), microsatellites, and other repeats (1). CNVs are imbalances that alter the diploid status of a locus and comprise deletions, insertions, duplications, and complex multi-site variants (2). CNVs are widespread in about 15% of the human genome and contribute substantially to population diversity (3). In the human genome there are CNV distribution hotspots: 1) genes involved in immunity and cell-cell signaling; 2) genes encoding proteins involved in interaction with the environment (e.g., immune response or perception of smell); and 3) retrovirus- and transposition-related protein coding genes (1). A substantial number of CNVs has significant impact on several human diseases, such as cancer (4), autism (5), systemic lupus erythematosus and similar inflammatory autoimmune disorders (6), schizophrenia (7), and even susceptibility to HIV (8). In cancer diagnosis and prognosis, CNVs are even more important.

They can be used as biomarkers to predict cancer status and locate cancer origin (9).

Microarray analysis enables simultaneous detection of CNVs in cancers at a genome-wide level and could be performed in two different ways, using: 1) two-color experiment also referred to as array Comparative Genomic Hybridization (aCGH) and 2) one-color experiment also known as single nucleotide polymorphism (SNP)-based microarray or SNP-array (10). SNP-arrays consist of SNP probes, provide both SNP genotype and CNV information and use reference intensity data built from a population of normal samples which are used as a reference for the patient sample (11-13). Genome-wide analysis for DNA (gDNA) extracted from formalin-fixed paraffin-embedded (FFPE) samples is technically challenging. This type of DNA is degraded into smaller fragments and the yield may be lower due to the amount of starting material available, especially in biopsy specimens (14). An additional biological variable is the cellular heterogeneity of tumor tissues where there is a presence of normal cells. To overcome these difficulties with FFPE tumor specimens, the OncoScan FFPE Assay Kit (ThermoFisher Scientific, Waltham, MA, USA)

was designed (15, 16). The OncoScan assay is an accepted cancer diagnostic microarray for detection of CNVs, loss of heterozygosity (LOH), and cancer-related somatic mutations (17).

II. Materials and instrumentation

Instruments

Safety cabinet (Thermo Scientific, ScanLaf), Veriti 96-well thermal cycler (Applied Biosystems), Microfuge for 1.5 mL Eppendorf vials (Biosan), Vortexer (Labnet International), Set of single/multi-channel pipettes (Eppendorf), Cold blocks for 96-well PCR plates and 24 microcentrifuge tubes (Sarstedt), Plate centrifuge (Hermle Labortechnik), Precision balances (Sartorius), Water bath TW8 (Julabo), Electrophoresis power supply (Bio-Rad), Gel imager (LI-COR Biosciences), GeneChip hybridization oven 645, Fluidics station 450 and GeneChip scanner 3000 7G (all from ThermoFisher Scientific, Waltham, MA, USA), Freezer, -20 °C (Liebherr), Refrigerator, 2÷8 °C (Liebherr).

Reagents

OncoScan CNV Assay (Cat. No. 902695) or OncoScan CNV Plus Assay (Cat. No. 902293) (Applied Biosystems, ThermoFisher Scientific Waltham, MA, USA), include modules: OncoScan CNV Buffer C/OncoScan Somatic Mutation Probe Mix 1.0, OncoScan CNV Copy Number Probe Mix 1.0 & Controls/OncoScan Copy Number Probe Mix 1.0 & Controls, OncoScan CNV Gap Fill and 1st Stage PCR/ OncoScan Gap Fill and 1st Stage PCR, OncoScan CNV 2nd Stage PCR and Post PCR Processing/ OncoScan 2nd Stage PCR and Post PCR Processing, OncoScan CNV Stain Reagents/ OncoScan Stain Reagents, Individual Bottles - Wash A, Wash B, OncoScan CNV Array/ OncoScan CNV Plus Array (12 pieces/package, 4 packages/kit). Other reagents: Low EDTA 1× TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0, Cat. No. 12090015, Invitrogen, ThermoFisher Scientific), Agarose SERVA for DNA electrophoresis, research grade (Cat. No. 1140405, Serva, Biotech), TBE buffer (Cat. No. T1507.0100, Duchefa Biochemie), GelRed nucleic acid stain, 10,000X in water (Cat. No.

41003, Biotium), Gel loading dye, Blue (6X) (Cat. No. B7021S, New England Biolabs), 50 bp DNA ladder (Cat. No. N3236S, New England Biolabs), 100% glycerol (Cat. No. G5516-100ML, Sigma), EDTA 0.5 M solution (Cat. No. 15575020, Invitrogen, ThermoFisher Scientific), Sodium hypochlorite solution reagent grade, available chlorine 10÷15% (Cat. No. 425044-1L, Honeywell, Fisher Scientific).

Plastic and glass

Laboratory bottle with screw-cap, capacity 1000 mL (Cat. No. B519220, Simax), Graduated cylinder, 500 mL and 10 mL (Cat. No. K001081, K001075, Kartell Labware), Microcentrifuge nuclease-free tubes, 1.5 mL (Cat. No. 1210-00, SSIbio), 8-tube strips without caps, 0.2 mL (Cat. No. 4316567, Applied Biosystems, ThermoFisher Scientific), Pipette tips (compatible with Eppendorf pipettes), 96-well PCR plates, high profile, unskirted (Cat. No. MLP9601, Bio-Rad), MicroAmp clear adhesive film (Cat. No. 4306311, Applied Biosystems, ThermoFisher Scientific), 15 mL conical tube (Cat. No. 91015, Techno Plastic Products), Pipette tips unfiltered, 200 µL (Cat. No. K000960, P-lab), 3/8" laser tough-spots (Cat. No. T-SPOTS-R, B, Diversified Biotech), Tube, safe-lock, 1.5 mL, natural (Cat. No. 0030123328, Eppendorf), Tube, safe-lock, 1.5 mL, amber (Cat. No. 0030120191, Eppendorf).

III. Procedures

Scheme of the process, material and devices

OncoScan CNV (Plus) Assay is suitable for DNA extracted from FFPE samples. The Cobas DNA Sample Preparation Kit for DNA purification could be used. After preparing the list of the samples, DNA isolation and DNA concentration measurement, samples are ready for use in copy number variant analysis assay. You need to prepare 10x TBE buffer in advance and provide enough amount of ultra pure water. The most appropriate timing for the procedure is starting at about 3:00 pm, on day 2 you will start at 8:00 am, washing and staining will start between 7:00-8:00 am.

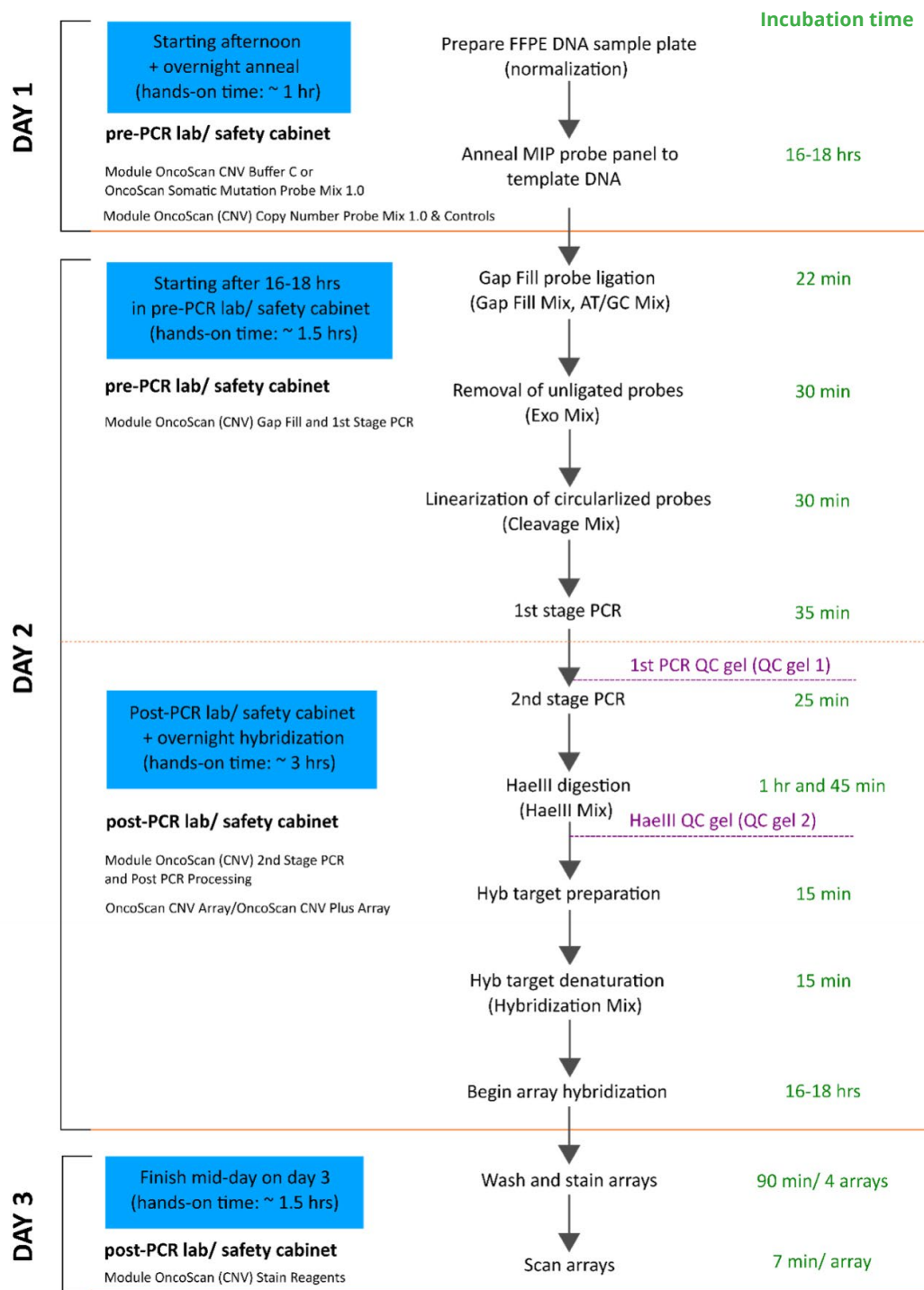


Figure 49.1. *OncoScan Assay workflow.*

DAY 1: Annealing of the MIP probes

1. Prepare the list of your samples. The most effective is to process 7 samples per run in total.
2. Measure the DNA concentration using the Qubit dsDNA HS.
3. Work in pre-PCR lab/ safety cabinet.
4. Normalize all gDNA samples to a single concentration of 12 ng/ μ L containing 6.6 μ L of DNA per well. Use OncoScan CNV Assay Kit (18) or OncoScan CNV Plus Assay Kit (19). Dilute DNA using 1X TE (pH 8.0) with reduced EDTA (0.1 mM EDTA). Dilute samples into 96-well plate. Cut this plate according to your usage and cool it down before pipetting. Mark the sample wells.
5. Thaw Buffer A, Copy Number Probe Mix 1.0 and Buffer C/ Somatic Mutation Probe Mix 1.0 at room temperature for 10-15 minutes. Vortex them at max speed for 3 sec, spin down briefly and place them on ice. Use module OncoScan CNV Buffer C/ OncoScan Somatic Mutation Probe Mix 1.0 and OncoScan CNV Copy Number Probe Mix 1.0 & Controls/ OncoScan Copy Number Probe Mix 1.0 & Controls.
6. Turn on the thermal cycler and allow the lid to heat up.
7. Prepare the Anneal Master Mix to a 1.5 mL tube on ice.
8. Add 3.4 μ L of Anneal Master Mix to your samples. Pipet up and down 3 times to rinse tips.
9. Now the current volume in the assay plate for annealing is 10 μ L.
10. Tightly seal the sample plate with a clear adhesive film. Vortex the plate at max

speed for 5 seconds. Spin down the plate at 2400 rpm for 60 seconds.

11. Place the plate on the thermal cycler and start the OncoScan Anneal program (Fig. 49.2).
12. After 6 minutes into the run, pause the program at 58 °C (1 min at 58 °C). Remove the plate from the thermal cycler and place on a chilled cold block for 1 minute. Ensure the seal is tight.
13. Spin down the plate at 2400 rpm for 30 seconds.
14. Place the plate back on the thermal cycler and press Start one more time to Resume.
15. Incubate the samples for 16 to 18 hours. Do not incubate samples for more than 18 hours.

DAY 2: Probe ligation, PCR reactions, *HaeIII* digestion, quality control of the products, hybridization

1. Work in pre-PCR lab/safety cabinet.
2. Place the Buffer A, dNTP AT Mix and dNTP GC Mix, Cleavage Buffer, water and PCR Mix on the bench top at room temperature for 10÷15 minutes to thaw. Use module OncoScan CNV Gap Fill and 1st Stage PCR/ OncoScan Gap Fill and 1st Stage PCR.
3. Prepare a 1.5 mL tube for Gap Fill Master Mix. Label it and place into cold block until ready to use. Mix reagent according to the table below to create Gap Fill Master Mix. Vortex between additions and spin down briefly in microfuge.
4. Remove the plate incubated overnight at

Anneal Master Mix	1 reaction	7 reactions (~60% overage)
Buffer A	1.53 μ L	17.1 μ L
Copy Number Probe Mix 1.0	1.37 μ L	15.3 μ L
Buffer C/ Somatic Mutation Probe Mix 1.0	0.5 μ L	5.6 μ L
Total Volume	3.4 μ L	38.1 μ L

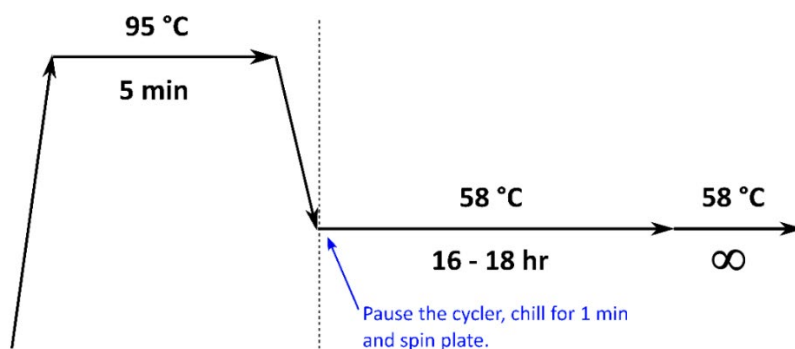


Figure 49.2. *OncoScan Anneal thermal cycler program.*

58 °C from the thermal cycler; place in a cold block for 1 min. Then spin the plate at 2400 rpm for 60 sec and return it to the cold block. As soon as you put the plate on ice, stop the OncoScan Anneal program and start the OncoScan Gap Fill program on the thermal cycler and wait for the thermal cycler to reach 58 °C, then pause the program.

5. Slowly and carefully remove the adhesive seal in order to avoid any contamination through sample splashing.
6. Add 14 μ L Gap Fill Master Mix to each reaction in the appropriate well. Pipet up and down 3 times to rinse tips. Gap Fill Enzyme Mix contain 50% glycerol, so pipet slowly.

7. Tightly seal the plate, vortex at high speed for 5 sec. Spin down the plate at 2400 rpm for 60 sec. Keep the plate on the cold block. The current volume in the plate is 24 μ L.
8. Prepare a fresh 96-well PCR plate, clearly marking the alternating AT row and GC row.
9. Transfer 10 μ L of each reaction from the assay plate with added Gap Fill Master Mix to the AT row and GC row in the new assay plate.
10. Tightly seal the plate with the samples split into AT and GC channel. Spin down at 2400 rpm for 60 sec. Load the plate on the thermal cycler and resume the Gap Fill program to run (Fig. 49.3.).
11. Meantime you can prepare AT Mix and GC

Gap Fill Master Mix	1 reaction	7 reactions (~20% overage)
Water	10.6 μ L	89.0 μ L
Buffer A	1.2 μ L	9.9 μ L
SAP, recombinant (1U/μL)	0.8 μ L	7.1 μ L
Gap Fill Enzyme Mix	1.4 μ L	11.8 μ L
Total volume	14 μ L	117.6 μ L

- Mix. It is highly recommended to prepare the AT Mix and GC Mix separately and using a new pair of gloves for each mix.
12. Prepare two 1.5 mL tubes labeled AT and GC for each dNTPs master mix. Place these tubes on ice until ready to use.
 13. To the tube labeled AT, add the reagents according to the table below. Rinse the pipette tips three times. Vortex the AT Mix tube for 3 sec at maximum speed. Spin down briefly in microfuge.
 14. To the tube labeled GC, add the reagents according to the table below. Rinse the pipette tips three times. Vortex the GC Mix tube for 3 sec at maximum speed. Spin down briefly in microfuge.
 15. After 11 min at 58 °C, press Pause on the thermal cycler and remove the plate. Place the plate in a cold block for 1 min. Spin down at 2400 rpm for 30 sec. Keep the plate on the cold block.
 16. Carefully remove the adhesive film to avoid any contamination between samples in wells.
 17. Add 4 µL of AT Mix to the samples in AT row and 4 µL of GC Mix to the samples in GC row. The current volume in the plate for each sample in the wells is 14 µL.
 18. Tightly seal the plate, vortex at high speed for 5 sec. Spin down at 2400 rpm for 60 sec.
 19. Place the plate back on the thermal cycler and press Resume (Fig. 49.3.).
 20. Set a timer for 10 min to be back and pause the thermal cycler at 58 °C immediately after the 11 min incubation. It is critical to remove the assay plate at 58 °C, before the cycler ramps down to 37 °C to avoid possible non-specific Gapfill.
 21. During the 58 °C incubation for 11 min, prepare the Exo mix. Starting from this step onward until the end of the assay, the number of reactions will double due to AT and GC channel split.
 22. Use a bench top cooler to carry the Exo Mix from the -20 °C freezer. Vortex for 1 second and spin down. Place back in cool block.
 23. When the thermal cycler temperature reaches the end of the 58 °C incubation, press Pause and remove the plate. Resume the program without a plate in the thermal cycler and allow it to ramp down to start of the 37 °C incubation. Pause thermal cycler at 37 °C.
 24. Place the plate in a cold block for 1 min. Spin down at 2400 rpm for 30 sec. Keep the plate on the cold block. Carefully remove the adhesive film.
 25. Add 3 µL Exo Mix to each reaction. Pipet up and down 3 times to rinse tips. The current

AT Mix	1 reaction	7 reactions (~20% overage)
Water	3.9 µL	113 µL
dNTP AT	0.07 µL	2.1 µL
Total volume	4 µL	115 µL

GC Mix	1 reaction	7 reactions (~20% overage)
Water	3.9 µL	113 µL
dNTP AT	0.07 µL	2.1 µL
Total volume	4 µL	115 µL

volume in the plate is 17 μ L per well.

26. Tightly seal the plate, vortex at high speed for 5 sec. Spin down at 2400 rpm for 60 sec.
27. Place the plate back on the thermal cycler and press Resume (Fig. 49.3.). Incubation of the samples with Exo Mix takes 30 min. Place the Cleavage Buffer on the bench top at room temperature for 10-15 minutes to thaw. And then keep on ice.
28. During the last 5 min at 95 $^{\circ}$ C on the thermal cycler, prepare the Cleavage Master Mix. Prepare a 1.5 mL tube for Cleavage Master Mix and place on ice until ready to use.
29. Use a bench top cooler to carry the Cleavage Enzyme from the -20 $^{\circ}$ C freezer. Vortex the Cleavage Enzyme for 1 sec. Spin down and keep on ice.
30. Mix the Cleavage Buffer and Cleavage Enzyme according to the table below. Pipet up and down 3 times to rinse tips. Vortex the tube with Cleavage Master Mix for 3 seconds at maximum speed and spin down. Keep on ice.
31. When the thermal cycler temperature reaches the start of the 37 $^{\circ}$ C step, press Pause and remove the plate. Place the plate in a cold block for 1 min. Spin down at 2400 rpm for 30 sec. Keep the plate on the cold block. Carefully remove the adhesive seal from the plate.
32. Add 25 μ L Cleavage Master Mix to each reaction. Pipet up and down 3 times to rinse tips. The current volume in each well is 42 μ L. Tightly seal the plate, vortex at high speed for 5 sec. Spin down at 2400 rpm for 60 sec.
33. Place the assay plate back on the thermal cycler and press Resume (Fig. 49.3). Set a timer for 25 min to leave 5 min to make the master mix for the next PCR step. Place the PCR Mix for the 1st PCR on the bench top at room temperature for 10-15 minutes to thaw. And then keep on ice.
34. Strew agarose into 1X TBE Buffer for at least 15 min to absorb water. Agarose gel will be better prepared. To prepare 10 $\times$ TBE Buffer dissolve 17.0 g powdered TBE in 1L of demineralized water. Put the bottle into water bath heated for 50 $^{\circ}$ C for better dissolving of the powder.
35. During the last 5 min at 95 $^{\circ}$ C on the thermal cycler, prepare the 1st PCR Master Mix. Prepare a 1.5 mL tube, label it and place on ice until ready to use. Use module OncoScan CNV Gap Fill and 1st Stage PCR/ OncoScan Gap Fill and 1st Stage PCR.
36. Using a bench top cooler, carry the Taq Polymerase for 1st PCR from the -20 $^{\circ}$ C freezer. Vortex the Taq Polymerase for 1 sec and spin down. Return to ice.
37. Mix the PCR Mix and Taq Polymerase according to the table below. Pipet up and down 3 times to rinse tips. Vortex the 1st PCR Master Mix tube for 3 seconds at maximum speed. Spin down. Keep on ice.
38. Once the OncoScan Gap Fill program completes, remove the assay plate and place on a chilled cold block for 1 min. After the 1 min, make sure the seal is tight on the plate, vortex and spin down the assay plate for 30 sec. Stop the OncoScan Gap Fill program and start the OncoScan 1st PCR program, pause program when thermal cycler reaches 60 $^{\circ}$ C.

Cleavage Master Mix	1 reaction	14 reactions (~25% overage)
Cleavage Buffer	25 μ L	438 μ L
Cleavage Enzyme	0.02 μ L	3.5 μ L
Total volume	25.2 μ L	441 μ L

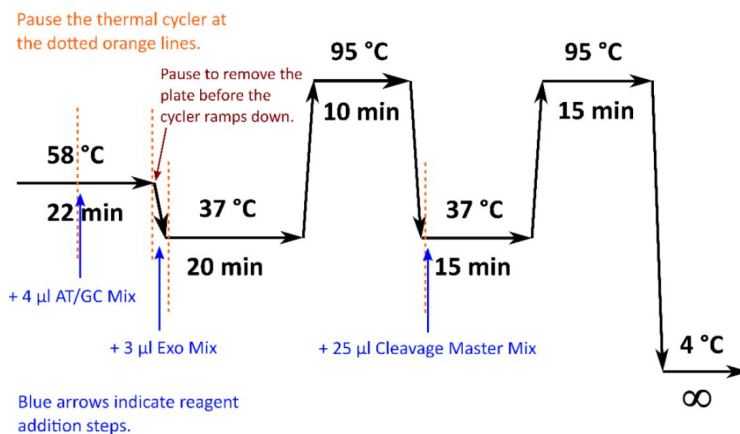


Figure 49.3. OncoScan Gap Fill thermal cycler program.

39. Add 25 µL 1st PCR Master Mix to each reaction. Pipet up and down 3 times to rinse tip. The currently volume in the assay plate is 67 µL per well.
40. Tightly seal the plate, vortex at high speed for 5 sec. Spin down at 2400 rpm for 60 sec. Place the plate on the thermal cycler and press Resume (Fig. 49.4). When the program has ended, transfer the sealed assay plate to the post-PCR lab/ safety cabinet and place on ice.
41. Prepare the 3% agarose gel for the QC gels. You need to prepare glycerol-EDTA buffer, running loading dye, running 50 bp ladder and 3% agarose gel.
42. Work in post-PCR lab/ safety cabinet. Clean your place in pre-PCR lab/ safety cabinet. Place the PCR Mix for the 2nd PCR on the bench top at room temperature for 10-15 minutes to thaw. And then keep on ice.
43. During the last 5 min of the OncoScan 1st PCR program, prepare the 2nd PCR Master Mix. Prepare a 1.5 mL tube, label it and place on ice until ready to use. Use module OncoScan CNV 2nd Stage PCR and Post PCR Processing/ OncoScan 2nd Stage PCR and Post PCR Processing.
44. Using a bench top cooler, carry the Taq Polymerase for 2st PCR from the -20 °C freezer. Vortex the Taq Polymerase for 1 sec and spin down. Return to ice.
45. Mix the PCR Mix and Taq Polymerase according to the table below. Pipet up and down 3 times to rinse tips. Vortex the 2nd PCR Master Mix tube for 3 seconds at maximum speed. Spin down. Keep on ice.

1st PCR Master Mix	1 reaction	14 reactions (~25% overage)
PCR Mix	24.4 µL	428 µL
<i>Taq</i> Polymerase	0.56 µL	9.8 µL
Total volume	25 µL	437 µL

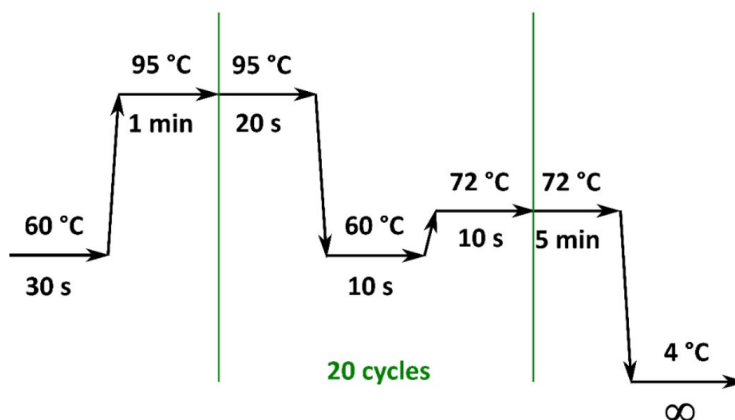


Figure 49.4. OncoScan 1st PCR thermal cycler program.

46. Prepare a new 96-well PCR plate for 2nd PCR and label it. Keep it in a cold block. Follow the same layout as for the previous plate, label row for AT channel and GC channel.
47. Add 25 μL 2nd PCR Master Mix to the appropriate wells in the new assay plate for 2nd PCR.
48. Once the OncoScan 1st PCR program completes, remove the assay plate and place on a chilled cold block for 1 min. After the 1 min, make sure the seal is tight on the plate, vortex and spin down the assay plate for 30 sec. Stop the OncoScan 1st PCR program and start the OncoScan 2nd PCR program, pause program when thermal cycler reaches 60 $^{\circ}\text{C}$.
49. Remove the seal from the assay plate with 1st PCR products carefully and add 2 μL from each well to the corresponding wells in the assay plate for 2nd PCR. Pipet up and down 3 times to rinse pipette tips. Cover the assay plate with 1st PCR products loosely with a seal until ready to use for QC gel procedure. Keep the plate on a chilled cold block. Total volume in each well is 27 μL .
50. Seal the assay plate for 2nd PCR. Vortex at max speed for 5 sec and spin down at 2400 rpm for 60 seconds. Place the assay plate for 2nd PCR on the thermal cycler and resume the OncoScan 2nd PCR program (Fig. 49.5). Place the Buffer B on the bench top at room temperature for 10–15 minutes to thaw and keep it on ice.
51. Run the first QC gel. The first quality control gel is used to check for first PCR products. Cut the prepared agarose gel in the half. Use one half for the first QC gel and the other half for the second QC gel put into the fridge.
52. Prepare tube strips without caps and label them. Aliquot 8 μL of the products from 1st PCR. Add 2 μL running loading dye. Pipet up and down three times to rinse the tips. Seal the assay plate with the 1st PCR products tightly and keep in a cold block on ice.
53. Load 10 μL of each reaction onto a 3% agarose gel.
54. Load 5 μL of running 50 bp ladder on both sides of the gel. Run the gel at 100 V for 15 min (for Sub-Cell Model 96 Cell, Bio-Rad). Adjust the gel running conditions based on the gel chamber used to run the gels. Run gels at 5 V/cm (5 volts $\times$ distance in cm between electrodes). Examine the gel in a gel imager to ensure PCR products are approximately 120 bp.
55. During the last 5 min of the 2nd PCR, prepare the *HaeIII* Master Mix. Prepare a 1.5 mL tube, label it and place on ice until ready to use.
56. Using a bench top cooler, carry the *HaeIII* and Exo enzymes from the -20 $^{\circ}\text{C}$ freezer. Vortex the enzymes for 1 sec and spin down. Return to ice.
57. Mix the Buffer B, *HaeIII* and Exo enzyme according to the table below. Pipet up and down 3 times to rinse tips. Vortex the *HaeIII*

Glycerol-EDTA buffer (50% glycerol and 50 mM EDTA)

Reagent	Initial concentration	Final concentration	Volume
100% glycerol	100%	50%	1000 µL
0.5 M EDTA	500 mM	50 mM	200 µL
Nuclease-free water	N/A	N/A	800 µL
Total volume			2000 µL

Running loading dye (1:10 dilution of 6X Gel Loading Dye)

Reagent	Volume
Glycerol-EDTA buffer	900 µL
NEB 6X loading dye	100 µL
Total volume	1000 µL

Running 50 bp ladder (dilution of the 50 bp ladder)

Reagent	Volume
Glycerol-EDTA buffer	830 µL
NEB 6X loading dye	100 µL
NEB 50 bp ladder	70 µL
Total volume	1000 µL

3% agarose gel (for Sub-Cell Model 96 Cell, BIO-RAD, with 2 electrophoresis combs)

Reagent	Amount/Volume
Agarose	3 g
1X TBE buffer	100 ml
GelRed Nucleic Acid Stain	5 µL

2nd PCR Master Mix	1 reaction	14 reactions (~25% overage)
PCR Mix	24.4 μ L	428 μ L
<i>Taq</i> Polymerase	0.56 μ L	9.8 μ L
Total volume	25 μ L	438 μ L

- Master Mix tube for 3 seconds at maximum speed. Spin down. Keep on ice.
58. Prepare a new 96-well PCR plate for *HaeIII* digestion and label it. Following the same layout as the assay plate for the 2nd PCR. Keep it in a cold block.
 59. Add 20 μ L *HaeIII* Master Mix to the appropriate wells in the new assay plate for the *HaeIII* digestion.
 60. Stop the PCR program after the completion of the 2nd PCR. Remove the assay plate from the thermal cycler. Place the assay plate with the 2nd PCR products on chilled cold block for 1 min. Ensure the seal is tight. Vortex for 5 seconds at max speed. Spin down at 2400 rpm for 60 sec. Return the plate to the cold block until ready to use. Stop the OncoScan 2nd PCR program and start the OncoScan *HaeIII* program, pause program when thermal cycler reaches 37 $^{\circ}$ C.
 61. Remove the seal from the assay plate with 2nd PCR products carefully and add 10 μ L from each well to the corresponding wells in the assay plate for *HaeIII* digestion. Pipet up and down 3 times to rinse pipette tips. Total volume in each well is 30 μ L.
 62. Seal the assay plate for *HaeIII* digestion tightly while the plate is in a chilled cold block. Vortex the plate at high speed for 5 sec. Spin down at 2400 rpm for 60 sec.
 63. Place the assay plate for *HaeIII* digestion on the thermal cycler and resume the OncoScan *HaeIII* program (Fig. 49.6).
 64. Set a timer for 85 min to pause the thermal cycler at 37 $^{\circ}$ C to remove the aliquot for the second QC gel. The assay plate with *HaeIII* digest needs to be paused before the 95 $^{\circ}$ C in order to observe the size of the DNA after *HaeIII* digest.
 65. It is recommended weekly clean the fluidics station using sodium hypochlorite bleach according to the Bleach_450 protocol. This protocol takes approximately one hour and forty minutes to complete. To avoid carryover, or cross contamination, from the bleach protocol, it is recommended to use dedicated bottles for bleach and

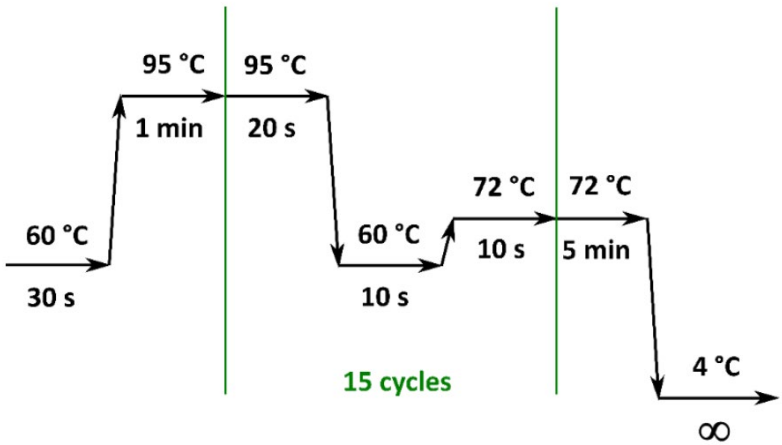


Figure 49.5. OncoScan 2nd PCR thermal cycler program.

water. Prepare 500 mL of 0.525% sodium hypochlorite solution using ultra pure water.

66. Prepare tube strips without caps for *HaeIII* digest QC gel and label them. Add 4 µL of 1X TE to the tubes for digested samples. Add 2 µL of running loading dye to each reaction, pipetting up and down 3 times to rinse the tips.
67. When the cyclor reaches 88 min at 37 °C, pause the cyclor. Remove the assay plate with *HaeIII* digest and place it on a cold block for 1 minute. Ensure the plate is tightly sealed. Vortex at max speed for 5 seconds. Spin down the plate at 2400 rpm for 60 seconds.
68. Remove 4 µL of *HaeIII* digest sample and add it to the tubes with TE and loading dye. Pipette up and down 3 times to rinse the tips. Seal the assay plate with *HaeIII* digest and place it back on the thermal cyclor. Resume the OncoScan *HaeIII* program. The assay plate with *HaeIII* digest can stay

in the thermal cyclor at 4 °C until agarose electrophoresis is done and you know which sample is good enough to load on the array.

69. Run the 2nd QC gel. Use the other half of the agarose gel from the step 51. Load 10 µL of each reaction onto a 3% agarose gel. Load 3.5 µL of running 50 bp ladder on both sides of the gel. Run the gel at 100 V for 15 min (for Sub-Cell Model 96 Cell, Bio-Rad). Examine the gel in a gel imager to ensure that a doublet bands around 40÷70 bp are observed. If any of the samples do not have obviously two bands or have a gel banding pattern similar to the negative control, do not hybridize these samples onto the arrays.
70. Preheat the hybridization oven. Set the temperature to 49 °C and rpm to 60. Allow approximately 30 minutes to 1 hour for the oven to preheat.
71. Allow the OncoScan arrays to equilibrate to

<i>HaeIII</i> Master Mix	1 reaction	14 reactions (~25% overage)
Buffer B	19.1 µL	321 µL
<i>HaeIII</i> enzyme	0.4 µL	6.7 µL
Exo enzyme	0.5 µL	8.4 µL
Total volume	20 µL	336 µL

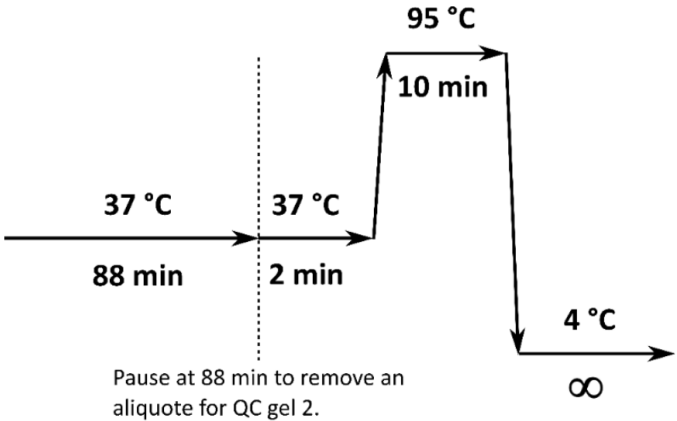


Figure 49.6. OncoScan *HaeIII* thermal cyclor program.

Reagent	Volume
Sodium hypochlorite solution	43.75 mL
Ultra pure water	456.25 mL
Total volume	500 mL

room temperature (10÷15 min). In another way the rubber septa may crack and the array may leak. Mark the front side of the array 1AT to 6AT (for AT channel) and 1GC to 6GC (for GC channel). Each sample uses two arrays so take care in labeling the array clearly to ensure that you know which sample is loaded onto each array.

72. Prepare the Hybridization Master Mix. Prepare a 15 mL conical tube, label it and place on ice until ready to use.
73. Mix the water and hybridization mix according to the table below. Pipet up and down 3 times to rinse tips. Vortex the Hybridization Master Mix tube for 3 seconds at maximum speed. Spin down. Keep on ice. The negative control sample that was processed until the *HaeIII* digest will not be hybridized on the arrays. Prepare only enough hybridization cocktail for the arrays that are being hybridized. The assay plate for hybridization must be prepared fresh right before hybridization.
74. Prepare a new 96-well PCR plate for hybridization and label it. Following the same layout as the assay plate for the *HaeIII* digestion without negative control. Keep it in a cold block.
75. Remove the assay plate with *HaeIII* digest from the thermal cycler after the program is complete. Ensure the seal is secure. Vortex at maximum speed for 5 sec. Spin down the plate at 2400 rpm for 60 sec. Place in a cold block.
76. Add 148 µL Hybridization Master Mix to the appropriate wells in the new assay plate for the hybridization. Any time fresh pipette tips are used, aspirate and dispense

three times to wet the tips prior to aliquoting Hybridization Master Mix.

77. Remove the seal from the assay plate with *HaeIII* digest carefully.
78. Transfer 22 µL of each reaction from the assay plate with *HaeIII* digest to the assay plate for hybridization. Pipet up and down 3 times to rinse tips. Total volume in each well is 170 µL. Tightly seal the assay plate for hybridization. Vortex at maximum speed for 5 sec. Spin down at 2400 rpm for 60 sec.
79. Place the assay plate for hybridization on the thermal cycler and start the OncoScan Hybridization program (Fig. 49.7).
80. Use Affymetrix GeneChip Command Console (AGCC) software (Samples-Quick Registration) to generate and enter information into a sample batch registration file. Scan the array barcodes. To ensure that the data collected during scanning is associated with the correct sample, mark the pairs of arrays by sample number. Save the Batch Registration File and close it.
81. Insert a 200 µL pipette tip (unfiltered tips are preferred) into the upper right septum of each array.
82. At the end of the 5 min incubation of the assay plate for hybridization at 49 °C while leaving the plate on the thermal cycler at 49 °C, carefully cut the adhesive seal to expose wells with samples.
83. Aspirate 160 µL from one well and slowly inject it into the lower left septum of the correspondingly marked array (AT array and GC array). Remove the pipette tip from the upper right septum of each array. Repeat this until all the arrays are loaded.
84. Cover both septa of the array with tough-

spots. Using larger Tough-Spots that overlap the window makes them easier to remove later on.

85. Place the arrays into hybridization oven trays. Load the trays into the hybridization oven.
86. Allow the arrays to incubate at 49 °C and 60 rpm for 16 to 18 hours.
87. Discard the Hyb Plate in a hazardous waste bin.
88. Clean your working place in post-PCR lab/ safety cabinet.

DAY 3: Arrays washing, staining and scanning

1. Use module OncoScan (CNV) Stain Reagents. Prepare at least 400 mL of wash buffer A, 300 mL of wash buffer B and 300 mL of ultra pure water. It is recommended to use dedicated bottles for each solution. Use the Affymetrix GeneChip wash A and wash B buffers that are designated for the OncoScan assay only. These wash buffers differ from the GeneChip expression buffers.
2. Put the lines to the appropriate bottle. Wash-A line in wash buffer A, wash-B line in wash buffer B and water line in ultra pure water. Ensure that the waste container has

been emptied.

3. Prime the fluidics station. Priming ensures the lines of the fluidics station are filled with the appropriate buffers. Affymetrix Launcher - AGCC Fluidics Control - Protocol: PRIME_450 - Probe Array Type:x - Check/uncheck all stations and modules:confirmed - Copy to Select Modules:Run all.
4. Prepare the following reagents into 1.5 mL tubes for each array (3 tubes with different solution for 1 array): 500 µL of stain buffer 1 into 1.5 mL tubes (use amber color tubes as stain buffer 1 is light sensitive), label it with number 1. 500 µL of stain buffer 2 into 1.5 mL tubes (clear tubes), label it with number 2. 1000 µL of array holding buffer into 1.5 mL tubes (clear tubes), label it with number 3.
5. Once the "prime" protocol is done, lift the needle lever and remove the vials used for priming.
6. For washing and staining the arrays select the OncoScan_450 wash protocol from the AGCC Fluidics Control Panel. Start the protocol and follow the instructions displayed on the LCD panel on the Fluidics Station module. Eject the wash block to avoid sensor time out.
7. When prompted to "Load vials 1-2-3": Place

<i>Hybridization Master Mix</i>	1 reaction	12 reactions
Water	30 µL	432 µL
<i>Hybridization Mix</i>	118 µL	1699.2 µL
Total volume	148 µL	2131.2 µL

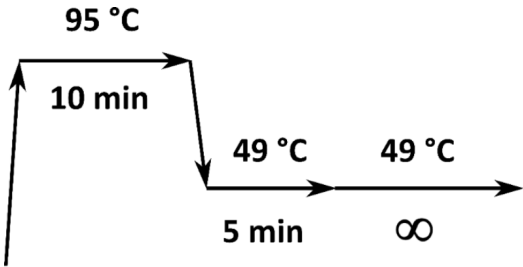


Figure 49.7. OncoScan hybridization thermal cycler program.

one vial containing 500 μ L stain buffer 1 in position 1. Place second vial containing 500 μ L stain buffer 2 in position 2. Place third vial containing 1000 μ L array holding buffer in position 3.

8. After 16 hrs of hybridization, remove four arrays that will be washed at this time. Leave the remaining arrays in the hyb oven at 49 °C until the fluidics station is ready for another run. Remove the tough-spots from the arrays that are ready to be washed and stained. Once the arrays are removed from the hybridization oven, quickly load them onto the fluidics station. Delays during this step will impact data quality.
9. Immediately insert the arrays into the designated modules of the fluidics station. The cartridge lever is in the down position. Pull the handle to engage the array in the washblock.
10. When the LCD panel instructs, press down on the needle lever to snap needles into position and to start the run. The fluidics protocol begins. The fluidics station dialog box at the workstation terminal and the LCD window display the status of the washing and staining steps. Watch the fluidics for 2 minutes to make sure the protocol begins. The user should watch for each module to start the process before walking away from the instrument. It is recommended to check on the fluidics stations every 30 minutes to make sure there are no errors while washing or staining and there enough wash buffers and water in the bottles.
11. When the wash and stain procedure is completed, remove the arrays from the fluidics station by pressing down the cartridge lever to the eject position. Washing and staining protocol lasts approximately 1 h 30 min.
12. Check the array window for bubbles or air pockets. If air bubbles are present insert a 200 μ L pipette tip into the upper right septum of the array. Using a P-200 pipette, remove the entire volume of the holding buffer from the array. Manually fill the array with 170 μ L of array holding buffer.
13. Carefully cover both septa with tough-spots. If necessary, clean the glass surface of the array with a non-abrasive towel or tissue before scanning. On the back of the array cartridge, clean excess fluid from around the septa, if present. When applying the tough-spots do not overlap the center area as the tough-spots should be flat against the array to avoid being pulled off inside the scanner.
14. If the array has no bubble, it is ready for scanning. If the arrays cannot be scanned promptly, store them at 4 °C in the dark until ready for scanning. Scan must be performed within 24 hours.
15. Pull up on the cartridge lever to engage wash block. Remove the microcentrifuge vials containing stain and replace with three empty vials as prompted. Next priming protocol washes the needles and lines.
16. Repeat wash and stain (step 9-14) for the next two batches of the arrays.
17. Turn on the GeneChip scanner at least 10 minutes before use.
18. Open the 'AGCC Scan Control' application from the 'Affymetrix Launcher'.
19. Load the arrays into the autoloader of the scanner.
20. Click the "Start" icon to initiate the scan. Select the check box "arrays in carousel positions 1÷4 at room temperature". If the arrays are not at room temperature, do not select this option. The scanner will wait 10 minutes before scanning begins to allow the arrays to reach room temperature.
21. Each array takes approximately 7 minutes to scan. The door is locked while the instrument is scanning. Do not attempt to open the door manually.
22. Scan all the arrays after washing and staining.
23. After automatical cleanout procedure remove vials and place the wash lines into a bottle filled with ultra pure water.
24. Using AGCC, choose the Shutdown_450 protocol for all modules. The Shutdown protocol is critical to instrument reliability. When the protocol is complete, turn the instrument off. Empty the waste bottle.
25. When not using the instrument, leave the sample needles in the lowered position. Each needle should extend into an empty vial. This will protect them from accidental damage.
26. Use OncoScan Console 1.3 (20) for quality control (QC) and data summarisation of your results. Download OncoScan Console 1.3 software on www.thermofisher.com and navigate to the following location: Home

→ Life Sciences → Microarray Analysis
 → Microarray Instruments, Software and Services → Microarray Analysis Software.
 Learn how to use this software.

27. Select array type: OncoScan or OncoScan_CNV, select analysis workflow: FFPE Analysis: NA33.
28. In Utility Action configure input sample path, output results path, central QC history path and library file path.
29. Add CELL files for analysis. Click Add CEL Files drop-down and choose AT Channel or GC Channel and find location of your AT CELL files or GC CELL files. Fill the column Result File Name.
30. In output result information box select outputfile folder. And press Submit. The workflow dashboard tab appears and processing begins.
31. After analysis is complete, click View results list. The results appear in the QC results tab.
32. A CELL file value that does not pass its threshold test is indicated with an orange background. The Threshold Test column displays outside bounds and is highlighted in orange.
33. A CELL file value that passes its threshold test is indicated with a blue background. The Threshold Test column displays within bounds and is highlighted in blue.
34. Click Select All to auto-select all your samples.
35. To export your QC results and reports click Generate Report and select Export All Data. Your previously assigned output folder file window appears. Click Save.
36. Discuss subsequent statistical analysis of your results with a statistician.

- Do not store enzymes in a frost-free freezer. Ensure that enzymes are kept at -20 °C until needed. When removed from the freezer, immediately place in a bench top reagent cooler that has been chilled to -20 °C.
- Use only the nuclease-free water that is supplied with the kit. Do not use any other water.
- The reagents are always added to chilled PCR plates (placed on the cold block) throughout the assay.
- Throughout the protocol, when carrying any 96-well plate to and from the centrifuge, carry the plate in the chilled cold block to keep the reactions as cold as possible.
- Spin down plates at room temperature or at 4 °C in a refrigerated centrifuge if available.
- Do not let the plate sit on the cold block for more than 10 minutes at any pre-PCR assay steps.
- Make sure all the required thermal cycler programs are set up on the thermal cycler before starting the assay (OncoScan Anneal, OncoScan Gap Fill, OncoScan 1st PCR, OncoScan 2nd PCR, OncoScan HaeIII, OncoScan Hybridization).
- DNA purified from FFPE blocks must not be eluted in water.
- Sample concentration determined by UV absorbance or NanoDrop must not be used at all in this assay.

IV. Comments

- The OncoScan FFPE Assay Kit is designed to process 24 samples (48 arrays) plus a negative control (the negative control is evaluated in the gel QC steps, but is not hybridized to arrays).
- You can freeze/thaw the reagents in the 24 reaction kit up to 4 times.
- Using positive and negative controls is strongly recommended to assess the performance of each run.

V. Pitfalls

Problem	Cause and cure
Faint or no PCR product visible on the 1st PCR QC gel image in both AT and GC channel for a given sample	• Low input starting material: recheck the quantification for the sample or purify DNA from fresh FFPE sample. • Sample type: sample might contain chemical or enzymatic inhibitors. If so, try cleaning sample over a column and starting assay from the beginning.
Faint or no PCR product visible on the 1st PCR QC gel image in AT or GC channel for a given Sample	• Pipetting error during gel QC: run the first QC gel for both channels again to ensure there was not a pipetting error in loading the gel. • Pipetting error in pre-PCR step: if the repeated gel electrophoresis shows the same faint/no PCR band in a given channel, there was likely a mis-pipetting step after the channel split in the pre-PCR stage. Repeat this sample from the beginning of the assay.
Smeared or multiple bands in QC gel.	• Low input starting material: recheck the quantification for the sample or purify DNA from fresh FFPE sample.
<i>HaeIII</i> gel smeared, no distinct double bands.	• Forgot to pause the thermal cycler at 88 min and pull an aliquot for QC gel: perform <i>HaeIII</i> digestion reaction once again and pause the cycler at 88 min.
Dim array.	• Fluidics stations needs bleach protocol run: dim arrays might indicate that the fluidics stations need the bleach maintenance performed.
The .CEL file is not generated.	• Signal from corner checkerboards is dim: ensure that GeneChip hybridization oven is calibrated and set to the correct temperature. Confirm that stain buffer 1 and stain buffer 2 are placed in the correct order on the fluidics station. Ensure that stain buffer 1 is stored in the dark when not in use. • One or more of the sub-grids in the .dat file image were not gridded by the software: follow the AGCC manual gridding procedure to fix the grids and regenerate the .cel file.

VI. Acknowledgment

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Determination of length of nucleic acid fragments by digital capillary electrophoresis

Lucie Kotková

I. Introduction

The precise size of nucleic acids fragments in the sample is key for many protocols used in a molecular and genetic laboratory - as quality control of DNA for gene panel testing, quality control of sequencing libraries, for PCR amplicon typing, RNA integrity, and degeneration monitoring (1). High-resolution fragment size determination also allows sensitive genotyping of insertions and deletions. Nucleic fragments sizing has an important role in other fields such as microbiology, biochemistry, forensic pathology or food processing industry (1, 2).

A basic method for determination of the length of nucleic acid fragments is polyacrylamide or agarose gel electrophoresis (2, 3). It allows separation of charged molecules in an electric field based on its mobility depending on the total charge and molecule weight. Short nucleic acid fragments move quicker through the gel matrix to the positive electrode and do not linger in the gel pores, while longer fragments are retained (4). In denaturation setting (mostly in the presence of sodium dodecyl sulphate), all molecules obtain a uniform negative charge and the following

separation is based only on the molecule weight. DNA molecules bear uniform negative charge to molecule mass ratio due to the presence of phosphate groups.

Other methods such as mass spectrometry or flow cytometry can be used for size determination of nucleic acids fragments (2). Flow cytometry allows measuring the length of every DNA molecule through the use of intercalating fluorescent dyes selectively binding to DNA – fragment length is determined based on fluorescence intensity. Both mass spectrometry and flow cytometry are more precise and faster compared to gel electrophoresis, but they are restricted to smaller fragments, more expensive, and less accessible in laboratories.

Although gel electrophoresis was long considered a gold standard (3), it is very time-consuming to perform, which limits its applications in dynamically developing high-throughput and automatized methods. Capillary electrophoresis mostly overcomes this disadvantage. It does not require tedious gel preparation, the analysis itself is significantly faster due to an application of high voltages and

sample requirements are lower (2, 3). Digital capillary electrophoresis can analyze up to 96 samples in one run without manual intervention and analysis of one to twelve samples takes only a few minutes depending on the chosen method and resolution (when using QIAxcel Advanced instrument, 6).

Due to digital processing and data analysis, no additional manipulation with gels and potentially dangerous chemicals during colouring, visualization and photographing of gels is needed. Hazardous waste handling is reduced as well.

Size determination of DNA fragments as quality control or for amplicon typing is described below.

II. Materials and instrumentation

Materials

- QIAxcel kit
 - QIAxcel DNA High Resolution kit (QIAxcel DNA High Resolution Cartridge) Cat No: 929002, Qiagen
 - QIAxcel DNA Screening kit (QIAxcel DNA Screening Cartridge) Cat No: 929004, Qiagen
 - QIAxcel DNA Fast Analysis kit (QIAxcel DNA Fast Analysis Cartridge) Cat No: 929008, Qiagen
 - QIAxcel RNA QC Kit v2.0 (QIAxcel RNA QC Gel Cartridge) Cat No: 929104, Qiagen
- QX Wash Buffer
Cat No: 929604, Qiagen
- QX Mineral Oil
Cat No: 929605, Qiagen
- QX DNA Separation Buffer
Cat No: 929603, Qiagen
- QX DNA Alignment Marker
Cat No: 929520, 929521, 929522, 929523, 929524, 929526, 929529, 929530, Qiagen
- QX DNA Size marker
Cat No: 929554, 929559, 929560, 929561, 929562, 929563, Qiagen
- QX DNA Dilution Buffer

Cat No: 929601, Qiagen

- QX RNA Separation Buffer
Cat No: 929603, Qiagen
- QX RNA Alignment Marker
Cat No: 929510, Qiagen
- QX RNA Size Marker
Cat No: 929580, Qiagen
- QX RNA Dilution Buffer
Cat No: 929602, Qiagen
- PCR strips and tubes 0,2 ml
Cat No: 929703, Qiagen
- tube racks
- nuclease-free PCR water for molecular biology. Cat No: 129114, Qiagen
- pipettes 5 µl – 5 ml and pipette tips

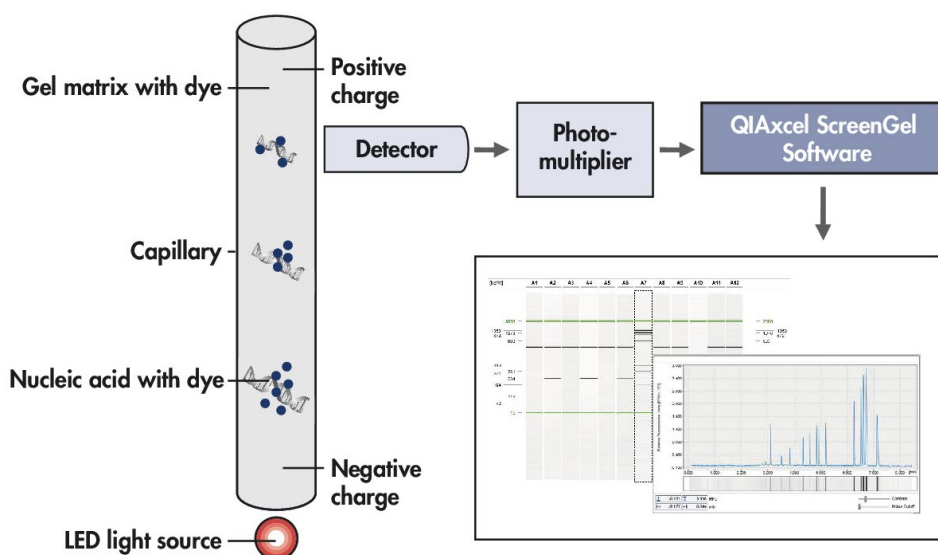
Instrumentation

- QIAxcel Advanced Instrument
Cat No: 9001941, Qiagen
- QX Nitrogen Cylinder
Cat No: 929705, Qiagen
- postPCR level laminar flow box
- centrifuge

III. Procedures

QIAxcel Advanced instrument contains an array of light-emitting diodes and micro-optical collectors that latch to QIAxcel cartridge containing twelve capillaries filled with the gel matrix.

Less than 0.1 µl of the sample is drawn to the cartridge, which reduces the sample consumption and leaves more sample for another downstream analysis. After the electric current application, nucleic acid fragments migrate through the gel matrix in the capillaries based on its size toward the positively charged terminus. Fragments pass excitation and detection spots. A signal from a detector is transmitted through a photomultiplier tube to the QIAxcel ScreenGel Software, where it is converted to an electropherogram and virtual gel image available for data interpretation.



Scheme 50.1.

Reagent preparation

Prepare new reagents every month. Store prepared reagents at 4 °C.

Buffer tray preparation

1. Wash the buffer tray using mild detergent and rinse it with deionized water.
2. Fill the buffer tray (Fig. 50.1)
 - 8 ml of QX Wash Buffer to WP position
 - 8 ml of QX Wash Buffer to WI position
 - 18 ml of QX DNA Separation Buffer to BUF position
3. Add QX Mineral Oil to prevent evaporation of buffers.
 - 2 ml to WP position
 - 2 ml to WI position
 - 4 ml to BUF position

Alignment marker preparation

1. Pipette 15 µl of proper QX DNA Alignment marker into each well of a 0.2 ml 12 tube strip. Add a drop of QX Mineral Oil to each well to prevent evaporation.

Size marker preparation

1. Prepare QX DNA Size marker in the 0.2 ml tube in a minimum volume of 10 µl.
2. Size markers are provided in concentration 100 ng/µl. It is possible to dilute them using QX DNA Dilution buffer to concentration 20, 10, 5, and 1 ng/µl.

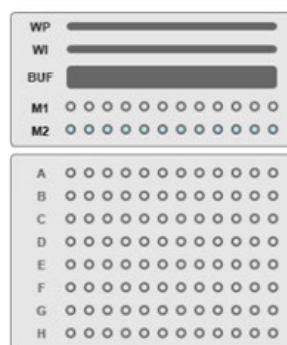


Figure 50.1. Buffer tray and sample holder.

Instrument preparation

1. Let all reagents and gel cartridge equilibrate to room temperature.
2. Open QIAxcel Screen Gel software.
3. With the sample door closed, move the buffer tray holder forward using the "Load position" button (Fig. 50.2) and load the buffer tray. Load tube strip with alignment markers to M1 position (Fig. 50.1).
4. Close sample door and move buffer tray holder back using the "Park position" button (Fig. 50.2).
5. Take the gel cartridge case and insert it to the instrument with the description label facing towards. Insert the smart key into the socket near cartridge. Close the cartridge door carefully, otherwise the cartridge will not be detected by the instrument and properly latched.

Notes: Gel cartridge must be stored in a vertical position at $2 \div 8^\circ\text{C}$ (temperature below 2°C can damage the cartridge). Capillary tips must be placed in the storing gel or submerged in QX Wash buffer to prevent drying out.

When removing the cartridge from the instrument, release it using the "Unlatch" button (clicking sound will be heard), remove the smart key from the socket, remove the cartridge, and return it to the cartridge case.

Setting the run

It is necessary to analyze whole 12-tube strip (11 samples and the size marker at a minimum). In case of fewer samples, load unused wells with 10 μl of PCR water or QX DNA Dilution Buffer.

1. Prepare the samples into the 0.2 ml tube strip in a minimum volume of 10 μl . High concentration or rare samples may be diluted using QX DNA Dilution buffer. Before the run, remove all air bubbles from the sample to prevent gel cartridge damage.
2. Load samples to buffer tray holder lines A-H.
3. Set the instrument in the "Process" interface (Fig. 50.2):
 1. In the "Process Profile" screen, use "Default High Res v2.0".
 2. In the "Run Parameters" screen (Fig. 50.3)

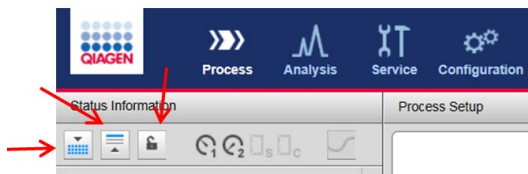


Figure 50.2. QIAxcel Screen Gel software. Red darts show (from the left) "Loading position", "Park position" and "Unlatch" buttons, in the upper part is the software interface selection.

- Left-click to mark the lines with samples loaded.
 - Right-click to mark the well with size marker loaded.
 - Set a proper analysis
 - Depending on the expected concentration of analyzed DNA
 - H – DNA concentration $> 100 \text{ ng}/\mu\text{l}$
 - M – DNA concentration $10 \div 100 \text{ ng}/\mu\text{l}$
 - L – DNA concentration $< 10 \text{ ng}/\mu\text{l}$
 - Depending on the needed resolution (Tab. 50.1)
3. In the "Analysis" screen, use "smear analysis" for analyzing fragmented DNA.
 4. In the "Marker" screen, set selected size and alignment marker.
 - Choose the closest size marker to the expected size of the sample DNA (sample DNA must be smaller than the biggest fragment of the size marker).
 - Choose alignment marker to cover all peaks of the size marker (size of the smallest and biggest peaks cannot be equal).
 - See recommended combinations in the Tab. 50.2. For analyzing of fragments with size around 200 bp (e.g. BRCA 1/2 testing) or sequencing libraries size marker $25 \div 500 \text{ bp}$ and alignment marker $15 \div 600 \text{ bp}$ is suitable.
 5. In the "Sample selection" screen, check positions of samples and size marker and correct it if needed.
 6. In the "Sample information" screen, write sample identifications.
 7. In the "Run check" screen, check and tick all notices regarding sample position, size marker and alignment marker. If no error or alert is displayed, start analysis pressing the "Run" button.

Notes: If some part of the interface turns yellow during the setting of the run, input data are incompatible. Most commonly they have incompatible sizes, wrong alignment marker, or wrong process profile.

If the whole alignment marker was already visualized on the gel, it is possible to shorten remaining analysis up to two minutes using "Separation time adjustment" bar. The same

way may be used to add up to two minutes to the analysis if alignment markers are not fully visualized.

It is necessary to let the analysis finish. If it was ended too early, data will be lost. Samples drawn into the gel cartridge that had not enough time to pass the detector can devalue the following analysis.

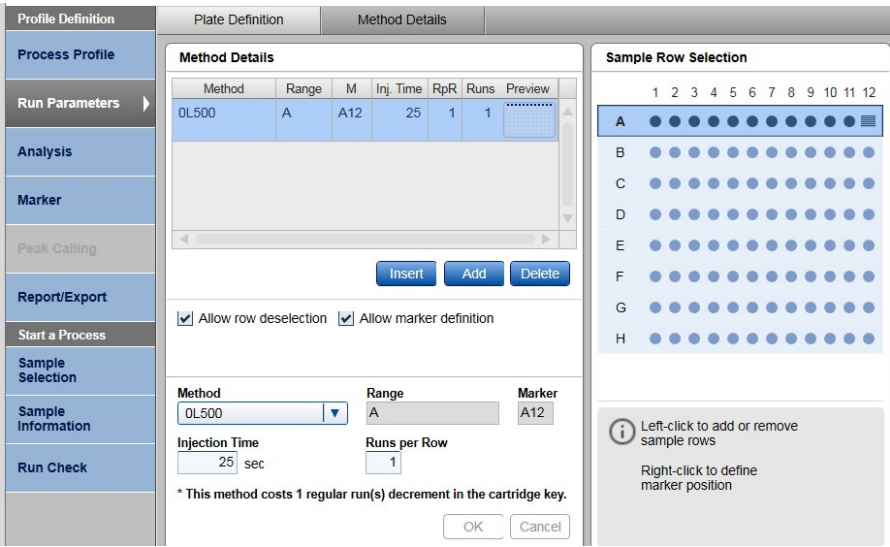


Figure 50.3. Setting the parameters of the run.

TABLE 50.1. Selection of optimal method depending on needed resolution.

Method	Fragment size			
	100–500 bp	500 bp – 1 kb	1–5 kb	5–10 kb
Best resolution				
OM400 OL400 OH400	20 bp	100 bp	500 bp	N/A
OM500 OL500 OH500	10 bp	50 bp	200 bp	N/A
OM800 OL800 OH800	3–5 bp	N/A	N/A	N/A
OM1200 OL1200 OH1200	N/A	N/A	500 bp – 1 kb	1–1.5 kb

TABLE 50.2. Recommended combinations of size and alignment marker.

Size marker	Cat. no.	Alignment marker	Cat. no.
QX DNA Size Marker pUC18/ <i>Hae</i> III (50 µl)	929550	QX Alignment Marker 15 bp/1 kb (1.5 ml)	929521
QX DNA Size Marker FX174/ <i>Hae</i> III (50 µl)	929551	QX Alignment Marker 15 bp/3 kb (1.5 ml)	929522
QX DNA Size Marker 25 bp – 500 bp (50 µl)	929560	QX Alignment Marker 15 bp/600 bp (1.5 ml)	929530
QX DNA Size Marker 100 bp – 2.5 kb (50 µl)	929559	QX Alignment Marker 15 bp/3 kb (1.5 ml)	929522
QX DNA Size Marker 50–800 bp (50 µl) v2.0	929561	QX Alignment Marker 15 bp/1 kb (1.5 ml)	929521
QX DNA Size Marker 250 bp – 4 kb (50 µl) v2.0	929562	QX Alignment Marker 50 bp/5 kb (1.5 ml)	929529
QX DNA Size Marker 250 bp – 8 kb (50 µl) v2.0	929563	QX Alignment Marker 15 bp/10 kb (1.5 ml)	929523

Data analysis

1. In left part of the "Analysis" interface, choose recently finished run, mark wells containing samples and drag it to the middle of the screen.
2. In the "Electropherogram" screen check the quality of the size marker. If the size scale could not be generated, redundant peaks must be removed manually (Fig. 50.4). Drag the dark blue horizontal line up to raise the baseline and embody redundant peaks into the noise. If this is not enough, mark the redundant peaks in the variant "Peak result" in the lower part of screen, right-click to open options and use "Delete selected peaks" (Fig. 50.5).
3. After redundant peaks removal, correct size of size marker fragments will be

calculated. In the upper part, choose the "Reference marker" tab and choose Apply (Fig. 50.6). Mark all samples, in the right part of the screen, choose the "Analysis" tab (most commonly Default DNA v2.0 or Default Smear Analysis) and click the "Start analysis" button.

Note: Marked samples are only those highlighted in blue. A number of marked samples is seen near the "Start analysis" button. The same applies later when exporting a report.

4. Alignment marker may be marked incorrectly at some samples (Fig. 50.7). In this case, it is necessary to manually remove all peaks before the real alignment marker peak the same way as at the incorrect size marker.
5. When working with low concentration

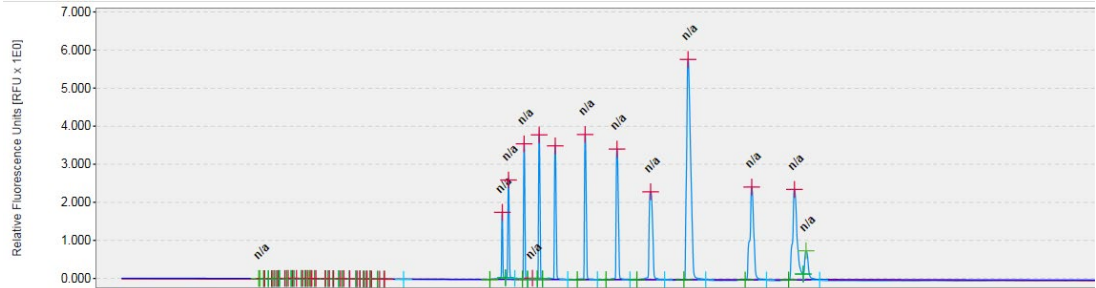


Figure 50.4. Electropherogram of improperly read size marker.

samples, it is possible to lower baseline by manually dragging the dark blue horizontal line down.

- When analyzing smear, it is possible to drag vertical purple lines from side to side to mark an area of interest, where from the

concentration of sample will be calculated.

- After completing the analysis, mark all samples, choose the "Report" tab in the right part of the screen, choose all needed parameters and generate a report pressing the "Start Report/Export" button.

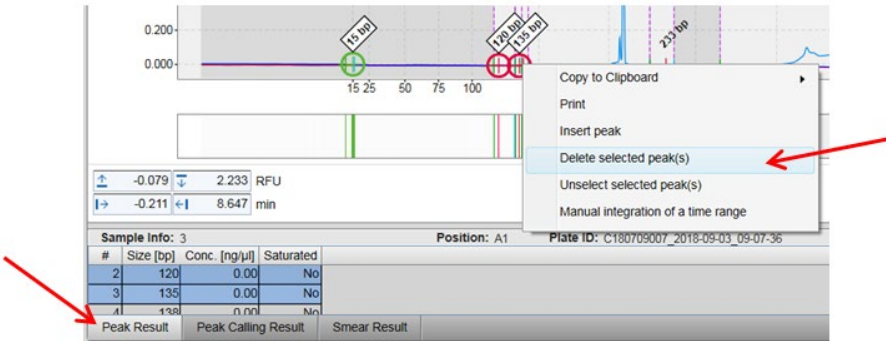


Figure 50.5. Manual removal of the redundant peaks.

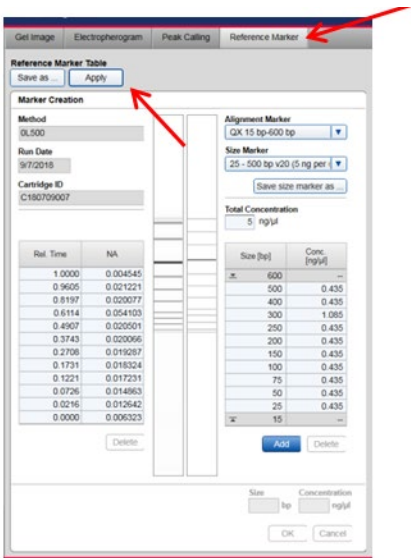


Figure 50.6. Editing of size marker.

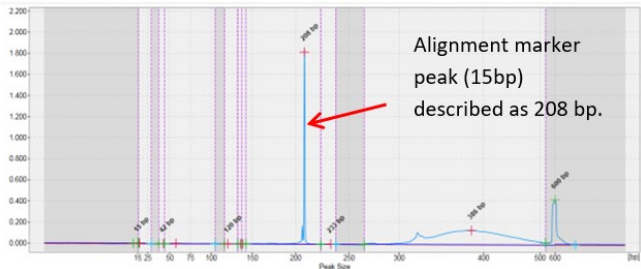


Figure 50.7. Electropherogram of improperly described alignment marker. Alignment marker peak of size 15 bp is described as 208 bp product peak due to redundant peaks before it. Measured size of the real product is wrong

IV. Comments

QIAxcel methods can reach a resolution of 3÷5 bp with use of DNA High-Resolution kit for fragments smaller than 0.5 kb. For applications with the lower required resolution, QIAxcel kits with faster and less expensive analysis are available (Tab. 50.3).

TABLE 50.3. QIAxcel Kit specifications

QIAxcel Kit	Size range	Best resolution				Run time for 12 samples [†]
		100÷500 bp	500 bp ÷ 1 kb	1÷5 kb	5÷10 kb	
QX DNA High Resolution Kit	15 bp÷10 kB	3÷5 bp	50 bp	200÷500 bp	1÷1.5 kb	7÷20 min
QX DNA Screening Kit	15 bp÷5 kB	20÷50 bp	50÷100 bp	500 bp	-	5 min
QX DNA Fast Analysis Kit	15 bp÷3 kB	50÷100 bp	100÷250 bp	250 bp÷1 kb*	-	3÷5 min
QX RNA QC Kit v2.0	15 bp÷6 kB	-	-	-	-	10 min

V. Pitfalls

Problem	Cause and cure
Gel cartridge does not latch	› Smart key is not inserted properly. › Lack of N₂ → change the N₂ cylinder (if N₂ cylinder lasts only couple of days, make sure it is tight and if problem lasts, contact Qiagen technical services).
Run cannot be started	› Some of set data are incompatible, check the Process interface (see Setting run part)
Broad or double peaks	› Prepare new reagents › Perform purge or long purge program of the gel cartridge in the "Service" interface
Size scale could not be calculated	› Check the size marker and delete redundant peaks (see Data analysis part)

Weak alignment marker bands	› Alignment marker is old and degraded → prepare new reagents
Small alignment marker peak	› Alignment marker peak was improperly marked, delete all redundant peaks before alignment peak (see Data analysis part)
Too low sample signal	› Too low DNA concentration → choose "L" method and increase injection time in the "Run parameters" screen › Too high salt concentration in the sample → desalt or dilute sample

VI. Acknowledgment

This work was supported by The Ministry of Education, Youth and Sports, project number LO1304 - Support of the sustainability of the Institute of Molecular and Translational Medicine.

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Bisulfite amplicon next-generation sequencing for methylation status assessment

Rastislav Slavkovský

I. Introduction

Methylation of DNA is a basic property of DNA connected to regulation of gene expression and chromatin structure. Typically, methylation in promoter area of a gene (near transcription site) acts as a repressor of gene transcription. DNA methylation is important during normal development and is associated with a number of processes including genomic imprinting, X-chromosome inactivation, and repression of transposable elements, aging, and carcinogenesis (1).

In mammals, cytosine is methylated most frequently within CG (also known as CpG) sites (Fig.51.1); in plants and other organisms methylation also in CHG and CHH (H=A, T, or C) sites was observed.

DNA methylation can be measured genome-wide by assays such as whole genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), probe based capture Methyl-Seq, methylated DNA immunoprecipitation (MeDIP), and methyl CpG capture based method. Also, small region or single site targeted methods such as methylation

specific PCR (MSP), bisulfite amplicon sequencing (BSS) using Sanger, pyro- or next-generation sequencing can be used. The latter provides the most flexibility when several sites are in question and many samples need to be analysed. In this chapter, will be described next-generation amplicon sequencing approach using most widespread sequencing platform – Illumina SBS. Amplicon bisulfite sequencing is quite straightforward (Scheme 51.2). When specific primers are ready, it takes usually 3 days to get from DNA to results.

II. Materials and instrumentation

- Personal sequencer (Illumina MiSeq, MiniSeq, NextSeq, or iSeq 100)
- PCR thermocycler (ideally with temperature gradient and fluorescence detection)
- Electrophoresis system (e.g. Agilent 2100 bioanalyzer or QIAGEN QiaXCEL)
- Spectrophotometer or fluorometer for DNA concentration measurement
- Laboratory magnetic stand for separation

of magnetic beads in 96 well plates (e.g. Magnetic-Ring Stand 96 well - AM10050, Invitrogen/ThermoFisher)

- Basic laboratory equipment including centrifuges, pipets, tips.
- BSS primer pairs (e. g. Sigma-Aldrich, Generi-Biotech).
- Nextera XT DNA library preparation kit (Illumina, FC-131-1024 or 1096).
- Nextera XT Index Kit v2 Set A (96 indexes, 384 samples, FC-131-2001 or similar)
- EZ DNA Methylation Gold Kit (Zymo Research, cat. no. D5005 or D5006)
- Human Methylated & Non-methylated DNA Set (Zymo Research, D5014-2)
- KAPA2G Robust HotStart PCR Kit (MERCK, 2GRHSKB or KK5522 SKU)
- Ampure XP or QIAGEN, QIAquick PCR Purification Kit
- Ethanol (>96%) molecular biology grade.

III. Procedures

Steps

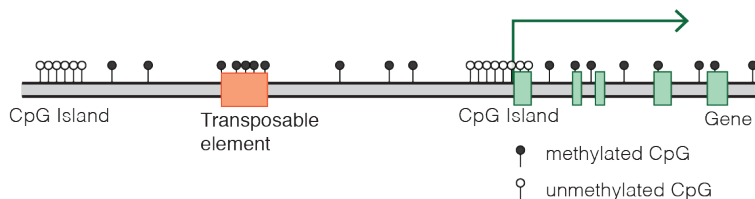
A. Primer design

1. Choose regions of interest. UCSC genomebrowser (<https://genome.ucsc.edu/cgi-bin/hgTracks>) or Integrative Genomics Viewer (IGV, <http://software.broadinstitute.org/software/igv>) could be of special use when searching target regions. Obtain

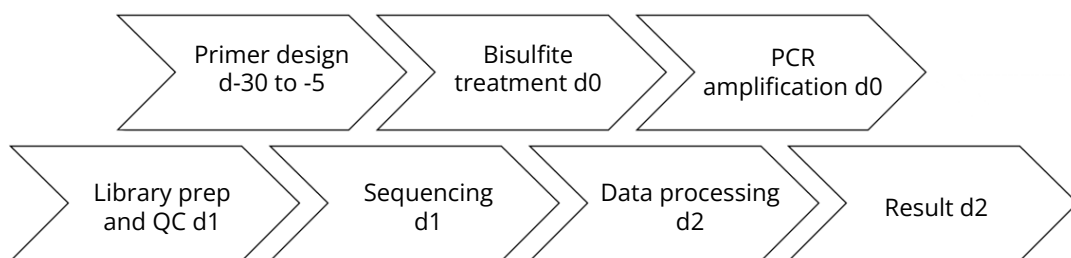
chromosomal coordinates of the region.

2. Obtain the raw sequence using web browser and link <http://genome.ucsc.edu/cgi-bin/das/hg19/dna?segment=chrN:start,stop> or <http://genome.ucsc.edu/cgi-bin/das/hg38/dna?segment=chrN:start,stop> where N is replaced with chromosome number and start, stop are coordinates of start and end of region, respectively.
3. Copy and paste sequence to a primer design software supporting BSP (bisulfite sequencing PCR) primers design e.g. MethPrimer2 <http://www.urogene.org/methprimer2/>, that supports variety of tuneable parameters (2) or Bisulfite Primer Seeker 12S (<https://www.zymoresearch.eu/bisulfite-primer-seeker-that-supports-just-basic-parameters>).
4. Set required parameters for primer design, notably target regions. Avoid designing primers with product size > 300 due to the fragmentation of DNA during BS treatment. Use product size < 250 especially when dealing with FFPE sample. Use degeneracy for primer selection when it is not possible to design proper primers due the abundant presence of CpG sites. Try to design at least 2-3 primer sets to the region of interest. Usually one third of primers have low performance. Primers for BSS are usually 5 bp longer (25÷30 bp) than usual primers due to lower CG content after BS treatment.

Typical mammalian DNA methylation landscape



Scheme 51.1.



Scheme 51.2.

B. Bisulfite treatment

1. Use EZ DNA Methylation Gold Kit for DNA bisulfitation. Please consult also the protocol supplied by the manufacturer (ref. 3).
2. Prepare fresh CT Conversion reagent mix by mixing the CT Conversion Reagent (a solid mixture) with 900 µl water, 300 µl of M-Dilution Buffer, and 50 µl M to original tube of CT Conversion Reagent.
3. Mix at room temperature with frequent vortexing or shaking for 10 minutes.
4. Add 4.8 ml of 100% ethanol to the 1.2 ml M-Wash Buffer concentrate.
5. Prepare 500 ng of DNA to PCR tube (20 µl @ 25 ng/µl DNA).

Note: This amount is sufficient usually for 25 PCR reactions per sample. Lower the input accordingly if lower number of PCR reactions is required. It is advisable to prepare also larger amount of bisulfited DNA from a sample for optimization of PCR reaction, especially when dealing with novel primer pairs. Use an optimization sample with larger amount of DNA available with similar characteristic as other experimental samples. If necessary, prepare multiple aliquots of bisulfite optimization DNA and mix them before use. It is advisable to measure DNA concentration using fluorescence based method. Especially DNA derived from FFPE samples overestimates concentration when using spectroscopic method in comparison to fluorescence based one. This artefact is most often caused by RNA presence in the sample and/or contamination with organic solvent.

Prepare also Universal Methylated Human DNA Standard (Zymo Research) and Universal

Non-methylated Human DNA Standard (Zymo Research) to check quality of the overall procedure.

6. Add 130 µl of the CT Conversion Reagent to 20 µl of your DNA sample in a PCR tube. Mix the sample by pipetting the sample up and down, and then centrifuge shortly the tube.
7. Place the sample tube in a thermal cycler and perform the following steps:
 - 98 °C for 10 minutes
 - 64 °C for 2.5 hours
 - 4 °C hold for up to 20 hours
8. Add 600 µl of M-Binding Buffer to a Zymo-Spin™ IC Column and place the column into a provided Collection Tube.
9. Load the sample into the Zymo-Spin™ IC Column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.
10. Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.
11. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.
12. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20 °C ÷ 30 °C) for 15÷20 minutes. After the incubation, centrifuge at full speed for 30 seconds.
13. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of M-Wash Buffer and centrifuge for an additional 30 seconds.
14. Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA. The DNA is ready for immediate analysis or

can be stored at or below -20 °C for later use. For long term storage, store at or below -70 °C. The elution volume can be > 10 µl notably when multiple PCR are planned. Usually 5 -20 ng should be used for PCR reaction.

15. Calculate theoretical DNA concentration according to the original DNA input and used elution volume.

C. Bisulfite sequencing PCR amplification

Note: DNA polymerase quality requirement is increased in this type of PCR reaction due to the specific properties of BS treated DNA and primers. Use robust polymerases such as KAPA2G Robust (Merck), Platinum Taq DNA Polymerase (Thermo-Scientific), EpiMark® Hot Start Taq DNA Polymerase (NEB) or ready to use polymerase master mixes Pyromark PCR Kit or HotStarTaq Plus Master Mix Kit or other polymerase of similar quality.

Note: In this example the mixing of mastermix for 96 PCR amplification is used. In total 12 biological samples is analysed for 8 genomic regions/targets each.

1. Prepare 8 primer mixes (PM) for each 12 reactions by mixing 10 uM primers (~4% safety margin) according to the Tab. 51.1.

Note: Increase the safety margin up to 10% if required.

2. Prepare mastermix (MM) for 100 reactions (~4% safety margin is included) according to Tab.51.1.
3. After mixing PCR buffer with MgCl₂, dNTP, polymerase, EvaGreen, and PCR water, vortex and spin shortly in 2 ml tube.
4. Prepare primer containing mastermix (PRMM1÷8) by distributing prepared mastermix to 8 tubes by 213 µl and add 19.6 µl of different primer mix to each tube. Vortex and spin shortly.
5. Distribute each PRMM to a separate line in PCR plate using 19 µl volumes.
6. Add 1 µl of converted DNA sample to each well of the column in PCR plate 10 ÷ 20 ng of DNA input is recommended.
7. Total volume of PCR reaction is 20 µl in each well.

8. Seal the plate and run the program in Tab.51.2 (* Annealing temperature 60 °C usually works. For QIAGEN mastermixes usually 55 °C ÷ 56 °C works well. If gradient PCR thermocycler is available for proper optimization, use PCR gradient experiment for each primers set in range 50 °C ÷ 66 °C beforehand with one selected optimization sample. Please change PCR program accordingly when using alternative polymerases. ** Do not use melting curve program when not using PCR thermocycler with fluorescence detection.)
9. Check the quality of PCR amplification using melting curve analysis and/or DNA electrophoresis.

Note: Check the quality and size of bands especially when using primer pairs for the first time. Presence of primer-dimer or non-specific products is not critical if also the specific band is present due to the fact that those artefacts will not be aligned properly to the regions of interest during further bioinformatics data analysis.

10. Pool the amplicons from same sample within each column and purify using SPRI select, Ampure XP beads, QIAquick PCR Purification Kit - (QIAGEN).

Note: It is advisable to pool PCR products according to semi-quantitative intensity of bands in gel or chip electrophoresis. For example: Pool 10 µl of amplicon for low intensity bands, 5 µl for medium and 2 µl for low intensity bands. Protocol for capillary gel electrophoresis using QIAGEN QIAxcel procedure is not provided in this protocol (please consult chapter 50). Also please consult protocols for your electrophoresis system and purification kit. Pooling and purification described in example presented in this chapter is optimized for 12 samples.

11. Measure the DNA concentration of each sample pool.

Note: Expected yield is ~ 20÷50 ng/µl when pooling 8 amplicons to ~ 40 µl pool and using 30 µl elution volume.

TABLE 51.1. Mastermix example for BSP amplification of converted DNA for 1 and/or 100 reactions. * as calculated from original input.

Reagent	Stock conc.	Final conc.	1 rxn [μl]	100 rxn [μl]
PCR buffer A (contains MgCl ₂ for 1.5 mM final concentration)	5x	1x	4	400
MgCl ₂ (additional)	25 mM	1 nM (from MgCl ₂ add.) + 1.5 nM (from buff.)	0.8	80
dNTP	25 mM	0.25 mM	0.2	20
Polymerase KAPA 2G ROBUST	5 U/μl	0,5 U/r	0.100	10
EvaGreen 20x (optional)	20x	0.5x	0.5	50
PCR H ₂ O			11.80	1180
Total MM (to be separated to 8 tubes)			17.4	1740
MM volume per one target (4% safety margin)				12 rxn [μl]
MM volume per one target (2% safety margin)				217.5
Primer FW + RW per one target	10 μM+10 μM	0.4 μM+0.4 μM	1.6	213.0
DNA		~10 - 20 ng/μl*	1	19.6
Total (MM + PM + DNA)			20	

D. Library preparation using tagmentation and QC

1. Please consult also original documentation for Nextera XT DNA library kit (Illumina, ref. 4)
2. Dilute the DNA on the concentration 0.5 ng/µl (range 0.25 - 0.5 ng/µl is allowed) Use following dilution chart to simplify DNA dilution (pool concentration: dil. factor: dil. volumes):
 - 6- to 12.5 ng/ul : 25x : 4 µl pool + 96 µl H₂O
 - up to 25 ng/ul : 50 x: 2 µl pool + 98 ul H₂O
 - over 25 ng/ µl : 100 x : 1 µl pool + 99 µl H₂O
3. For each sample add following from Nextera XT kit to new well of PCR plate or strip: 5 µl Tagment DNA buffer 2.5 µl amplicon pool DNA sample (0.25÷0,5 ng/µl) 2.5 µl ATM mix
4. Mix carefully by pipetting up and down and

TABLE 51.2. Universal BSP program.

Program	Time	Temperature [°C]	
polymerase activation	3 min	95	
denaturation	15 s	95	
annealing	30 s	55-62 *	50 cycles
elongation and signal capture	45 s	72	
final elongation	5 min	72	
melting curve program		60 -> 95 **	
cooling	5 min	60	

TABLE 51.3. Combination table of indexes (bold numbers) and samples. Eg. sample S14 has indexes i5 = 6 and i7 = 2.

	1	2	3	4
1	S1	S9	S17	etc.
2	S2	S10	S18	
3	S3	S11	S19	
4	S4	S12	S20	
5	S5	S13	S21	
6	S6	S14	S22	
7	S7	S15	S23	
8	S8	S16	S24	

spin shortly.

5. Incubate the PCR strip or PCR plate in thermocycler at 55 °C for 5 min then set to cool immediately at 10 °C until ready to process
6. Add 2.5 µl NT buffer (kit Nextera XT) to neutralize tagmentation reaction. Mix carefully by pipetting up and down and spin shortly.
7. Incubate the strip or plate for 5 min in room temperature.
8. For library PCR amplification add to tagged DNA (total volume = 12.5 µl) the following reagents:
 - 7.5 µl NPM (PCR master mix from Nextera XT kit)
 - 2.5 µl index primers i5
 - 2.5 µl index primers i7 and mix carefully by pipetting up and down and spin shortly.

Note: Transfer index primers to strips beforehand and use multichannel pipette when dealing with high number of samples. Indexes i5 are pipetted from left to right to every column. Indexes i7 are pipetted from top to bottom to every line (Tab.51.3). Carefully note which index were used for each sample.

9. Transfer tubes to a thermocycler and run following program:
 - 72 °C / 3 min
 - 95 °C / 30 s
 - 12 ÷ 16 cycles:
 - 95 °C / 10 s
 - 55 °C / 30 s
 - 72 °C / 30 s
 - 72 °C / 5 min, hold 10 °C

10. Purify each sample separately using QIAquick PCR Purification Kit or SPRI select magnetic beads and quantify the DNA concentration using Qubit HS or similar fluorometric method. PCR product has the volume approximately 25 µl.

Note: For experienced user it is possible to pool and co-purify all samples together in one spin column or well, supposing only small variability in sample DNA concentrations.

E. Preparation for sequencing and sequencing

Sequencing libraries should appear as smear in region 170 bp ÷ 400 bp. Usual average size is around 230 bp ÷ 250 bp.

1. Calculate molarity of DNA pool library from mass concentration assayed from Qubit HS using formula $c \text{ [nM]} = \frac{\rho_i \text{ [ng/}\mu\text{l]} \cdot 106 / (660 \cdot 230)}{\rho_i \text{ [ng/}\mu\text{l]} \cdot 6.6}$ where:
 - ρ_i is mass concentration of DNA
 - 230 is approximate average size of DNA molecule in bp.
 - 660 g/mol is average molar weight of one base (1 bp).
2. Dilute the pooled library to 2 nM concentration.
3. Calculate the required capacity for sequencing.

Note: Capacity (C, bp) = samples (n) x amplicons(m) x required depth (D) x average

TABLE 51.4. Example of multiple libraries pooling using 300-cycle MiSeq Reagent Kit v2 with typical capacity of 5 Gb.

Library	Required capacity	Percentage	Library volume for pool µl
Library 1	2.5 Gb	50 %	10
Library 2	2.25 Gb	45 %	9
Library 3	0.25 Gb	5 %	1
Total	5 Gb	100 %	20

amplicon size (A). E.g. for $n = 12$, $m = 8$, $D = 10000$, $A = 250$: $C(\text{bp}) = 12 \times 8 \times 10\,000 \times 250 = 240\,000\,000$ bp; $C(\text{Gb}) = 0.24$ Gb. 300-cycle MiSeq Reagent Nano Kit v2 sequencing flow cell would provide enough capacity for this pool (capacity = 0.3 Gb).

4. If other libraries are required to be sequenced, dilute also other libraries to 2 nM and pool them in ratio required for sequencing according to the example in Tab.51.4.
5. Denature DNA using freshly prepared 0.2 M NaOH.
6. Add 10 µl of denatured pool and 990 µl HT1 to obtain 10 pM dilution of sequencing library.
7. Apply sample to sequencing flow cell.
8. Prepare sample sheet and run sequencing experiment at Illumina Sequencer.

Note: As the tagmentation is used, fragmentation of DNA is present and the random fragments are sequenced. Pair-end read or single-end read with two indexes is supported. Read length from 50 to 150 bp is possible.

F. Data processing and visualization

1. Locate the fastq files in the data folder of your sequencer or download the FASTQ files using Illumina BaseSpace online service. For MiSeq use path `D:\Illumina\MiSeqOutput\<date_and_run_identification> \Data\Intensities\BaseCalls\`.
2. Align obtained fastq files using Bismark tool (5) and Bowtie2. Note: Consult www.bioinformatics.babraham.ac.uk/projects/bismark for the documentation to Bismark. Command line example: `bismark --bowtie2 --multicore 2 --non-directional -p 4 --path_to_bowtie /path/to/bowtie/bowtie2-2.2.8-n1-l128 /path/to/reference_sequence/ -1 filenameR1_001.fastq.gz -2 filenameR2_001.fastq.gz`.

When running Bismark for the first time prepare reference genome using command `bismark_genome_preparation --path_to_bowtie /usr/bin/bowtie2/ --verbose /path/to/ /reference_sequence`

3. Extract methylation calls using Bismark methylation extractor.
Command line example: `bismark_methylation_extractor -p --multicore 10 --merge_non_CpG aligned_data_files.bam`. Bismark_methylation_extractor script operates on Bismark result files and extracts the methylation call for every single C analysed. The position of every single C will be written out to a new output file, depending on its context (CpG, CHG or CHH), whereby methylated Cs will be labelled as forward reads (+), non-methylated Cs as reverse reads (-).
4. Evaluate and visualize methylation status data using Seqmonk. Note: SeqMonk is available under the GNU General public license. Download the software at <https://www.bioinformatics.babraham.ac.uk/projects/download.html#seqmonk>.
5. Run SeqMonk software and prepare new project (File → New or Ctrl-N) and select reference genome. Use the same version as used for alignment.
6. Import the .txt data output file from bismark_methylation_extractor. Follow this sequence at top menu: File → Import data → Text (Generic). Fill the data import option with description as shown in Figure 51.3.
7. Import annotation of your regions of interest (e.i amplicons) using tab or comma delimited text file e.g "coordinates_regions.txt" with following columns: chromosome, start, stop, region(gene) name. These regions are now denominated as features.
8. Search coordinates of your region of interest using Goto Position icon (Ctrl-G) or search gene of the interest using Find Features icon from top menu (or Ctrl-F).
9. Generate single nucleotide probes using "Read Position Probe Generator" (Data → Define Probes → Read Position Probe Generator). Select all your samples holding shift. Modify parameters according to your experimental needs and press Run Pipeline. Do not modify "Valid positions per window" parameter from default value 1, if single base resolution is required. After probes are generated close "Define Quantitation" window. Probes in this case are areas of interest at the position of C nucleotide from CpG.

10. Quantify methylation level using pipeline selected from top menu: Data → Quantitation pipelines → Bisulphite methylation over features.

- Mode A. for single nucleotide resolution quantification choose value [Existing probes] in menu "Feature to quantify". Example of result is shown in Figure 51.5.

Note: Probes need to be generated beforehand using ReadPosition Probe Generator in menu "Feature to quantify".

- Mode B. for quantification of custom regions of interest, choose the name of your region file, e.g. "coordinates_regions.txt" in menu "Feature to quantify". For both modes, modify parameters according to your experimental needs and press "Run Pipeline".

Note: Please note that using quantification mode B, any artefacts data would be included in quantification. It is recommended to inspect data using mode A beforehand. Mode A and mode B cannot be displayed simultaneously.

11. Filter the artefact using top menu Filtering-> Filter on values -> Individual probes. Select analysed samples while holding the shift key. Value must be between 0 and 100 for at least Y of the X selected samples. Y/X should be at least 50% e.g. 6 out of 12. Increase the ratio value for more stringent filtering. This filtering will filter out the probes (CpG positions) with high ration of missing data with sample cohort. Adjust parameters according your data quality and experimental requirement. Press Run Filter and name the list.

12. Change visualization of data using <Data Track Display Option> button or select View → Data Track Display from the top menu. Options that were used for visualization of figures 51.6 and 51.7 are displayed in Figure 51.8.

13. Generate quantification report using top menu Reports → Feature report. Select Annotate with "coordinate_regions.txt" with "any overlapping" probes and "Include" features with no probes. Note: This quantification uses all the valid probes. Probes that were filtered out will not be used

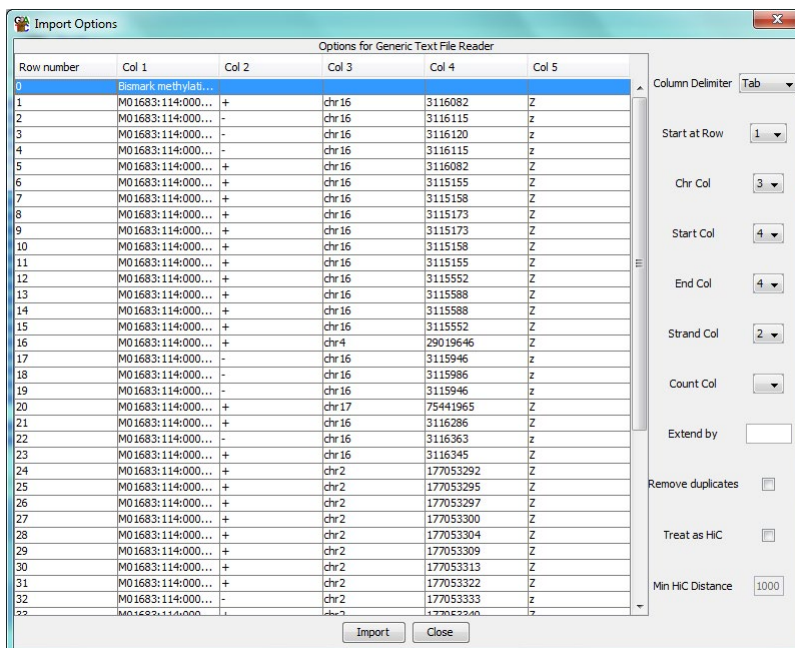


Figure 51.3. SeqMonk data import options.

for quantification. Each defined regions will be quantified using all the data present in probes (GpGs). Mean, No. of probes, and StDEv is calculated for each sample.

14. Generate quantification report using top menu Reports → Annotated probe report. Select Annotate with "coordinate_regions.txt" with "any overlapping" probes and

"Include" features with no probes.

Note: This quantification uses all the valid probes. Probes that were filtered out will not be used for quantification. Each probe (one CpG) will be quantified separately for each sample.

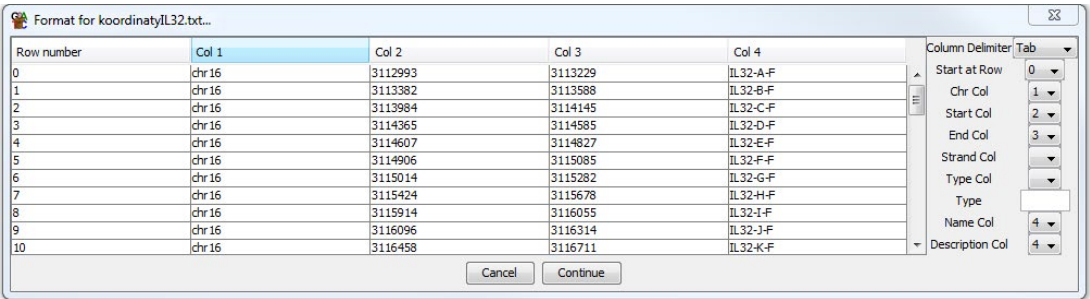


Figure 51.4. SeqMonk data annotation import options window.



Figure 51.5. Single nucleotide CpG methylation quantification visualisation. Each bar represent one C out of CpG. Higher bars show higher methylation level, lower bards lower methylation levels. At the same time, low temperature colours (blueish) show higher methylation and high temperature colours (reddish) show lower methylation status.

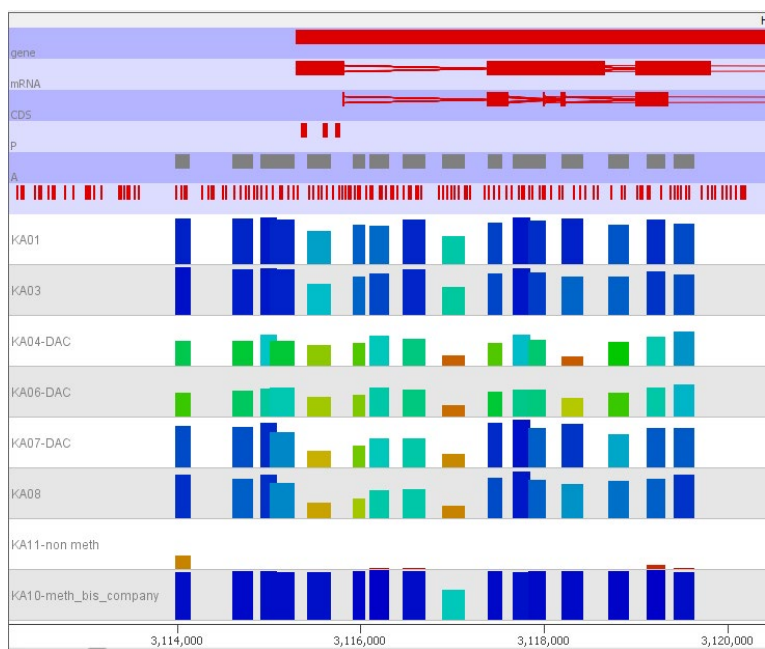


Figure 51.6. Regional CpG methylation quantification visualisation in custom features. Each bar represents data averaged from all C out CpG present in the selected regions. Higher bars show higher methylation level. At the same time low temperature colours (blueish) show higher methylation and high temperature colours (reddish) show lower methylation status.

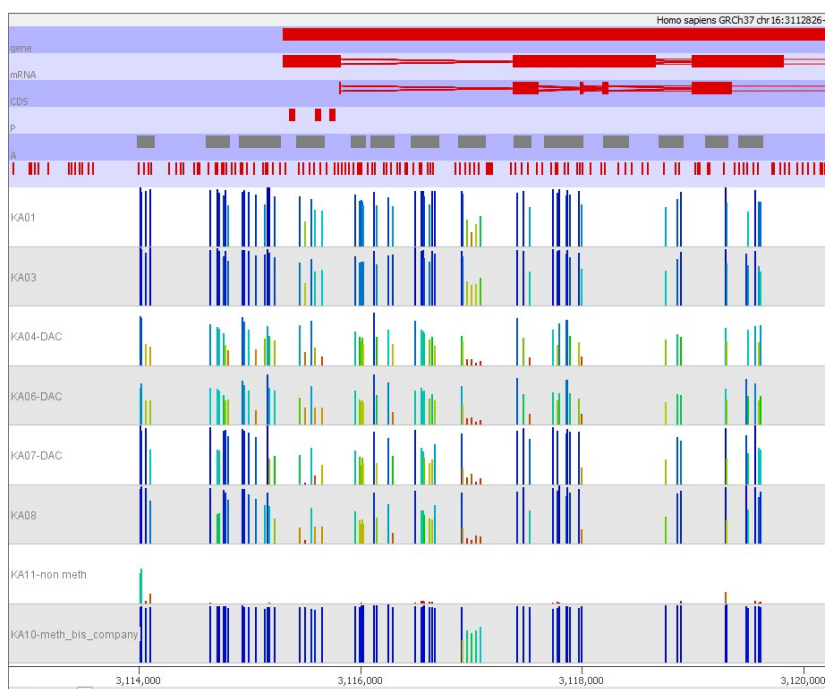


Figure 51.7. Single nucleotide CpG methylation quantification visualization with filtering of artefacts. Please compare to Figure 51.5.

IV. Comments

The user can use other open source bio-informatics pipelines such as:

- **bicycle** (bisulfite-based methylcytosine caller, java based), ready to use docker package (6).
- **methKit** (comprehensive R package for analysis of DNA methylation profiles, ref. 7)
- **gemBS** (python based high throughput analysis of DNA methylation data, ref. 8)

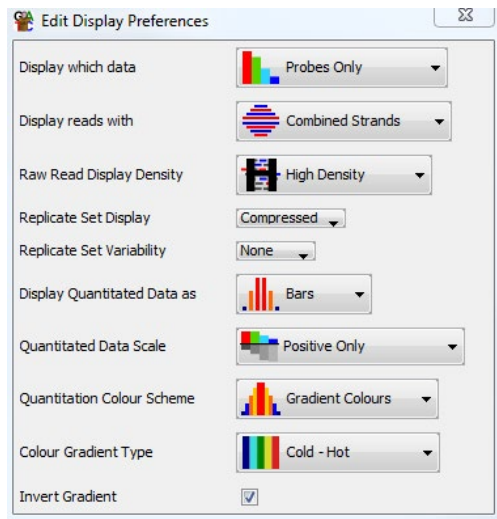


Figure 51.8. Data track display options are highly modifiable.

V. Pitfalls

Problem	Cause and cure
Significant methylation signal in non methylated DNA control	• When this problem is observed in the majority of areas of interest, this symptom is most likely caused by incomplete bisulfite conversion. Repeat the process of conversion, check the reagents. • When effect is observed in particular areas of interest, this symptom is most likely caused by PCR artefacts. Redesign the primers in the areas where this effect was observed.
Low methylation signal in methylated DNA control	• Most likely this symptom is caused by failure in primary amplification. Check the quality of the DNA or redesign the primers in the areas where this effect was observed.

VI. Acknowledgement

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Detection of methylation by pyrosequencing for biological age estimation

Karolína Bartáková

I. Introduction

Aging is a process of becoming older associated with various molecular modifications in cells, such as epigenetic transitions, which create changes in gene expression that affect the systems responsible for maintenance, repair and defence responses against internal and environmental assaults that induce cumulative damage at various levels (8). Epigenetics is the study of heritable changes in gene expression (and thus usually in phenotype) that are not due to DNA nucleotide alteration but to DNA modification (by methylation of one of the DNA strands of cytosine) or chromosome scaffold modification (for example by histone acetylation). Many studies indicate DNA methylation as an important element of aging. In addition to inherited, genetically-engineered changes in DNA methylation, a number of environmental factors influence the methylation status and make a set of sensitive markers of epigenetic drift from human DNA of methyloma (6). The difference between biological and chronological age may be due to a number of clinical and environmental factors and can also serve as an indicator of increased risk of disease and mortality (9).

With the advent of high performance sequencing technologies, the study of epigenetic changes became more accessible and led to the successful identification of changes in age-related DNA methylation (5, 6, 11). These discoveries led to the development of age prediction models. Methods for determining age based on epigenetic analyzes were mainly based on DNA isolation from blood, but other sources of DNA were investigated as well (7).

For years, forensic sciences needed a relevant biological marker to determine the age of offender from biological footprints and to resolve criminal offenses (10). The AgePlex Kit presented in this chapter was developed to estimate human age by determining the methylation level of epigenetic markers markers *ELOVL2*, *C1orf132*, *TRIM59*, *KLF14* and *FHL2* isolated from human blood (12).

II. Materials and instrumentation

Laboratory equipment

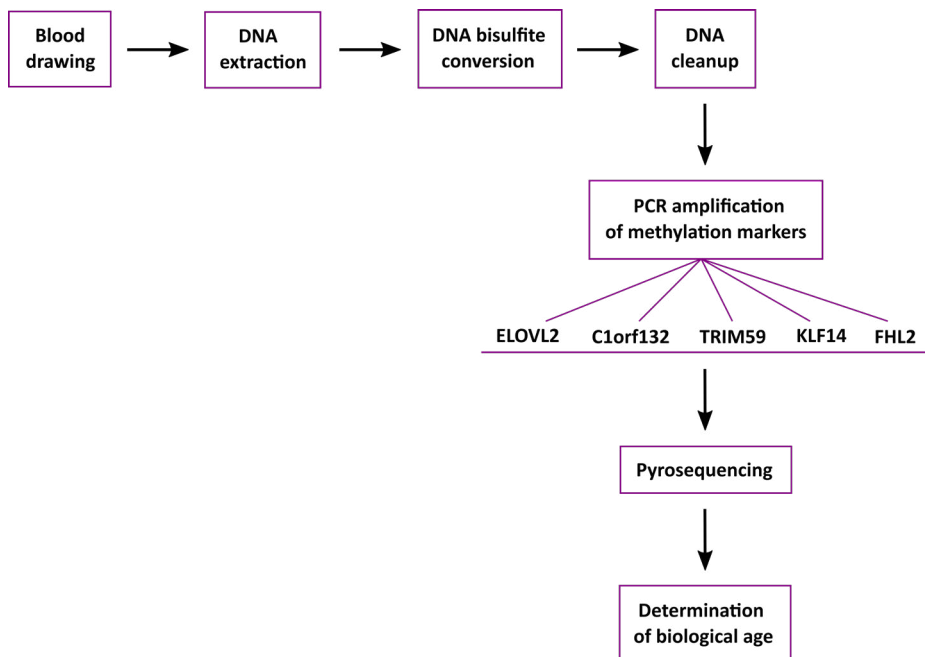
- Safety cabinet, Mars Safety Class 2 (ScanLaf)
- Thermal cycler, CFX96 Real-time System (BioRad)
- Thermal cycler, Mastercycler nexus (Eppendorf)
- Microfuge for 1.5 mL tubes, MiniSpin (Eppendorf)
- Vortexer (Labnet International)
- Set of single/multi- channel pipettes on stand (Eppendorf)
- Electronic multi-dispense pipette, Pipetman 2÷20 µL (Gilson)
- Cold blocks for 96-well PCR plates (Sarstedt)
- Robotic workstation QIAcube (Qiagen)
- Plate centrifuge 5430 (Eppendorf)
- QIAxcel Advanced Instrument (Qiagen)
- Pyromark Q48 Autoprep, sequencer (Qiagen)
- Freezer, -20 °C (Gorenje)
- Refrigerator, 2 °C ÷ 8 °C (Gorenje)

Chemicals

- MagCore Genomic DNA Bisulfite Kit (Cat. No. MGB400-02), RCBioscience, KRD
- EpiTect Fast DNA Bisulfite Kit (Cat. No. 59824 or 59826), Qiagen, Dynex
- PyroMark PCR Kit (Cat. No. 978703 or 978705), Qiagen, Dynex
- PyroMark Q48 Advanced CpG Reagents (Cat. No. 974022), Qiagen, Dynex
- AgePlex Mono Kit (Cat. No. BVE-200-202), BioVectis, Dynex, contains:
 - 5 PCR primer pairs
 - 5 Pyrosequencing primers
- PyroMark Q48 Magnetic Beads (Cat. No. 974203), Qiagen, Dynex
- High-purity water (from Cell biology lab)

Plastics and glass

- Microcentrifuge nuclease-free tubes, 1.5 mL (Cat. No. 1210-00), SSIbio, P-lab
- 8-tube strips without caps, 0.2 mL (Cat. No. 4316567), Applied Biosystems, ThermoFisher Scientific



Scheme 52.1.

- Pipette tips (compatible with pipettes), Eppendorf
- Filter-Tips, 200 μ L, prefilled tip rack (Cat. No. 990332), Qiagen, Dynex
- Filter-Tips, 1000 μ L, prefilled tip rack (Cat. No. 990352), Qiagen, Dynex
- Elution Tubes 1.5 mL, Safe Seal Conical Tube (Cat. No. 72706), Sarstedt, Dynex
- Sample Tubes RB, 2.0 mL safe-lock microcentrifuge tubes (Cat. No. 990381), Qiagen, Dynex
- PyroMark Q48 Disc (Cat. No. 974901), Qiagen, Dynex
- PyroMark Q48 Absorber strip (Cat. No. 974912), Qiagen, Dynex

Bisulfite conversion

1. Thaw purified DNA from blood sample isolated, e.g. by MagCore Genomic DNA Whole Blood Kit. Check that the A_{260}/A_{280} ratio of the sample DNA is between 1.7 and 1.9 and concentration at least 10 ng/ μ L.
2. Prepare EpiTect Fast DNA Bisulfite Kit (2): upon arrival, store the MinElute DNA spin columns, DNA Protect Buffer, and Buffer BD at 2 °C ÷ 8 °C. All other buffers including the Bisulfite Solution should be stored at room temperature.
3. Prepare Buffer BW by adding 120 mL of 96 ÷ 100% ethanol to the 52 mL Buffer BW concentrate and store at room temperature. Invert the bottle several times before starting the procedure.
4. Prepare Buffer BD by adding 27 mL of 96 ÷ 100% ethanol to Buffer BD and draw a mark using ethanol-resistant pen. Invert the bottle several times before starting the procedure. Avoid transferring precipitates of the Buffer BD-ethanol mix to the MinElute DNA spin column.
5. Equilibrate samples and buffers to room temperature. Make sure the Bisulfite Solution is completely dissolved. If not, heat the Bisulfite Solution to 60 °C in heating block and vortex until all precipitates are dissolved. Do not place dissolved Bisulfite Solution on ice.
6. Prepare the bisulfite reactions in 200 μ L PCR tubes according to the Tab. 52.1 (for one reaction). Add each component in the order listed.
7. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature. DNA Protect Buffer should turn from green to blue after addition to the DNA-Bisulfite Solution mixture. It indicates sufficient mixing and correct pH for the bisulfite conversion reaction. Do not put the DNA Protect Buffer into the freezer. If so, do not use.
8. Perform the bisulfite DNA conversion using Eppendorf thermal cycler or program the thermal cycler according to Tab. 52.2. Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated

TABLE 52.1. Components of bisulfite reactions

Component	Final concentration of DNA 200 ng
DNA	Variable fractions (together 20 μ L)
RNase-free water	
Bisulfite Solution	85 μ L
DNA Protect Buffer	35 μ L
Total volume	140 μ L

TABLE 52.2. Thermal conditions of bisulfite conversion

Step	Time	Temperature
Denaturation	5 min	95 °C
Incubation	20 min	60 °C
Denaturation	5 min	95 °C
Incubation	20 min	60 °C
Hold	indefinite	20 °C

lids are suitable for this procedure. If using a thermal cycler that does not allow you to enter 140 µL reaction volume, set the instrument to the largest volume setting available. Converted DNA can be left in the thermal cycler overnight at room temperature without any loss of performance.

Cleanup of bisulfite converted DNA in QIAcube

1. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions. Then transfer the complete (140 µL) bisulfite reactions to 2.0 mL safe-lock microcentrifuge tubes (sample tubes RB). Up to 12 samples can be processed in QIAcube per one Cleanup run.
2. Set up QIAcube for Cleanup protocol. Open the door of the QIAcube to gain access to the worktable (Fig. 52.1). If you need more information, see the manufacturer’s user manual (4).
3. Prepare Reagent Bottle Rack. Follow the instructions for each solution position according to the picture below (Fig. 52.2). Remove the lids from the reagent bottles before placing on the worktable. Reagent bottles designed for use with the QIAcube must be used, otherwise errors may occur during liquid detection. If Buffer BL contains

- precipitates, dissolve them by heating (maximum 70 °C) with gentle agitation.
4. Place prefilled tip racks holding 200 µL filter-tips and 1000 µL filter-tips to the place in the worktable dedicated for tips.
 5. Load the 2.0 mL safe-lock microcentrifuge tubes (RB tubes) with bisulfite converted DNA into the Shaker according to the loading chart (Fig. 52.4). Use shaker adapter for 2 mL safe-lock microcentrifuge tubes (marked with ”2”). Lids of sample tubes must be securely placed into slots at the edge of the shaker rack. Do not place the shaker rack plugs into the empty slots at the edge of the shaker rack (plugs in empty slots will confuse software counting the number of samples during load control).
 6. Prepare Rotor Adapters (Fig. 52.3) and place them into the Centrifuge swing-out buckets according to the loading chart (Fig. 52.4). Use Sarstedt Micro Tube 1.5 mL, Safety Cap as collection tube. For high process safety, the rotor adapters are designed so that they fit into a centrifuge bucket only in the correct orientation. All centrifuge buckets must be mounted, even if fewer than 12 samples are to be processed. The robotic gripper transfers spin columns into the collection tubes during sample preparation.
 7. Close the instrument door and switch on the QIAcube power switch.
 8. Select the Cleanup application from the main menu by pressing the icon.
 9. Select the correct kit name, sample material

and type of protocol. Scroll through the list using up and down arrows and select *EpiTect Fast DNA Bisulfite, Converted DNA* and *Up to 100 ng DNA* respectively. To confirm the selection, press the button Select. This protocol is designed for cleanup of up to 100 ng of bisulfite converted DNA (the DNA concentration is dramatically reduced after the bisulfite conversion).

10. Open the worktable door again and follow the instructions displayed in the touchscreen for loading the worktable with samples, reagents, and labware. Complete and confirm the required actions or confirm what has already been done.
11. Close the QIAcube door and press *Start*. Cleanup procedure takes about 90 min if processing 12 samples.
12. When the protocol run has finished, follow the instructions in the touchscreen for worktable cleanup.
13. Centrifuge the MinElute spin column once more in a benchtop centrifuge for 1 min at 10,000 x g (9,594 rpm at Hettich Rotina 420R centrifuge with rotor radius 97 mm, type 4790-A) to elute the rest of DNA. The volume of DNA after Cleanup protocol is 15 µL.
14. You can store purified DNA at 2 °C ÷ 8 °C for up to 24 h, or at -20 °C for longer time.

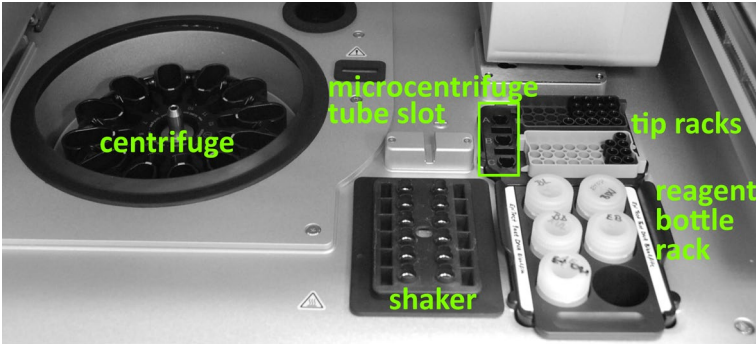


Figure 52.1. QIAcube worktable

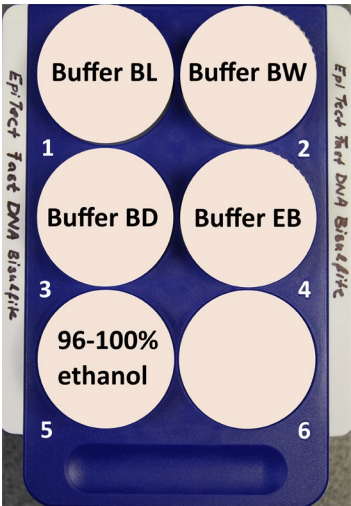


Figure 52.2. Reagent Bottle Rack and positions of reagent solutions.

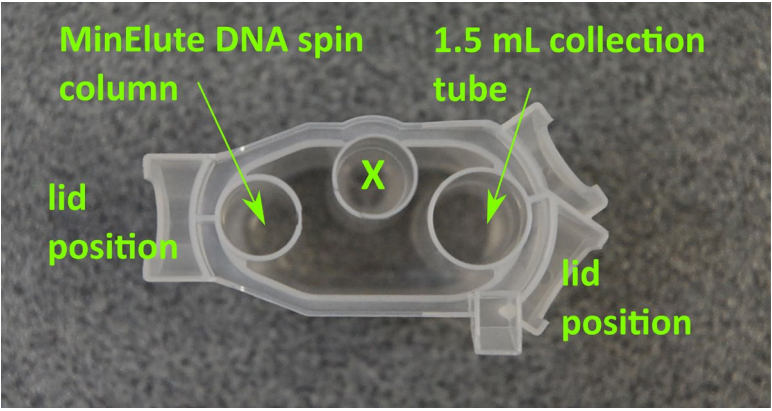


Figure 52.3. *Placing of tube and spin column in Rotor Adapter for cleaning protocol in QIAcube.*

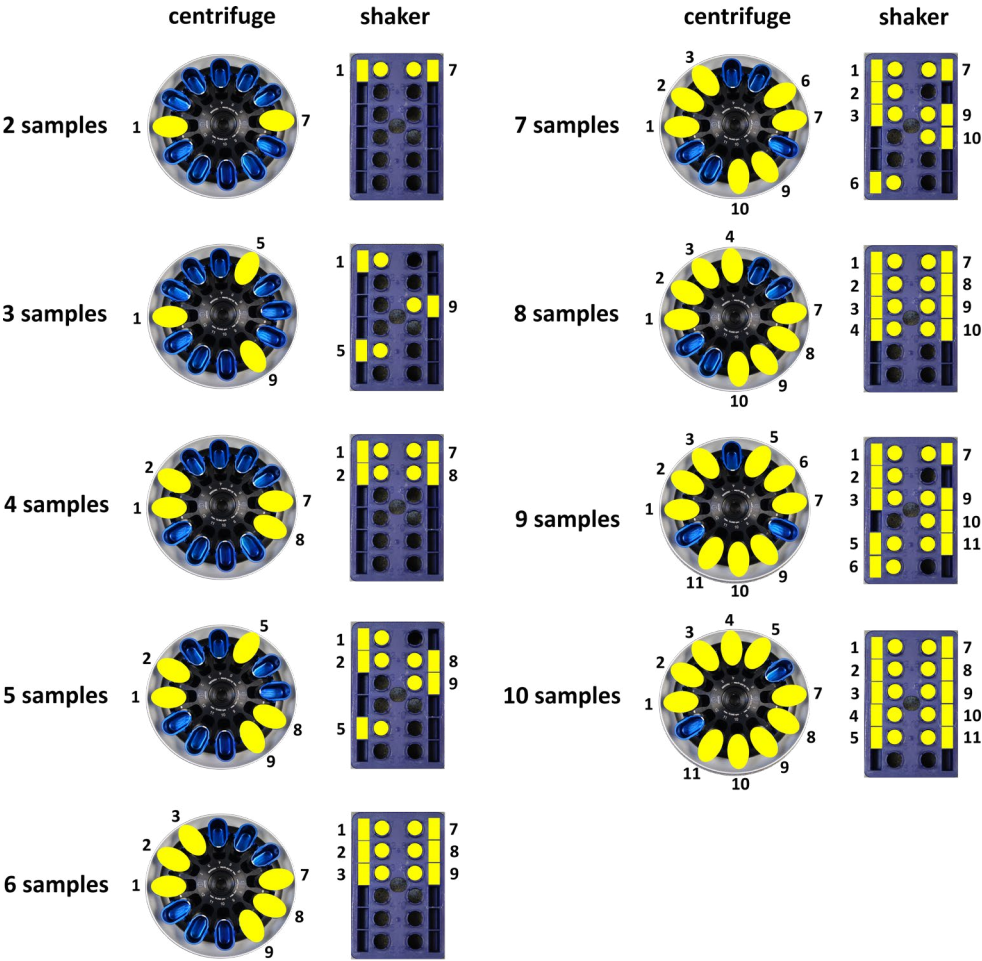


Figure 52.4. *Loading chart for the different number of processed samples.*

PCR amplification of AgePlex methylation markers

1. Use PyroMark PCR Kit (PCR reagents) (1) and AgePlex Mono Kit (PCR primers) for methylation markers PCR amplification. One of the primers is biotin-labeled to enable immobilization to magnetic streptavidin-coated beads during the preparation of a single-stranded DNA template in sequence run.
2. Thaw the PyroMark PCR Master Mix, primer solutions, 25 mM MgCl₂ and RNase-free water at room temperature. Vortex and short spin the reagent tubes and place them on a chilled cold block.
3. Prepare 8-tube 200 µL strip/s and set up the reaction according to the Tab. 52.3. Prepare 5 PCR reactions for 1 sample using five different primers for markers: *ELOVL2*, *Clorf132*, *TRIM59*, *KLF14*, and *FHL2*. Set up for 4 reactions (1 PyroMark sequencing run) and for 12 reactions (when QIAcube
4. Gently pipette the master mix up and down for thorough mixing and dispense 19.5 µL of the reaction into 200 µL PCR tubes.
5. Perform steps 3 and 4 for each AgePlex marker (five times).
6. Add 3 µL of purified bisulfite converted DNA to each of 200 µL PCR tubes with different AgePlex marker. Vortex and spin down the tubes.
7. Perform the PCR amplification reaction using BioRad thermal cycler (program name AGEPLEX_PROG) or program the thermal cycler according to the Tab. 52.4. Use a thermal cycler with a heated lid and Ramp speed setup to 1 °C/s.
8. After amplification, samples can be stored overnight at 2 °C ÷ 8 °C or at -20 °C for longer storage.

TABLE 52.3. Components of methylation markers PCR amplification

Component	1 reaction	4 reactions	12 reactions
PyroMark PCR Master	12.5 µL	58.5 µL	175.5 µL
25 mM MgCl ₂	1.0 µL	4.7 µL	14.0 µL
AgePlex PCR Mono	2.0 µL	9.4 µL	28.1 µL
RNase-free water	4.0 µL	18.7 µL	56.2 µL
Total volume	19.5 µL	91.3 µL	273.8 µL

TABLE 52.4. Thermal conditions of methylation markers PCR amplification

Step	Time	Temperature
Hot start	15 min	95 °C
Denaturation	30 s	94 °C
Annealing	60 s	56 °C
Extension	60 s	72 °C
Final extension	10 min	72 °C
Cooling	indefinite	4 °C

Pyrosequencing in PyroMark Q48

1. Use PyroMark Q48 Advanced CpG Reagents. Dissolve the lyophilized Enzyme and Substrate Mixtures in 660 µL each of PyroMark Advanced Annealing Buffer. Do not vortex. After reconstitution, the Mixtures are stable for at least 5 days at 2 ÷ 8 °C. Frozen reagents should not be subjected to more than 6 freeze-thaw cycles.
2. Forty eight positions on the Pyro Mark disc can be occupied several ways. You can analyse 4 samples with 5 sequencing primers in duplicates during one sequencing run (40 positions occupied) or 9 samples individually in one sequencing run (45 positions occupied). For higher precision, you can repeat the sequencing run with the same samples. For more information, please refer to the manufacturer's manual (3).
3. Create *Run Setup* file for the current sequencing run based on a previous run by right-clicking the processed run file (blue box with check mark) in the shortcut browser and selecting *Copy and Rerun* from the context menu. Only the run setup, not the run and analysis data, will be copied. Select standard protocol type, mark manual primer loading, and edit sample ID according to the LCM numbers used in each run by double-clicking to the corresponding cell. Save the file as *Ageplex_Olomouc_date_operator* (date in

format yymmdd). It is possible to print the disc setup by selecting *Pre Run Information* from the *Tools* menu.

4. If you want to set up a *New CpG Assay*, see Tab. 52.5 with the necessary information (where A= adenine, C= cytosine, G= guanine, T= thymine, R= G or A, Y= T or C). Create CpG Assay for each marker of methylation. Enter the Sequence to analyze from the chart into the *Sequence Before Bisulfite Treatment* text box in PyroMark Q48 Autoprep Software. Mark *Forward Orientation*. You can lock the assay for editing by clicking the *Lock Assay* button at the bottom of the assay setup window (it will not be possible to edit the analysis parameters or results after the assay has been processed). Create disc setup according the instructions in step 3.
5. Insert the USB stick containing the run file created using PyroMark Q48 Autoprep Software into the USB port at the front of the PyroMark Q48 Autoprep instrument.
6. Prior to the first pyrosequencing run of the day, injectors (Fig. 52.5) must be cleaned with high-purity water: select the *Cleaning* button on the Home screen and all injectors for cleaning. A symbol of a drop will appear indicating selection. Insert an absorber strip into the instrument (Fig. 52.6). Pipet 200 µL of high-purity water into the reservoirs of the injectors selected to be cleaned. Close

TABLE 52.5. CpG assays for methylation markers involved in age estimation

Sequence name	Sequence to analyze	Variable positions	Location (GRCh38)
ELOVL2	CCRTAAACRTTAAACCRCCRCRCRAAACCRAC	C7	Chr6:11044661
C1orf132	AAATCTACRCAAACRACRATAAATAATCC	C1	Chr1:207823681
TRIM59	GGTTTGGYGYGGGAYGAGGYGAAGYGTYGG TGGTYGAYGGTTTTTGAGGAATTATTTTTATTT	C7	Chr3:160450199
KLF14	TYGYGTTTTTTTTTTGTGYYGAGTTAGGTA ATGGTAATAGAG	C1	Chr7:130734355
FHL2	AGTTATYGGGAGYGTGTTTTYGGYGTGGG TTTTYGGGYGYGAGTTTYGGAYGAGTTTGGG	C2	Chr2:105399288

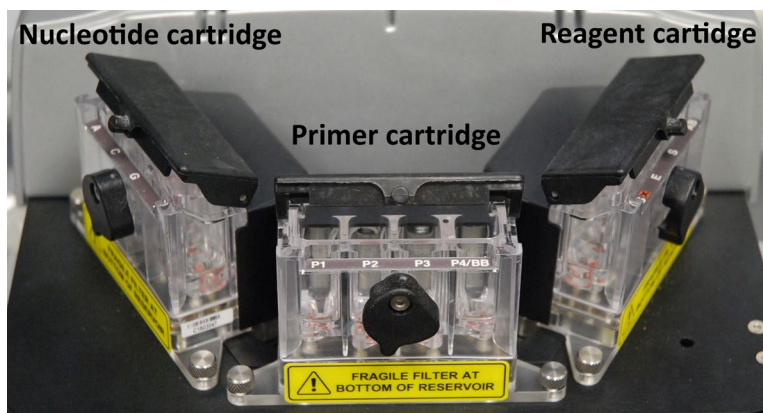


Figure 52.5. *Injector cartridges. Nucleotide cartridge: A- dATPaS, C- dCTP, G- dGTP, T- dTTP. Primer cartridge: P1- Primer 1, P2- Primer 2, P3- Primer 3, P4/BB- Primer 4/Binding buffer. Reagent cartridge: DS- Denaturation solution, E- Enzyme, S- Substrate, AB- Annealing buffer.*

and lock the cartridge lids. Select the *Start* button to begin the first clean. Then follow the instructions on the screen and wash the injectors once more.

7. To start a sequencing run, select *Sequence* on the home screen and load the run via USB stick. Start the run setup by clicking on its name and the arrow appearing behind the run name.
8. Insert a new absorber strip into the instrument (Fig. 52.6). The absorber strip has a maximum capacity of 8 mL. Software will inform the user to replace the strip prior to conducting another run, prime, or clean. The chamber cavity can be cleaned with ethanol to remove any excess waste left behind.
9. Prime the injectors. Select injectors to prime by touching on the injector box on the touchscreen of the PyroMark Q48 instrument. Selected injectors will display a droplet symbol. If there is only one run per day, mark *Select if this the last run today* and the software will calculate the minimum volumes required to achieve one run only.
10. Manually pipette each reagent into the designated injector, according to the volumes shown on the instrument touchscreen. Ensure that the tip of the pipette does not touch the filter at the bottom. Close and lock the cartridge lids using the lid locks.
11. Select the *Start* button. Injectors with a green tick indicate that they have been primed and do not require injector priming. Injectors with a question mark do not need to be primed. Load DNA template (PCR product) and magnetic beads into the disc, omitting positions with Ø symbol that correspond to waste wells.
12. Insert a PyroMark Q48 Disc into the instrument (Fig. 52.6): insert the disc index pin on the rotor hub into the disc index hole (outer hole near well B1). Pin must be in the hole to allow the disc detection system to recognize the disc. Lock the disc into position by screwing down the lock nut.
13. Load DNA template (PCR product) and magnetic beads into the disc, omitting positions with Ø symbol that correspond to waste wells.
14. Vortex the magnetic bead slurry to homogeneity before dispensing and pipette 3 µL magnetic beads into the required wells of the disc using electronic multi-dispense pipette. Magnetic beads fall out of suspension very quickly after vortexing. Repeat the resuspension step between each pipetting step.
15. Pipette 10 µL of PCR product into the correct wells of the disc, following instructions on the screen.
16. Begin the run by selecting the *Start* button.

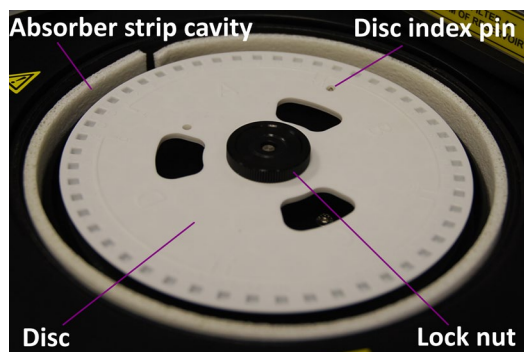


Figure 52.6. Chamber

Preparation of single-stranded DNA includes evaporation by heating step (chamber lid will be open for this step) to reduce the volume in the well.

17. After 20 min, manually add sequencing primers.
18. Pipette 2 μL of the required sequencing primer (4 μM) into the designated sample. Then select the *Next* button to proceed.
19. Once the run has completed, the lid will automatically open and the data will be transferred onto the USB flash (the user will be notified by message *Run Complete*).
20. Select the *Home* button to return to the home page. There, you can begin another run or start cleaning the injectors.
21. After the last run, remove the disc from the instrument and clean the injectors by selecting the *Cleaning* button. Notification to start the cleaning procedure will automatically appear, if the last run of the day was selected.
22. Follow the step 6 to perform the cleaning protocol after sequencing run is done. Close and lock the cartridge lids. Change an absorber strip and select the *Start* button.
23. Remove the USB flash drive with transferred data and analyze the data on the PyroMark Q48 Autoprep Software. Make sure that the point is set as decimal separator on your computer's operating system.
24. In PyroMark Q48 Autoprep Software, open the run file by selecting *Open* in the *File* menu and select *CpG analysis* mode in the toolbar. All wells on the disc with an *Assay Setup* corresponding to the selected analysis mode

will be automatically analyzed.

25. Analysis of the selected wells can be initiated by clicking the *Analyze Selected Wells* button (blue box with check mark) in the *Overview* tab. It is also possible to right-click the selection and select *Analyze Selected* from the context menu. During the analysis, a progress dialog box is shown. This dialog box contains a progress bar, a stop button, and the name of the well that is being analyzed. The progress dialog box closes when the analysis is finished or by clicking *Stop*. The run will be automatically saved in the background after the analysis is complete.
26. View the analysis results. Select an analyzed (light blue) well in the *Overview* tab, the corresponding Pyrogram is displayed in the *Pyrogram area* and the well information is listed in the *Well Information area*. If several wells are selected in the disc overview, information for the well with the orange selection frame is shown.
27. The quality assessment is displayed by the background color of the result (methylation percentage, or average methylation percentage displayed in the Pyrogram). Blue= Passed, yellow= Check, red= Failed.
28. To determine the methylation level of each methylation marker, count the mean methylation level derived from two independent pyrosequencing runs. For individual methylation marker use value of methylation of CpG site 7 for *ELOVL2*, CpG site 1 for *C1orf132*, CpG site 7 for *TRIM59*, CpG site 1 for *KLF14*, and CpG site 2 for *FHL2*.
29. You can export *Report* for CpG from *Reports* menu. Select *CpG Analysis Results* or *CpG Full Report*.
30. To save the analysis, click the diskette icon in the toolbar.
31. To estimate the age of the person who provided sample, go to the web-based *Age Calculator* available at <http://biovectis.com/forensic1/age-calculator>. Follow the instructions on the web site to calculate the biological age. The latest algorithms will be applied.

IV. Comments

- Unlike EpiTect Fast DNA Bisulfite Conversion Kit, common bisulfite procedures usually require high amounts of input DNA to compensate for DNA degradation during conversion and DNA loss during purification.
- Buffer BL contains a guanidine salt that is incompatible with disinfectants containing bleach (they can form toxic fumes - thiocyanogen chloride or chlorine gas).
- Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure.
- Ethanol-based disinfectants can be used for disinfection of QIAcube surfaces, such as the worktable or inside the centrifuge but not QIAcube door. Clean the QIAcube door with distilled water only.
- DNA-ExitusPlus can be used to remove nucleic acid contamination on working surfaces, worktable items, centrifuge rotor, and waste drawer in QIAcube.
- Do not submerge buffer bottles (QIAcube) in 70% alcohol. The blue ring is not ethanol resistant.
- If you are using less than 100 ng of genomic DNA template, it is necessary to use Carrier RNA during DNA cleanup step. Dissolve lyophilized Carrier RNA (310 µg) in 310 µL of RNase-free water to obtain a 1 µg/µL solution. Split the dissolved Carrier RNA into conveniently sized aliquots (e.g., 50 µL) and store at -20 °C. Prepare only enough Buffer BL-Carrier RNA solution to process your samples (in a ratio of 1:100). This solution should be prepared fresh, and is stable at 2 ÷ 8 °C for up to 48 hours.

V. Pitfalls

Problem	Cause and cure
Low conversion rate after bisulfite reaction	• Poor DNA quality (i.e., protein contamination) Check that the A_{260}/A_{280} ratio of the sample DNA is between 1.7 and 1.9. • Amount of DNA used outside recommended range Increase or decrease the amount of starting DNA material to stay within the range of 1 ng to 2 µg for purified DNA. • Bisulfite Solution contains precipitates Heat the Bisulfite Solution to 60 °C and vortex until all precipitates are dissolved again.
Poor results in downstream PCR applications	• Failure of bisulfite conversion reaction Sample DNA was degraded before modification reaction. Ensure that sample DNA is handled and stored correctly.
Little or no DNA recovery in DNA cleanup step	• <i>Carrier RNA not added to Buffer BL</i> • Prepare carrier RNA and add to Buffer BL, as described in Comments. • <i>Buffer BL contains precipitates</i> • Check Buffer BL for precipitate. Dissolve by heating (maximum 70 °C) with gentle agitation.

**Poor or faulty
Pyrosequencing run**

- **Background of contaminating sequence from PCR**

Check your PCR product on agarose gels to confirm that you only have one specific product.

**Errors in pyrosequencing
related to analysis**

- **Reagents incorrectly dissolved or stored**

Be sure to follow the instructions in this chapter. Include an empty well (Annealing Buffer only) in the run setup to check whether background peaks are coming from nucleotides.

- **Dispensation error**

Perform the injector test (see section 8.5 in PyroMark Q48 Autoprep User Manual). If the problem remains, contact QIAGEN Technical Services.

**Loaded wrong nucleotide
into injector position in
PyroMark Q48 Autoprep
instrument**

- Run Injector Clean for that injector according to the step 6 in section *Pyrosequencing in PyroMark Q48*.

**Pump of the PyroMark Q48
Autoprep will not reach
pressure**

- Check the cartridge lids are closed. Check cartridges are firmly seated.

**Injector Clean/Priming
failure**

- Check to see if there is sufficient liquid in the cartridge reservoir. Potential issue with drop sensor or injector. Run injector test (see section 8.5 in PyroMark Q48 Autoprep User Manual).

**Liquid or beads outside of
the wells**

- Ensure that the disc index pin is situated in the disc index hole (near well B1) to allow the disc detection system to recognize the disc positions.
- Ensure that the well capacity volume (20 µL) is not exceeded.

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