Antigen detection by Western Blotting II.

Blotting and antigen imaging

Aim

To learn the technique of Western Blotting for the detection of a specific protein.

Introduction

Western blotting or protein immunoblotting is a very sensitive and analytical method that involves detection of a specific protein in a complex mixture. Protein samples are first separated using SDS Polyacrylamide gel electrophoresis (SDS-PAGE) followed by the immobilization of proteins on nitrocellulose or PVDF membranes. The transfer of proteins from the gel to the membrane is done electrophoretically. The transferred protein is detected by immunodetection using specific primary antibody and secondary enzyme labeled antibody and substrate. This method utilizes the principle of antigen-antibody interaction for identification of specific antigens by monoclonal or polyclonal antibodies(Picture 1)

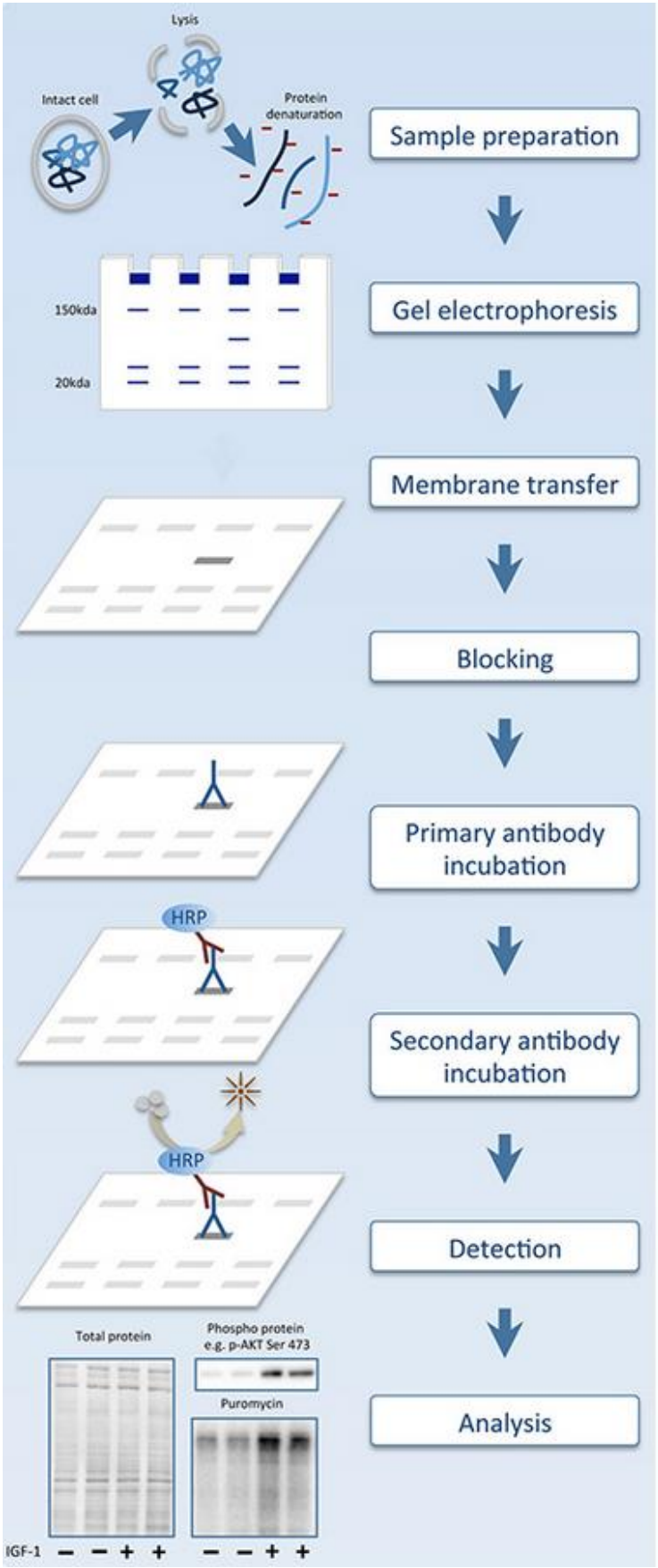
Principles

Western blotting or immunoblotting is a method used for identifying a specific protein in a complex mixture along with determination of its molecular weight. Protein samples are first electrophoresed on SDS-PAGE. In this process proteins migrate through the gel and they are separated according to their size and charge. These separated proteins are electrotransferred onto nitrocellulose/PVDF membrane for further analysis. To detect the protein (antigen) blotted on the membrane it is incubated with an antibody (primary) specific for the protein of interest. The membrane is then incubated with a second antibody (secondary) which is specific for the first antibody. The secondary antibodies are covalently attached to an enzyme, e.g. alkaline phosphatase or horseradish peroxidase. These enzymes form a coloured precipitate upon reacting with a chromogenic substrate. As a result a visible band can be seen on the membrane where the primary antibody is bound to the protein.

Immunodetection: After electrotransfer, proteins bound to the membrane are detected immunologically. This process is known as Immunodetection or Immunoblotting. A suitable blocking reagent (non-fat dry milk/BSA) is used to block the unoccupied sites on the membrane. Then the membrane is probed with a primary antibody specific to the protein of interest. The primary antibody binds to the protein (antigen) and an antigen (Ag)-antibody (Ab) complex is formed on the membrane. The membrane is washed to remove excess unbound primary antibody. It is then treated with an enzyme-labeled (Alkaline phosphatase/Horseradish peroxidase) secondary antibody which attaches

to the primary antibody of the Ag-Ab complex. Finally, the membrane is incubated in a solution containing phosphatase or peroxidase substrate which results in a visible coloured band on the membrane where the Ag-Ab complex is formed. As a result the molecular weight of the protein of interest can be determined.

https://precisionbiosystems.com/western-blot-troubleshooting-guide/



Picture .1 Western Blotting (https://precisionbiosystems.com/western-blot-troubleshooting-guide/)

Reagents

Clarity Western ECL Substrate Kit

Criterion 4-15% TGX Stain-Free Gels

Dithiothreitol (DTT)

Lambda Protein Phosphatase

Laemmli Sample Buffer

Precision Plus All Blue Standard

Precision Plus Unstained Standard

1x TBS 1% Casein Blocking Buffer

10x TGS Running Buffer

Trans-Blot Turbo RTA Mini/Midi Transfer Kit,

10x Tris-Buffered Saline (TBS)

10x Tris/Glycine/SDS (TGS; running Buffer))

10% Tween 20

Materials

Glass wares: Conical flask,

Measuring cylinder

Beaker

Petri dish, staining tray

nitrocellulose membrane

PVDF membrane

container for washing the gel

Distilled water to dilute the washing solution

Measuring cylinder for the preparation of the washing solution

micropipettes,

Glass pipettes

Microtubes

Pipette Tips

eppendorf tubes

filter paper/cellulose wadding to dry the strips after washing

permanent pen

Appliances

Mini Protean Biorad

Mini Trans Blot Module

Shaker

Procedure

1. Phosphatase aplication

1.1.Prepare 20 ml of 1x phosphatase buffer per gel, add 2 µl of 5 M MnCl2 and 10 µl of 2 M DTT per 10 ml of buffer

Prepare the phosphatase buffer just before use.

1.2. Label the chambers of the 6-well mini strip absorbent box as follows:

Spot number:

– Chamber 1 A protein antibody, false

– Chamber 2 A protein antibody, treated with PP

– Chamber 3 Phospho-specific antibody, false

– Chamber 4 Phospho-specific antibody, treated with PP

1.3. Add 3 ml of 1x phosphatase buffer to each chamber of the blotting box.

1.4. Add 5 µl of distilled H2O to each chamber that will contain a membrane that is not phosphatase treated (false).

1.5. Add 5 µl lambda protein phosphatase (LambdaPP)

into each chamber that will contain the membrane to be

treated with phosphatase.

1.6. Cut the membrane between each set of standard stripes.

1.7. Place each strip in the corresponding chamber of the absorbent box and incubate for 2 hours at 37°C per cradle.

1.8. Pour out phosphate buffer

2. Western blotting

2.1. Dilute all required primary antibodies to the desired working dilution (use a 1:1,000 dilution) in Casein Tween blocking buffer.

2.2. Carefully drain the last remnants of TBST buffer from each chamber and add 3 ml of diluted primary antibody to the box.

2.3. Incubate overnight at 4°C on a cradle set at 30 rpm.

2.4. The next day, carefully pour out the primary antibody to avoid contamination between the chambers.

2.5. Add 10 mL of TBST wash buffer and incubate at room temperature for 5 minutes on a shaker set to 150 rpm.

2.6. Drain the washing solution and wash a total of 5 times.

2.7. Prepare the appropriate secondary antibody for the required working dilution in blocking buffer with casein supplementation.

2.8. Add 3 ml of diluted secondary antibody to the appropriate chamber of the absorbent box and incubate for 1 hour at room temperature.

2.9. Carefully pour out the secondary antibody, avoiding contamination from chamber to chamber.

2.10. Add 10 mL of TBST Wash Buffer to each chamber and

Incubate at room temperature for 5 minutes on a shaker.

2.11. Drain the washing solution and repeat step 2.10 a total of 5 times.

3. Antigen immunodetection

3.1. Mix the components of the Clarity Western ECL Substrate Kit in a 1:1 ratio in a clean tube. A total of 1 ml of mixed substrate is needed for each membrane strip.

3.2. Lift the membrane with clean pliers and shake gently until the excess TBST wash buffer drains off.

3.3. Gently apply 1 ml of mixed ECL substrate directly to the top of each membrane, covering the membrane evenly if possible.

3.4. Allow to incubate for 5 minutes at room temperature.

3.5. The result is a visible band on the membrane, only at the site where the primary antibody is bound to the protein.

Test evaluation

A/ Draw and describe the position of proteins on the membrane.

B/ Insert the picture of the membrane after blotting.

Questions

A/ What type of antigen detection method is it?

B/ Why is it necessary to prepare phosphatase buffer just before use?

C/ What is the function of the ECL substrate in this method?