Introduction

Protocol for Western blot procedure and the sample preparation. With this technique we can detect the presence of the desired protein. To quantify it, we must apply the SDS-PAGE protocol.

Materials

- Samples
- NuPAGE® Bis-Tris Gels (Invitrogen)
- Novex Tris-Acetate SDS Running Buffer (20X)
- Blotting Pads
- PVDF Membrane
- XCell IITM Blot Module (Invitrogen)
- XCell SureLock® Mini-Cell gel running tank (Invitrogen)
- TBS (10X) (Axil)
- Tween-20
- Milk (Common stock)
- Primary & secondary antibodies
- Sodium azide (Life)
- Pierce™ ECL Western Blotting Substrate
- Plastic sheet
- BIORAD ChemiDoc™ Imaging System

Procedure

Boiling Sample Preparation

In an Eppendorf mix 20 µL of cell lysis with 5 µL of loading buffer for each sample and boil at 95 ºC for 5 minutes. Spin down at maximum speed for 1 minute. Loading buffer should be added to the samples under the hood.

Urea Sample Preparation

In an Eppendorf mix 20 µL of cell lysis with 10 µL of loading buffer and 10 µL of urea for each sample and incubate at 37ºC for 15 minutes. Spin down at maximum speed for 1 minute. Loading buffer should be added to the samples under the hood.

Set up Gel Tank

1. Remove the withe tape near the bottom of the polyacrylamide gel and place it in the cuvette.
2. Fill the tank with running buffer and once filled get rid of the comb to expose the wells.
3. Load samples.
4. Connect the tank to the power supply and run the gel at 120 V until the front dye is at the very bottom of the gel and the ladder has separated enough.

Dry Transferring

1. Once the gel has run, with a spatula break the plastic case of the gel by carefully inserting the spatula in the lateral grooves and prying.

2. Cut the gel well fringes and the excess gel. Place the gel in a cuvette and wash it with distilled water for a few seconds.

Prepare the iBlot2 dry transfer kit:

3. Open the kit and place the bottom layer with the plastic into the iBlot2.

4. Lay down the transfer paper, use rolling pin to avoid any bubble.

5. Lay down the running gel centred, use rolling pin to avoid any bubble.

6. Wet-activate the paper layer in distilled water and lay it on.

7. Put the last metal layer and the filter making sure that the anode and cathode of the layers are touching its respective parts of the machine.

8. Run the iBlot2 with the 7 minutes program.

9. Discard everything BUT the transfer paper. Mark the top side with a pencil.

10. Cut the sides of the paper (without touching the transferred region) so that it fits into the cuvette.

Ponceau staining

1. Place the transfer paper in a cuvette and add the Ponceau staining solution (reusable).

2. After couple of minutes, proteins will begin to stain. This indicates protein presence. Cell lysis was done correctly.

3. Return the Ponceau staining solution to the original Falcon.

Fixing and Antibody incubation

1. Prepare 1L of TBST. (in our case 500 mL)

2. In a moving cuvette, wash the transfer paper 3 x 5minutes changing the TBST in between.

3. Prepare the fixing solution (TBTS 4% milk) as follows:
   - In a 50 mL falcon add 40 mL of TBST.
   - Add 1.6 g of milk powder (4%) and mix well.

4. Incubate, in a moving cuvette, the transfer paper with the fixing solution for 15 minutes.

5. Discard the liquid and wash, in a moving cuvette, the transfer paper 3 x 5 minutes changing the TBST in between.

6. Add the primary antibody solution (10 mL of the TBST 4% milk + primary antibody 1:5000).

7. Incubate, in a moving cuvette, for 4 hours (or overnight at 4ºC) with the primary antibody.

8. Wash, in a moving cuvette, the transfer paper 3 x 5 minutes changing the TBST in between.

9. Add the secondary antibody solution (10 mL of the TBST 4% milk + primary antibody 1:1000).

10. Incubate, in a moving cuvette, for 1 hour with the secondary antibody.

11. Wash, in a moving cuvette, the transfer paper 3 x 5 minutes changing the TBST in between.

Western Developing

1. Prepare cassette – line interior with an A4 transparent plastic wallet cut to size, tape to hold.

2. Prepare the ECL reagent.
   - Pipette equal volumes of solutions A & B of the ECL reagent into a clean 1.5 or 15 ml tube – mix by pipetting.
   - For 1 membrane, a total volume of 500ul is enough.
   - Do not leave mixed reagent sitting for too long, exposure signal will be lost.

3. Remove membrane from 1X TBS and dab on paper towel to remove excess buffer.

4. Place membrane, the right way up, on the bottom layer of plastic sheet lining the cassette.

5. Pipette mixed ECL reagent and dispense over membrane.

6. Lower the top layer of plastic sheet and flatten it over the membrane to remove air bubbles and excess solution.

7. Wipe dry spill over at the edges.

8. Remove ALL air bubbles from membrane.

9. Close the cassette lid and proceed with exposure in the dark room.

10. Wait for 6 minutes and develop with with the imaging system.