Western Blot

Protein extraction from adherent cells

1. Wash cells in the tissue culture flask or dish by adding cold phosphate buffered saline (PBS) and rocking gently. Discard PBS.
2. Add PBS and use a cell scraper to dislodge the cells. Move the mixture into microcentrifuge tubes.
3. Centrifuge at 1500 RPM for 5 minutes and discard the supernatant.
4. Add 180 µl of ice cold cell lysis buffer with 20 µl fresh protease inhibitor cocktail. (Make sure the protein concentration is high enough, if not it is advised to repeat the procedure with a higher proportion of protease inhibitor cocktail)
5. Incubate for 30 minutes on ice, and then clarify the lysate by spinning for 10 minutes at 12000 RPM at $4^{o}C$.
6. Transfer supernatant (or protein mix) to a fresh tube and store on ice or frozen at -20 ^{o}C or -80 ^{o}C.
7. Measure the concentration of protein using a spectrophotometer.

Sample preparation

1. Put 50µg of protein extract in each well
2. Add 5 µl sample buffer to the sample, and make the volume in each lane equalized using ddH_2O. Mix the buffer and the sample.
3. Heat the samples with dry plate at $100^{o}C$ for 5 minutes.

Gel preparation

1. After preparing the 10% stacking gel solution, assemble the rack for gel solidification.
2. Add stacking gel solution until the level Is equal to the green bar holding the glass plates. Add H_2O to the top. Wait for 15-30 minutes until the gel solidified.
3. Overlay the stacking gel with the separating gel, after removing the water.
4. Insert the comb, ensuring that there are no air bubbles.
5. Wait until the gel solidified.

Electrophoresis

1. Pour the running buffer into the electrophorator.

2. Place the gel inside the electrophorator and connect to a power supply.
3. Make sure the buffer covers the gel completely, and remove the comb.
4. Load 6µl of marker and 15 µl of samples into each well.
5. Run the gel with low voltage (60 V) for separating gel ; higher voltage (140 V) for stacking gel.
6. Run the gel around an hour, or until the dye front runs off the bottom of the gel.

Electrotransfer

1. Cut 6 filter sheets to fit the measurement of the gel, and one polyvinylidene fluoride (PDVF) membrane with the same dimensions.
2. Wet the sponge and filter paper in transfer buffer, and wet the PDVF membrane in methanol
3. Separate glass plates and retrieve the gel
4. Create a transfer sandwich as follows:
   Sponge, 3 Filter Papers, Gel PVDF, 3 Filter Papers. (Make sure there is no air bubbles between the gel and PVDF membrane)
5. Relocate the sandwich to the transfer apparatus, which should be placed on ice to maintain 4oC. Add transfer buffer to the apparatus, and ensure that the sandwich is covered with the buffer. Place electrodes on top of the sandwich, ensuring that the PVDF membrane is between the gel and a positive electrode.
6. Transfer for 90 minutes.

Recipe

1. Dissolve the following in 800 ml of ddH_2O
   - 8.8 g of NaCl
   - 0.2 g of KCl
   - 3 g of Tris base
2. Add 500µl of Tween-20
3. Adjust the pH to7.4
4. Add ddH_2O to 1L
5. Sterilize by filtration or autoclaving

Reference:

Tahrin Mahmood and Ping-Chang Yang. “Western Blot: Technique, Theory and Trouble Shooting”. *North American Journal of Medical Sciences*