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**Příloha 4: Protokoly pro praktika z buněčné a molekulární biologie (AJ)**

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**1. FLUORESCENT STAINING OF MICROFILAMENTS AND DNA IN CANCER CELLS**

**1.1 PERMEABILIZATION OF CELLS**

**1)** Pour the total volume of Triton X solution (about 5 ml) from the tube into a Petri dish containing the coverslip with fixed cancer cells. You permeabilize the cells by that. Incubate for 10–15 minutes at room temperature.

*If we do not permeabilize the cells, we can stain them only on a surface. In this way, inner cell structures can be stained as well.*

*(To avoid cell lysis during sample staining we use isosmotic solution, e.g., PBS - phosphate-buffered saline).*

**2)** Pour out Triton X solution from the Petri dish to the waste pot **without a plastic bag inside**; the coverslip will be attached to the bottom of the Petri dish.

**3)** Wash the coverslip with cells in the Petri dish by PBS solution.

Washing the cells by PBS means: Pour into the Petri dish approximately 5 ml of PBS (see the scale on the tube, estimate the volume), incubate approximately 5 minutes, and then pour out the PBS from the Petri dish into the waste pot **without a plastic bag**.

**Repeat this procedure at least three times.**

*During these washing steps, exceed Triton X is removed from the sample, so it does not loose membrane more and alternatively does not affect the staining of the cells.*

**1.2 STAINING OF CELLS**

**1)** Pour out PBS as previously and dry the area surrounding the coverslip in the dish by cellulose (do not touch coverslip, you would lose the cells!!!). Then pipette a drop of staining solution (approximately 60 μl) containing the conjugate (phalloidin-TRITC) directly on the cell on the coverslip. Put a square of folium on the drop, spread it by gently pressing and incubate about 20 minutes.

The folium decreases the used volume of staining solution and suppresses evaporation of staining solution.

*Phalloidin specifically binds to polymerized actin (F-actin), covalently linked fluorophore TRITC (tetramethylrhodamine isothiocyanate) emits red light after excitation. Actin microfilaments with the bound complex of phalloidin-TRITC conjugate will be therefore shinning red in microscope.*

**2)** Wash the coverslip with cells in the same way as in step **1.1 3)**at least three times (5 minutes each step) by PBS.

*By washing the cells, we get rid unbound fluorescence conjugate that otherwise causes non-specific fluorescence of the sample*.

**3)** Detach the coverslip with cells from the bottom of the dish by a needle. Turn the coverslip upside down and carefully put **cells down** into a drop of mounting medium on a slide.

*Mounting medium contains DAPI (4', 6-diamidino-2-phenylindole) that binds double- stranded (ds) DNA. DAPI is excitable by UV light and emits blue light just after binding to dsDNA.*

Observe cells by fluorescent microscope.

**Questions and tasks:**

**1) Describe briefly a shape of cells and cell nuclei. Based on the description, determine if**

**you have stained growing or non-growing cell population.**

* **Some cells are irregularly shaped with round nuclei. These cells normally grow.**
* **Some cells are round-shaped and with fragmented nuclei. These cells are not growing or are even dying.**

**2) Decide, if the cells that you have stained are sensitive or resistant to the effect of the**

**tested cytostatic.**

**3) What can you say about the effect of the tested cytostatic on sensitive cancer cells**

**(what is a ratio of growing and dying cells)?**

**USED SOLUTIONS:**

**PBS (phosphate-buffered saline pH 7,4)**

**Solution of 0,1% Triton X-100 in PBS**

**Staining solution containing phalloidin-rhodamine**

**Commercial mounting medium (Vectashield) containing DAPI**

**2. Analysis of protein levels in samples** - **part I**

**2.1 ISOLATION OF PROTEINS**

**1)** Sign four 1.5ml microtubes (**on their caps**) with the number of your study group, letter of your working group (the letter on the pipette holder), and type of the sample:

**B**one muscle…**B** **M**ilk…**M**

**L**iver…**L** **P**urified serum…**P**

**2)** Pipete 900 μl of lysis buffer into microtubes signed “**B**“ and “**L**”

**3)** According to the instructions, collect small pieces of skeleton muscle and liver samples by scalpel knife. Transfer them into corresponding microtubes with 900 μl of lysis buffer.

*Lysis buffer contains the detergent SDS (sodium dodecyl sulfate) that lyses cell membranes.*

*The disintegration of the tissue to small pieces is necessary for proper contact of the tissue with lysis buffer.*

**The steps 3) and 4) can be made simultaneously**

**4)** Next,pipete 200 μl of breast milk-simulating solution into microtube signed “**M**”. Dilute purified serum 2 times into lysis buffer (final volume will be 200 µl).

**5)** Mix the contents of microtubes by vortex device.

*Vortexing facilitates the disintegration of the tissue and makes the lysis more effective.*

**6)** Incubate the samples in microtubes on ice for 10–20 minutes, vortex the microtubes from time to time during the incubation.  
*Cell proteases released from the tissue during lysis are inhibited when samples are incubated on ice.*

**7)** Centrifuge the microtubes containing samples of bone muscle and liver with 14 000 rpm for 15–25 minutes at 4°C.

*The supernatant will contain proteins; non-lysed rests of tissues and cells will drop to pellet.*

**8)** Sign two new 1.5-ml microtubes in the same way as sample “**B**” and “**L** in step **2.1 1**)

**9)** Transfer 400 μl of the supernatants, or protein suspensions, from centrifuged samples (avoid transferring of any pieces of tissue!) to these newly signed microtubes.

**10)** see part **2.2 (opposite page)**

**11)** After the determination of protein concentration, put protein suspensions to the paper box. The solutions will be frozen and stored at *-*20°C.

*(Samples can be stored at -20°C for several weeks)*

**USED SOLUTIONS: lysis buffer containing SDS**

**2.2 Determination of protein concentration by the Bradford ASSay**

**PRINCIPLE:**

*This method is based on colorimetric reaction running after mixing of Bradford reagent and solution containing proteins. Bradford reagent contains Coomassie Brilliant Blue that binds to basic and aromatic amino acid residues in proteins.*

*(Complex of proteins and the dye has the absorption maximum at 595 nm).*

*When we create a dilution scale, it is then possible to arrange calibration curve from known values of protein concentrations and their corresponding absorbance. From the curve, concentration of proteins in unknown samples based on the knowledge of their absorbance can be assessed.*

**1)** Sign four new 0.5 ml microtubes as in step **2.1 1**) Pipete 95 μl of distilled water to the labeled microtubes.

**2)** Mix your protein lysates well. Pipette 5 μl from corresponding protein lysates to signed 0.5 ml microtubes. How many times are the solutions diluted then? These diluted solutions will be used for the determination of protein concentration by Bradford method.

*Samples must be diluted so their concentration could be measured in the detection limits of the method.*

**3)** Sign four new microtubes with 1 ml of Bradford reagent that will be given to you like in step **2.1 1**)

**4)** From 0.5 ml microtubes, take 20 µl of diluted samples and pipete this into corresponding microtubes containing Bradford reagent. Vortex the microtubes well.

**5)** Incubate tubes at least 5 minutes (maximum 45 minutes) at room temperature.

**6)** According to the instructions, transfer the samples to a cuvette and measure samples absorbance at 595 nm on a spectrophotometer (NanoPhotometer IMPLEN).

*Spectrophotometer software calculates sample concentration from sample absorbance using the calibration curve that was created previously by lectors.*

*(The values of absorbance correlate to the amount of proteins present in the sample).*

**The result has to be multiplied by the previous dilution for the calculation of concentrations of undiluted solutions.**

**Note down the concentration of the sample in ug/ul.**

**7)** According to the measured concentration of proteins, calculate the volume of each sample so

it contains 6 and 60 μg of proteins. These volumes will be loaded onto gel on the next part of

the practice. **Note down also these volumes.**

**USED SOLUTION: Bradford reagent**

**2. COMPARISION OF PROTEIN LEVELS IN SAMPLES - part II**

**2.3. PROTEIN ELECTROPHORESIS - SDS PAGE**

**2.3.1 Preparation of samples for loading into the gel**

**1)** Sign four new 0.5 ml tubes (write letter of your working group and type of the protein solution: **B**one muscle, **M**ilk, **L**iver, **P**urified serum) and pipette 30 μl of the Sample buffer to each microtube.

**2)** First, mix well protein sollutions prepared in the first part of practice (**B**one muscle, **M**ilk, **L**iver, **P**urified serum). Add 30 μl of the corresponding protein sollution to the microtubes that contain 30 μl of the Sample buffer (see previuos step).

**3)** Heat your samples at 95°C for at least 5 minutes in a heated thermoblock.

*Polypeptides take on a rod-like shape and a uniform charge-to-mass. Proteins are thus separated only according to their molecular weights.*

*Proteins are denatured by heating in sample buffer containing SDS and S-S bonds-reducing agent such as 2-mercaptoethanol.*

**4)** Use such volumes of the samples to load **30 μg** of proteins of **B**onemuscle sample, and **3** and **30 μg** of proteins of **M**ilk**, L**iverand **P**urified serum. Do not forget sollutions were mixed into the sample buffer.

**2.3.2 Loading of samples to the gel**

**1)** Prepare the electrophoresis unit for loading of the samples and course of the electrophoresis according to the instructions.

**2)** After electrophoresis unit is assembled, fill the inner space between glasses with electrolytic buffer (Running buffer) until buffer overflows into the bath and finally the level of the buffer will be close to the top of the bath (approximately 1 liter of the buffer will be poured within).

*If the level of the buffer is lower than the top of the glass with spacers, the electric power cannot run through the gel, and the separation of proteins cannot run.*

**3)** This is the order of samples in the well:

**1.** Marker of molecular weights

**2.** Sample of your **B**onemuscle

**3.** ─ **8.** Your samples of the **L**iver**,** **M**ilk, and **P**urified serum in pairs (**3** and **30 μg** of proteins), load calculated volumes of samples, see step **2.3.1 4)**

**9.** The sample of the brain of the patient X (**XB**)

**10.** Sample of the serum of the patient X, one month after chemotherapy (**XS**)

***Markers of molecular weight*** *are used for precise determination of the molecular weight of separated proteins.* ***The molecular weights*** *we use are* ***25,******37****,* ***50, 75, 100, 150 and 250******kDa****.*

*They also allow monitoring of* ***separation of proteins in the gel****, because they are pre-stained with covalently attached dyes.*

**2.3.3Start and end of protein separation by electrophoresis**

**1)** Place the lid on the electrophoresis unit after samples loading. Insert the electrical leads into a suitable power supply (200 V) with the proper polarity. Begin electrophoresis and run this approximately 45 minutes (watch the course of electrophoresis).

*The bromphenol blue which is a component of the loading dye has the highest electric mobility of all components loaded on the gel.*

***When it reaches the end of the gel, electrophoresis should be stopped to avoid the release of proteins to the running buffer!***

**2)** After electrophoresis is complete, turn off the power supply and disconnect the electrical leads. Take out the inner part of the unit, and then release the glasses with the gels.

**2.4 STAINING OF THE GEL AND ANALYSIS OF THE SAMPLES**

**1)** Separate the glasses by the **opener** and let the gel to be attached to one of the glasses.

**2)** Take the gel carefully and put it into the **Staining solution**. Stain with mild agitation approximately for 5─10 minutes.

*Coomassie Blue present in the Staining sollution is a protein-binding substance that enables visualization of proteins.*

**3)** Subsequently, change the **Staining solution** for the **Destain solution** to wash out unbound Coomassie blue. After 10 min. pour out the **Destain solution** and continue in distaining with hot water.

*Particular bands of proteins become visualized by slow distaining of the nonspecific gel coloration.*

**Questions:**

**1) According to markers of molecular weight identify actin and myosin in the muscle**

**sample.**

***Actin and myosin are highly conservative proteins. The molecular weight of actin is***

***approximately 43 kDa. Myosin consists of a heavy chain with a molecular weight of 210 kDa***

***and several light chains of 15─20 kDa.***

**2) Decide, if your samples of liver, milk, purified serum, and samples XB and XS contain**

**tested cytostatic**

***As positive controls use your serum sample, as a negative control use your skeleton***

***muscle sample.***

**3) If the cytostatic is present in some samples, determine roughly the amount of the cytostatic.**

***Compare the level of the cytostatic in a sample with the levels in the serum samples.***

**2.5 PREPARATION OF GELS FOR NEXT STUDY GROUP**

**2.5.1 Preparation of the 10% separating gel**

**1)** Take the plate with stuck spacers and the short plate and clean them by gauze with ethanol. Prepare the plates according to instructions and put them in the cassette for pouring of gels.

**2)** Pour a small amount of ethanol by squirt into the space between the plates to check the right position of the plates. If there is no leaking, remove ethanol to washbasin and rest of ethanol suck out by a square of tissue.

*After this leakage test, the gel cassette is ready for pouring the gel.*

**3)** Take the tube with premixed 10% separating gel and add **3,5 μl** of TEMED and **35 μl** of 10% APS. Mix by gentle inverting the tube several times. **Do not vortex!**

*By adding APS and TEMED polymerization of the gel is started. From this moment it is important to work quickly to avoid polymerization of the gel before pouring between plates.*

**4)** Pipette the liquid gel to the space between glasses with a pipette with the blue tip to the level **indicated by lectors** of the practice, you will approximately pipete four times 800 μl. Avoid forming bubbles.

**5)** Immediately overlay the gel solution with ethanol by the squirt.

*Ethanol prevents the contact of polymerizing gel with oxygen in the air (it inhibits the polymerization process). Ethanol also removes bubbles from the surface of the gel and helps to form a straight horizontal level of the gel.*

**6)** Allow the gel to polymerize for 30─45 minutes.

*The course of gel polymerization can be observed in the tube. When the rest of the gel in the tube gets a solid structure, you can start with the stacking gel preparation.*

**2.5.2 Preparation of 4% stacking gel**

**1)** After complete polymerization of the 10% gel, remove ethanol as previously, see point **2.5.1** **2)**

**2)** Add **3 μl** of TEMED and **20 μl** of APS to the tube with 4% premixed stacking gel. Mix by inverting the tube several times. **Do not vortex!**

**3)** Pipette the 4% liquid stacking gel to the space between plates on the top of polymerized 10% gel until the top of the plate with spacers is reached. Avoid forming of bubbles.

**4)** Place a green plastic comb to the space between glasses to form wells in the gel. Again, avoid creating of bubbles.

**5)** Allow the stacking gel to polymerize for 30─45 minutes.

*Complete polymerization of the gel can be expected when the rest of the gel in the tube has a solid structure.*

*For separation we use two gels with different concentration of acrylamide and different pH.*

*The stacking gel enables proteins to reach the interface between both gels at the same time so that the zone of proteins loaded to the wells becomes sharp.*

*Thus, the mobility of separated proteins is finally dependent only on their molecular weight.*

**6)** Leave the gels you have just made in apparature; they will be used by next study group. You used the gels, which were prepared for you by the previous study group.

**USED SOLUTIONS:**

**Sample buffer**

**Running buffer**

**Staining solution**

**Destain solution**

**10% polyacrylamide gel**

**4% polyacrylamide gel**