WESTERN BLOT PROTOCOL

Written by: Ruhina Rafiq

Protocol Sections:

A. Sample Preparation
B. Gel Electrophoresis
C. Protein Transfer
D. Blocking & Antibody Incubation
E. Protein Imaging

Tools and Reagents:

House-made Solutions (for each section):

A. 2X Sample Buffer
- 0.09M Tris-HCl, pH 6.8
- 20% Glycerol
- 2% Sodium Dodecyl Sulphate (SDS)
- 0.02% Bromophenol Blue

B. 10X Tris-Glycine-SDS (Running Buffer)
- 30g Tris Base
- 144g Glycine
- 10g SDS
- Adjust pH to 8.3
- dH_2O to 1L

1X Running Buffer
- 100 mL 10x Tris-Glycine-SDS
- dH_2O to 1L

C. 10X Tris-Glycine (Transfer buffer):
- 30 g Tris Base
- 144 g Glycine
- Adjust pH to 8.3
- dH_2O to 1L

1X Transfer Buffer
- 200mL 10x Tris/Glycine

- 400mL methanol
- dH_2O to 2L

D. Tris Buffered Saline + 0.1% Tween (TBST):

- 50mM Tris, pH 7.4
- 150mM NaCl
- 0.1% Tween 20

Tools and Reagents (for each section):

A. B-Mercaptoethanol (BME), (320, fridge)

B. Precision Plus Protein Kaleidoscope Ladder (321, -20 top shelf, 2nd rack, "Rosenthal Lab WB" labelled Box)
Mini-Protean TGX gradient gel/Criterion TGX gradient gel (320 fridge)
Buffer Dam (WB drawer, 319 side)
Mini-Protean Tetra Cell/Criterion Electrophoresis Cell (Top shelf, 319 side)
Bio-Rad Power Pac Basic/HC (Top shelf, 319 side)

C. Gel Plate Opener
Assembly Tray
Gel Holder Cassette
2 Filter pads
2 Criterion Blot Filter papers
0.45um nitrocellulose/PVDF membrane
Roller
Blunt end forceps for handling membrane
Container to hold gel and membrane
(WB drawer, 319 side)
Criterion Blotter Buffer Tank (Top shelf, 319 side)
Ice pack (321, -20 bottom drawer)
Bio-Rad Power Pac HC (Do not use Basic, cannot take the load!)
**Methanol (ONLY if using PVDF membrane, Flammable chemicals cabinet)

D. Different sized containers to hold membrane pieces (WB drawer, 319 side)
Ponceau S Solution, (white chemical cabinet, 3rd shelf)
Superblock T20 (TBS), (320, fridge)
Primary Antibody (321, -20 top shelf, 2nd rack, "Rosenthal Lab WB" labelled Box)
Secondary Antibody (320, fridge)

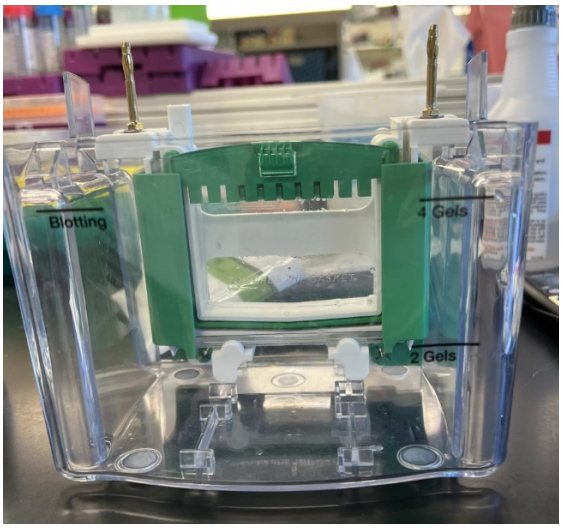
E. Super Signal West Femto Kit (320, fridge)
1 Criterion Blot Filter Paper
2 clear plastic pieces (WB drawer, 319 side)
Flash Drive
Imager (Lillie 330)

Steps:

A. SAMPLE PREPARATION

1. Aim to load 5ug of protein per well. Max. 30uL can be loaded per well. Calculate sample and buffer volumes accordingly.
2. Add BME in 2x Sample Buffer at a 1/20 dilution. Perform in fume hood as BME is a hazardous chemical.
3. Mix protein sample and 2X Sample Buffer w/ BME in a 1:1 ratio. Vortex and spin briefly, then incubate at 95C for 10-30 mins.

B. GEL ELECTROPHORESIS



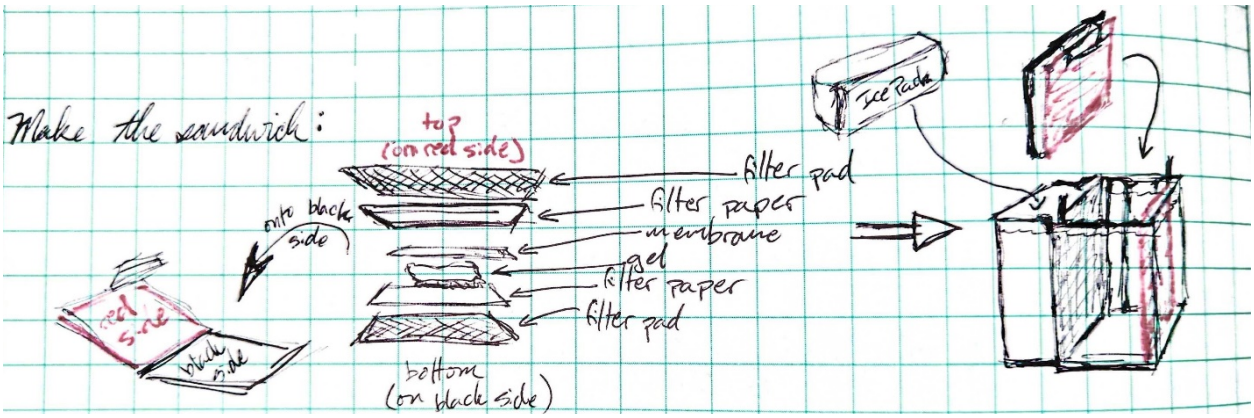
https://www.youtube.com/watch?v=XnEdmk1Sqvg

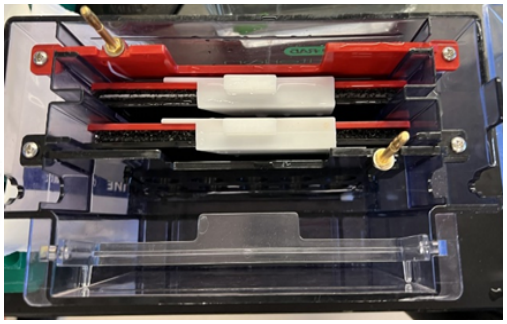
1. Select Mini-Protean TGX Gel (10 wells) or Criterion TGX Gel (18 wells). If selecting Mini-Protean option, remove green blocking sticker at the bottom.
2. Fit into the electrode assembly module with comb side facing inwards towards the gasket. The goal is to create a closed system, so the other side can either have another gel or a

buffer dam in place. Lock the assembly frame and place inside the Mini-Protean Tetra Cell. (Enure the black and red colored electrodes match its placement in the cell). For Criterion, one gel is enough to create a sealed chamber, separate cell and electrode assembly module set up is used.

3. Pull comb out, maintaining a straight and upwards motion to minimize distorting the well walls.
4. Pour fresh Running Buffer into the inner chamber created by the assembly frame. Pause to check if any solution is leaking to ensure a tight seal has been established. If so, fill to the top with buffer.
5. Can use recycled running buffer to fill the outer chamber up to the 2-gel mark.
6. Use pipette tips to straighten any well walls that got distorted during comb removal.
7. Pipette up and down in each well with the running buffer to wash out any excess polyacrylamide debris in them.
8. Load 5uL of Precision Plus Protein Kaleidoscope ladder and appropriate volume of samples. (Be slow and gentle when loading as sample can easily spill over to the next well when loading large volumes).
9. Run at 160V for 45 minutes. Check to see bubbles. (Ideally the visible blue band should have run till the bottom of the gel without running off when stopping).

C. PROTEIN TRANSFER





1. Set up the assembly tray, with the larger chamber having the black side of the transfer sandwich cassette facing down and the red side facing outwards. (It will fit into the dish in only one orientation). In the other chamber place the roller, 2 filter pads and 2 filter papers (per 1 gel) soaked in transfer buffer.
2. Cut out the nitrocellulose membrane in the desired size for the gel and place in a separate container in transfer buffer to equilibrate first, for at least 5 minutes. If using PVDF membrane, soak in Methanol for 5 minutes first to activate it. ONLY handle membrane with blunt ended forceps touching the edges.
3. Take out gel plate and rinse in dH_2O to remove any SDS. Pry open the plate with the gel opener directed at the well side. Place the plate side with the gel on it in a container with transfer buffer and the gel will peel off. Can tap the plate on the edge of the container to help gel peel off. Let it equilibrate for at least 5 minutes. (Handle gel with gloved hands as handling with forceps can lead to it tearing off).
4. Assemble set up, starting with the black side of the cassette, followed by a filter pad, a filter paper and then the gel.
5. When placing the gel, as it will flip during the transfer, make sure the first lane with the ladder is on the rightmost side. Use the roller to squeeze out any air bubbles and straighten its positioning. Add transfer buffer on top with a transfer pipette to keep gel from drying out.
6. Cut the top edge of the membrane diagonally to serve as a marker and place the membrane on top of the gel with the cut edge aligned with the first lane of the gel (rightmost side of the set up). Use roller again to squeeze out any air bubble forming between the membrane and the gel. Add some transfer buffer on top keep membrane damp. Place filter paper on top, repeat the rolling process. Place the filter pad on top, then close the gel holder cassette.
7. Place it inside the Criterion Blotter Buffer Tank (use the one with the metal plate as it will run faster) and add Transfer Buffer to it. Place ice pack inside tank and top it off with Transfer Buffer until the gel holder cassette is fully submerged. Make sure red side (membrane) of the cassette faces red and black side (gel) faces black in the buffer tank. The current will move through the gel and towards the membrane which will move the proteins and "transfer" them onto the membrane.
8. Run at 90V for 30 minutes or 80V for 45 minutes. (May recycle transfer buffer 3 times).

D. BLOCKING & ANTIBODY INCUBATION

1. Disassemble the sandwich and take out the membrane with blunt forceps, touching only the corner. The cut-out side of the membrane being on the left side should be the correct orientation. Place it in an appropriately sized container and wash 5 minutes on a shaker plate (orbital shaking for optimal wash coverage) at RT, with just enough Ponceau S

solution to submerge the membrane. (Careful not to touch the membrane or let it dry out).

2. Rinse it out in dH_2O to wash off residual Ponceau S solution. Verify that the protein transferred, as the Ponceau S will stain the protein. Trim membrane if necessary to only have the protein samples on it (make it as small as possible) and cut membrane into appropriate segments for incubation with different antibodies.
3. Wash for 5 minutes in TBST on orbital shaker plate at RT to remove Ponceau S stain.
4. Block membrane for 30 minutes in Superblock T20 (TBS) on orbital shaker plate at RT.
5. Dilute primary antibody *e.g.* 1:2500 in blocking buffer (ratio can change depending on the antibody, this is the dilution used for Anti-FLAG antibody). Volume should correspond to the size of the sealed container that will contain the membrane, making sure the membrane stays fully submerged in the solution overnight. (*e.g.* 1ul in 2.5mL for small containers, 2ul in 5mL for medium containers)
6. Incubate membrane with primary antibody overnight on orbital shaker plate at 4°C
7. Next day: wash 6X for 5 minutes in TBST on orbital shaker plate at RT.
8. Dilute secondary antibody *e.g.* 1:20000 in blocking buffer (*e.g.* Anti-FLAG antibody takes the HRP goat anti-mouse secondary).
9. Incubate for 1 hour in secondary antibody on orbital shaker plate at RT.
10. Wash 6X for 5 minutes in TBST on orbital shaker plate at RT.

E. PROTEIN IMAGING

1. Prepare substrate from Super Signal West Femto kit. Mix equal parts of the two reagents and vortex briefly. Measure membrane size and make 50uL substrate per cm^2 of membrane.
2. Remove membrane from TBST, dab edge of membrane on a filter paper, holding the side of the membrane carefully with a blunt forcep to get excess TBST off. Place membrane back into a fresh container and promptly pipette the substrate over the membrane dropwise so all the surface area is covered.
3. Incubate membrane and substrate for 5 minutes at RT w/o shaking.
4. Pick membrane up by the corner and dab edge on a filter paper to get the substrate off then place it on top of a clear piece of plastic. Place another transparent plastic piece on top to create a sandwich. Make sure to squeeze out any air bubbles forming on the membrane.
5. Substrate is photosensitive so keep membrane covered when transporting it to the imaging station.
6. Imager is in Lillie 330 (Room is locked after 5pm, need keys to enter after hours). Click “Resume” and wait for “CCD Status” on screen to turn from orange to green. Use the chemiluminescence setting with the colorimetric marker option.

7. Use the black tray with the white insert. Place membrane within the square marking on the tray and insert into imager.
8. Image first with the "Auto" setting for exposure. Save image on a flash drive, which can be attached directly to the equipment. Follow up with adjusting exposure time manually to get image of desired quality.
9. Discard membrane or if interested in reprobing then strip membrane overnight in TBST on an orbital shaker at 4^{o}C and return to Section D of the protocol or store in a sealed plastic bag at 4^{o}C.