Task 1B: Basic electrophoretic and immunochemical methods for protein analysis

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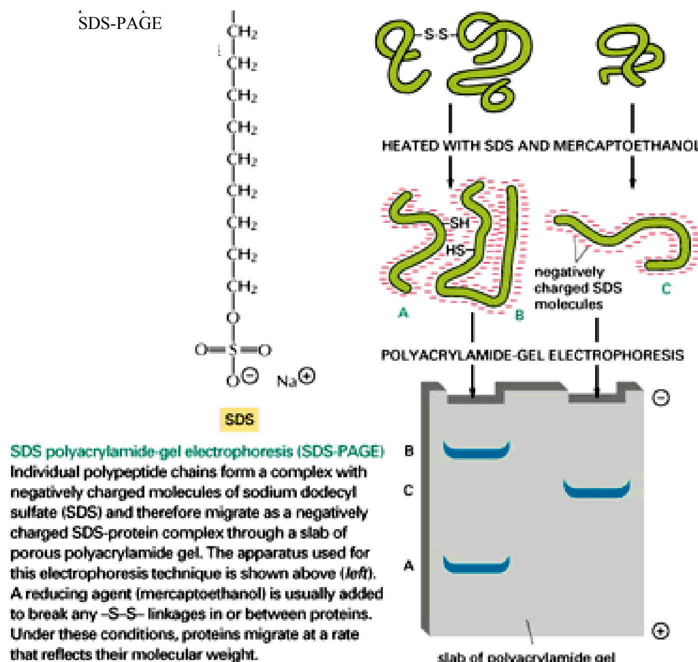
I. Electrophoresis of proteins in the presence of SDS – SDS-PAGE

Objective: *Determination of molecular weight of p53 family proteins using electrophoresis in the presence of SDS*

Principle:

Polyacrylamide gel electrophoresis (PAGE) is the most common method of protein separation. Proteins migrate in an electric field based on their shape (globular proteins migrate faster than fibrous) and the charge density (the ratio of charge to the unit of mass). To determine the molecular weight we use electrophoresis in the presence of SDS. SDS (sodium dodecyl sulfate) binds to protein at a ratio of 1.4 g of SDS per 1 g of protein, the charge of protein-SDS complex is proportional to its molecular weight. Disulfide bonds are often abolished by reducing agents (beta-mercaptoethanol) and protein denaturation is completed by boiling. As a result, the sole factor determining the mobility of proteins in a denaturing SDS-PAGE is the molecular weight. SDS-PAGE is simple, fast and reproducible method for qualitative characterization and comparison of proteins. By comparing the relative mobilities of an unknown protein and standards, it is possible to determine its molecular weight.



Img.1 Principle of SDS-PAGE method

Procedure:

1. Preparation of polyacrylamide gel

a. Clean the glass plates using distilled water and ethanol and then dry the glass, for each gel one 1.5 mm glass with spacer and one glass cover, similarly prepare the comb (1.5 mm).

b. Preparation of **running** gel using the table: prepare running gel according to the table in written order → stir and pour between glass plates to the height of about 5 cm → add few drops of butanol

	1 gel	2 gels
10% PAGE	**10 ml**	20 ml
10% APS	**50 µl**	100 µl
TEMED	**25 µl**	50 µl

c. Preparation of **stacking** gel using the table → suck up butanol with filter paper → stir the mixture → pour between glass plates and insert comb

	1 gel	2 gels
5,0% PAGE	**2,5 ml**	5 ml
10% APS	**25 µl**	50 µl
TEMED	**12,5 µl**	25 µl

d. After the gel has polymerized (check polymerization in the unused mixture), carefully remove the comb and rinse the starts with electrophoretic buffer to get rid of residual unpolymerized acrylamide.

e. Put the gel in an apparatus and pour 1x RB electrophoretic buffer (10xRB: 0,25 M Tris; 1,92 M glycine; 1% SDS, pH 8,3).

2. Preparation of samples

a. také 10 μl of protein sample, add 3 μl 4 x CSB (100 mM Tris-HCl, pH 6,8; 200 mM β–mercaptoethanol; 4% SDS; 0,1% bromphenol blue; 20% glycerol).

THE SAMPLES WILL BE PIPETTED ACCORDING TO THE TABLE

sample		µl	4x CSB (µl)
1	SPL1	10	3
2	SPL2	10	3
3	SPL3	10	3
4	SPL4	10	3
5	M	10	3
6	SPL1	10	3
7	SPL2	10	3
8	SPL3	10	3
9	SPL4	10	3
10	Standard p53	10	3

a. Boil mixture in thermoblock at 99 °C for 5 min, centrifugate it.

b. Load the samples on gel. M is marker of molecular weight (BioRad).

3. Electrophoresis

a. Electrophoretic apparatus with loading samples is closed by cover with connectors and connected to the source of voltage.

b. Electrophoresis takes 15 min at 70 V and 45 min at 150 V, until forehead represented by bromophenol blue reaches the bottom edge of the gel.

c. Switch off source, take out gel and carefully separate glass plates.

List of necessary equipment and chemicals:

- Storage solution for preparation of running gel: 10% acrylamide:bisacrylamide 19:1; 0,375 M Tris-Cl,H 8,8; 0,1% SDS
- Storage solution for preparation of stacking gel: 5% acrylamide:bisacrylamide 19:1; 0,125 M Tris-HCl, pH 6,8; 0,1% SDS
- 10% ammonium peroxydisulfate (APS), TEMED (N,N,N´,N´-tetramethylethylendiamine)
- RB (Electrode buffer): (25 mM Tris; 0,192 M glycine; 0,1% SDS, pH 8,3)
- Samples from tumour cell lines, Protein standards BioRad
- 4 x CSB: 50 mM Tris-HCl, pH 6,8; 100 mM β-mercaptoethanol; 2% SDS; 0,1% bromphenol blue; 10% glycerol
- Microtubes, Pipettes, Tips, Thermoblock, Centrifuge, Source of voltage, Shaker

II. Transfer of proteins to a membrane (blotting)

Objective: *Transfer of proteins from SDS-PAGE, PAGE or agarose gel to a membrane*

Principle:

Transfer of protein to a membrane is used to identify proteins by antibodies and usually follows after the separation of proteins by SDS-PAGE, or after separation of protein-DNA complexes on native PAGE or agarose gel. Because the probe-antibody for the protein generally cannot be used directly in the gel, proteins and their complexes with the DNA are transferred to a membrane after electrophoresis (using nitrocellulose, PVDF and others).

Depending on the transmitted macromolecules we distinguish:

-Southern blot: transfer of DNA;

-Northern blot: transfer of RNA;

-Western blot: transfer of proteins.

According to the method of transfer of macromolecules to a membrane we can divide blotting into:

-electrophoretical transfer, in which use is made of the electric field;

-vacuum transfer in which the macromolecules are transferred via buffer (10X SSC) sucked by the vacuum pump;

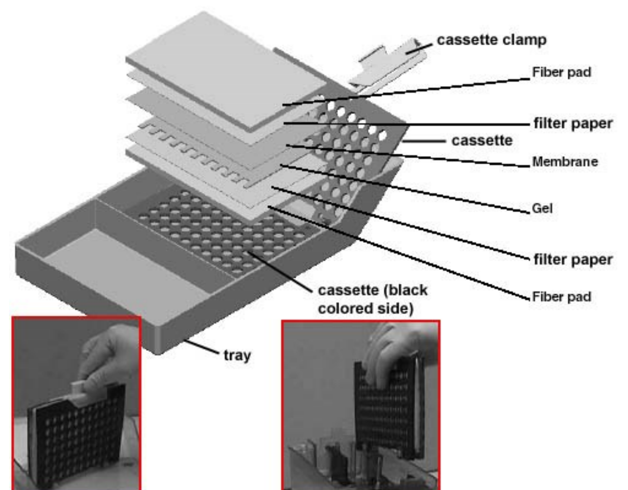
-capillary transfer, where the capillary forces are used to penetrate the gel and the membrane and pull down the nucleic acids or proteins. Wicking of buffer is provided by putting absorbent material to the membrane.

Img.2 Apparatus for vacuum transfer and electroblotting

Procedure:

a) transfer of protein from SDS-PAGE to a membrane

1. prepare a nitrocellulose membrane, mark one side, cut Whatman paper on the size of membrane
2. cut off *stacking* gel and soak *running* gel in 1x BB
3. construct sandwich: washcloth, 2x whatman, gel, membrane, 2x whatman, washcloth (black side down)
4. pour 1x BB + 100 ml MetOH (to 1 l)
5. transfer lasts 90 min at constant 150 mA



Img. 3 Building of blotting apparatus

b) detection of proteins on the membrane by Ponceau S staining

1. soak the membrane in the solution of Ponceau S for 5-10 min
2. destain with distilled water from bottle
3. take a photo of the membrane

Material and chemicals:

- gel with transfered protein or nucleic acid
- blotting apparatus
- filter paper, watman paper
- nitrocellulose or PVDF membrane

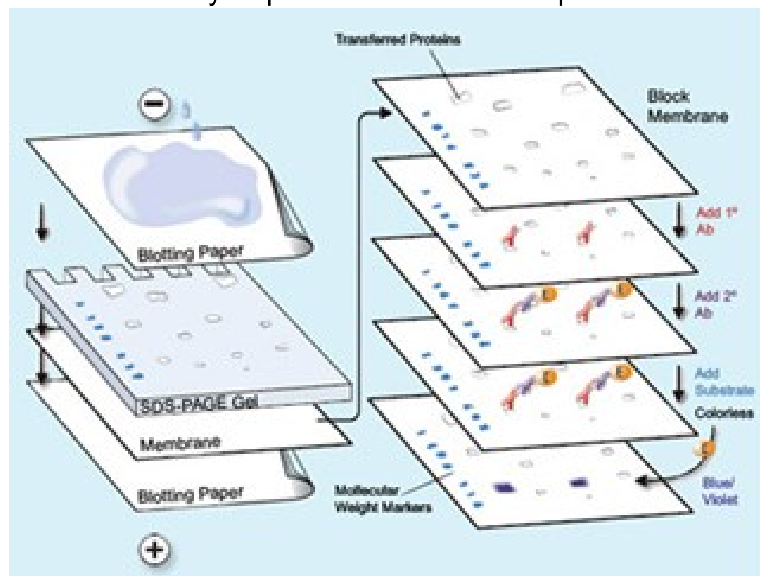
- 10x **Blotting buffer** (1 L 30.3 g Trizma base (= 0.25 M), 144 g Glycine (= 1.92 M) pH 8.3), 1x BB (100-200 ml MeOH + 100 ml 10xTB + water)

III. Immunodetection of proteins on the membrane

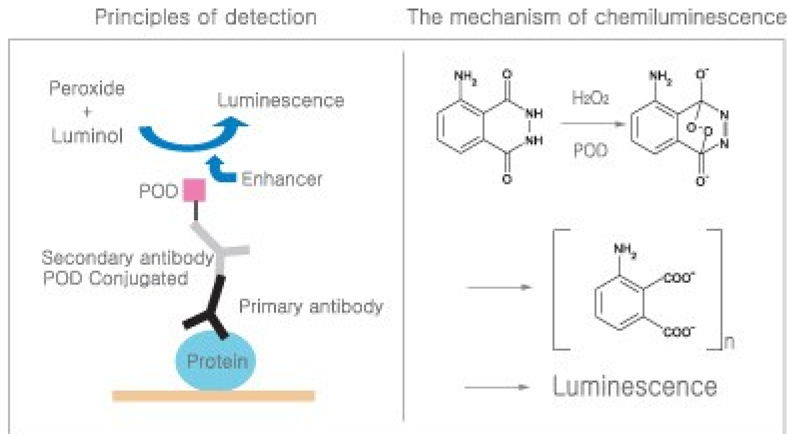
Objective: *Immunodetection of p53 protein after transmission from SDS-PAGE gel to the membrane*

Principle:

Protein detection is frequently performed by immunochemical methods. After blotting followed by saturating free binding sites by albumin or milk proteins, the membrane is incubated in a solution containing antibody against the studied protein. This primary antibody is prepared to exhibit a strong specificity for the studied protein. In the next step, the secondary antibody is used. The secondary antibody is species-specific, which means that it recognizes the primary antibody according to the type of organism in which it was produced. The detection is performed using reaction catalyzed by the enzyme conjugated to the secondary antibody (alkaline phosphatase, peroxidase, luciferase, etc.). Specific substrate is added to the membrane so that its conversion to colored product or emission of light occurs. Since the protein-Ab1-Ab2-enzyme complex is anchored to the membrane, the reaction occurs only in places where the complex is bound. Its position on the membrane is indicated by staining the membrane. The intensity of signal can determine the amount of protein in given location.



Img.4 Principle of transmission and immunodetection



Img. 5 Principle of chemiluminiscence detection using ECL substrate with HRP (horseradish peroxidase)

Procedure:

1. mark the membrane after transfer of proteins and let it shake in 5% low-fat milk (5 g of milk powder + 100 ml 1x PBS) for 1 h (30 min)
2. primary antibody diluted in milk (1:10 000) → let shake over night at 4 °C (30 min)
3. wash in 1x PBS + tween → 3x 5 min on shaker
4. secondary antibody (conjugate) diluted in milk (1:5000) → let shake 1 hour at RT (30 min)
5. wash in 1x PBS + tween → 3x 5 min on shaker
6. insert the membrane into plastic foil
7. prepare solution for chemiluminiscence (ECL, 1:1): 1 ml of reagent 1 + 1 ml of reagent 2 (on the membrane of size 8 cm * 6 cm) → pour over whole membrane and wait 1 min
8. suck up solution → take a photo on chemiluminiscence detector

Material and chemicals:

- 1 x PBS
- 5% low-fat milk in 1 x PBS
- primary antibody
- secundary antibody with conjugated enzyme (HRP)
- 1 x PBST (100 ml 10xPBS, 0.5 ml Tween 20 in 1l)
- shaker

Solution for chemiluminiscence:

RPN2106 Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare)