Antigen detection by Western Blotting I.

Gel electroforesis

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.(Picture 1)

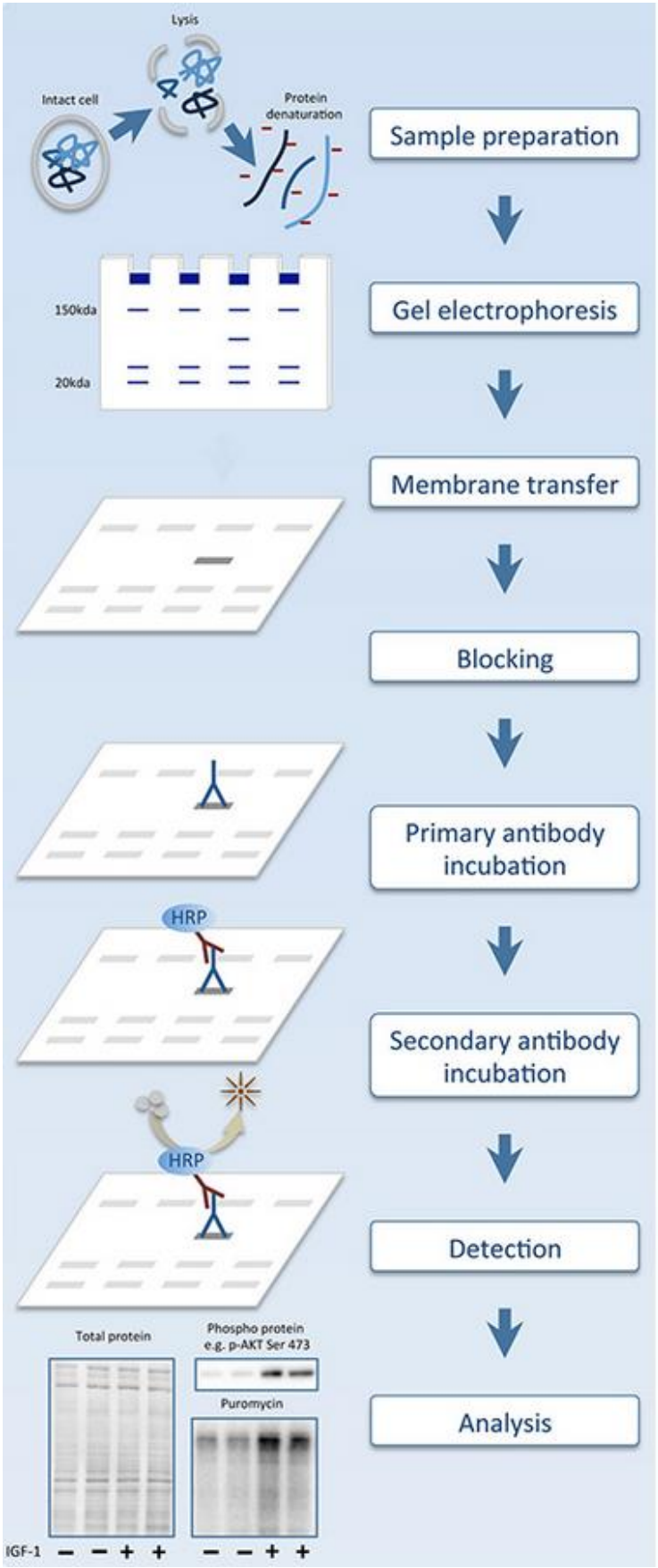
The entire procedure can be divided into following steps:

SDS-PAGE: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in biochemistry, genetics and molecular biology to separate proteins according to their molecular weight. The electrophoretic mobility of proteins depends upon their size. The purpose of SDS-PAGE is to separate proteins according to their size. As proteins are amphoteric compounds, their net charge can therefore be determined by the pH of the medium in which they are suspended. Therefore, at a given pH and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and

charge of molecules. As proteins are high molecular weight molecules, it needs porous gels to get separated. Polyacrylamide gels are those which provide a means of separating proteins by size as they are porous.

Western blotting: Immunoblotting or Western blotting is the electro transfer of resolved proteins from the polyacrylamide gel to the nitrocellulose/PVDF membrane in presence of a specific buffer called transfer buffer. For this transfer procedure, the gel is placed on the membrane and both of them are sandwiched between two filter papers as shown in Figure 1: 4 Fig1: Arrangement of the gel and membrane for electrotransfer This set is placed between two sponge pads and then placed in a plastic cassette. The entire set is then placed inside a gel tank filled with cold transfer buffer. The resolved proteins are transferred to the corresponding positions on the membrane after the electrotransfer. The protein of interest is immunodetected on the membrane.

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. Lysate and reagents preparation

1.1. Prepare TBST Wash Buffer (1x TBS with 0.1% Tween 20 in a 1:1 ratio)

Use 10% Tween 20, 10x TBS, and distilled water

1.2. Prepare Casein Tween Blocking Buffer (1x Casein Blocking Buffer + 0.1% Tween 20), use 1x TBS, 1% Casein Blocking Buffer and 10% Tween 20

1.3. Prepare phosphate buffer (mix 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM MnCl2, 2 mM DTT pH 7.9 at 25°C)

1.4. Prepare 1x TGS by diluting 10x TGS 1:10

1.5. Prepare Transfer Buffer (included in Trans-Blot Turbo RTA Mini/Midi Transfer kit.

1.6. Prepare antigen lysates by diluting with 0.5 mL of PBS

2. SDS PAGE electroforesis

2.1. Prepare 1x TGS Running Buffer by diluting 10x TGS Running Buffer with distilled water 1:10

2.2. Remove the 18-well Criterion 4-15% TGX Stain-Free gel from the packaging (securing the green comb and remove the white tape), rinse the gel wells with dest. H2O to ensure that any air bubbles are removed.

Place the gel in the chamber. Fill the inner chamber with 1x TGS Running buffer to ensure that the wells are covered.

2.3. Mix and paste Precision Plus All Blue Standards and Precision Plus unstained standards in a 1:1 ratio and load 10 µL per well into suitable gel wells.

2.4. Place the lysates in the appropriate gel wells.

2.5. Connect the Criterion Cell to the power supply and set the voltage to 300 V. Continue to power until the front of the dye reaches the bottom of the gel (approximately 20-25 min).

2.6. Remove the gel from the cartridge. Use gel separators.

2.7. Cut well the "fringe" from the top of the gel and the "legs" from the bottom of the gel. Place the gel in the large absorbent opening of the box with 1x TGS Running buffer.

3. Trans blotting of membrane

3.1. Spotless UV activation of the gel is required for imaging.

3.2. Use the Trans-Blot Turbo RTA Midi Transfer Kit

which includes the PVDF membrane needed to image the blot for the total protein without coloration

3.3. Standardisation procedure :. Using a low fluorescent PVDF membrane instead of normal PVDF or nitrocellulose – allows visualization of the fluorescence emitted from an activated tri-halo compound bound to the Tryptophan residues of proteins on the gel that have now been transferred to the membranes.

3.4. When the transfer is complete, take a picture of the membrane

4. Blotting membrane preparation

4.1. Place the membrane in a large absorbent box with 15 mL of blocking buffer to replenish casein.

4.2. Use a pencil to mark the corner of each membrane so that you can distinguish between them later. Incubate 30 min at room temperature (RT).

4.3. The membrane is now ready for blotting

Test evaluation

A/Describe the location of the wells on the membrane

B/ Insert the membrane image before blotting

Questions

A/ What type of detection method is it?

B/ Why is it necessary to prepare the bald patches on the spot?

C/ What is the function of the enzyme in this method?