Western Blot

NOTE: Dilution of working stock NE cell lines is done with the same buffer it is extracted with, which is Nuclei Lysis Buffer +PI's. NLB will sometimes form cloudy precipitate due to SDS. This does not affect use. Just make sure you mix thoroughly when used.

1) For WCNE made in this protocol, make 1mL of RIPA +PI's on ice (see previous recipe). For stock Nucelar Extracts for various cell lines, make 1mL of Nuclei Lysis Buffer (NLB) +PI's (see recipe below).

1mL Nuclear Lysis Buffer + PI's:

Final Concentration	Stock Concentration
50mM Tris pH 8.1 ----------	50µL 1M Tris pH 8.1
10mM EDTA ----------	20µL 0.5M EDTA
1% SDS ----------	50µL 20% SDS
Aprotinin ----------	13µL
Leupeptin ----------	1µL
PMSF ----------	10µL
H2O ----------	856µL

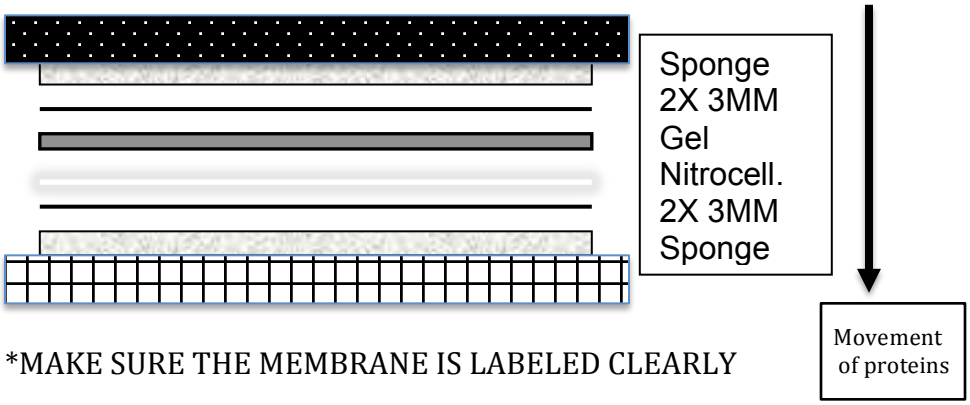
1) Prepare sample NE or WCNE for Western Blot pre-cast gels. Make appropriate volume amount for either 15-well or 12-well precast gel. Load 40µg of NE or WCNE per well. Range can be between 10µg to 40µg.

Final Solution	**12-Well Precast Gel Working Solution (20µL)**
1x Laemmli Sample Buffer (LSB) ----------	5µL 4x LSB
10% **β**ME ----------	2µL 100% **β**ME
40µg NE or WCNE in NLB/RIPA+PI's ----------	13µL

Final Solution	**15-Well Precast Gel Working Solution (15µL)**
1x Laemmli Sample Buffer (LSB) ----------	3.75µL 4x LSB
10% **β**ME ----------	1.5µL 100% **β**ME
40µg NE or WCNE in NLB/RIPA+PI's ----------	9.75µL

2) Heat at 95 degrees for 5 minutes. Longer denaturing at this time can lead to aggregates. **You may freeze samples at this point and continue at a later time.**
3) While samples are boiling, prep the gel box for running a protein gel and thaw Protein Marker.
    a. Obtain proper gel box and make up 1X TGS Running Buffer from 10X TGS. (800mL for 1-2 gels or 1100mL if running at >200V; 1100mL for 3-4 gels)
    b. Obtain gel, remove the comb & REMOVE TAPE!
    c. Place into gel box with shorter wall facing middle of box. Make sure the sides line up with the seal (ask Heather if you don't know exactly what this means! It's hard to explain on paper and better in person) and that the gel is seated correctly at the bottom.
    d. Rinse the wells with running buffer and straighten the sides of the wells if needed.
    e. Add appropriate amount of **1xTGS** Running Buffer (not TBS buffer). (~200mL in chamber; 550mL for 1-2 gels or ~1100mL for 3-4 gels or 1-2 gels being run at >200V in the outer chamber)
4) Once samples have boiled for 5 minutes and cooled down, quick spin, load all of samples into appropriate wells and 10µL Protein Marker (possibly more when protein target is 20kDa or lower).
5) Run at 100V for ~90 min (200V for ~40 min or 300V for ~20 min). Stop run when dye band runs past the reference line, which is the black line near the bottom of the gel.

6) While the gel is running, prepare for transfer by obtaining whatman paper roll, transfer box, transfer black/white sandwich clamp, membrane roll, something to roll out bubbles and glass dishes. Label the membrane clearly to avoid confusion later.
    a. Prepare 1xTransfer Buffer (Tris/Glycine) with 10% methanol and get cold ahead of time. Make 1700mL for each transfer box which will allow excess buffer to soak the items below. If your protein is very large or small, you can add SDS and/or change the percentage methanol. We use Nitrocellulose with **0.2µm pores, so the potential of smaller sized proteins transferring through membrane is greatly reduced.** Check the Abcam website for recommendations at: http://www.abcam.com/ps/pdf/protocols/WB-beginner.pdf
    b. Cut 4 pieces of whatman paper slightly larger than gel and one piece of membrane to size of gel.
    c. In a container with Transfer Buffer, soak pieces in the following order:
    i. Sponge
    ii. 2 whatman
    iii. membrane (labeled clearly – tears easily)
    iv. 2 whatman
    v. sponge
    d. Use gel opening tool to open plastic enclosing the gel and remove the top of gel with wells and sides of the gel with green plastic cutting tool.
    e. Place pieces in the following order on top of WHITE. You may place stack on black/white plastic sandwich clamp with WHITE part on the bottom after sandwich from sponge to sponge is done.



*MAKE SURE THE MEMBRANE IS LABELED CLEARLY

    f. Close black/white sandwich with clamp.
    g. Place in box following color guidelines and fill with same cold 1x Transfer Buffer to appropriate level. Place ice pack in box to help with reducing heat.
    h. Place in cold room and run at 90V for 1 hour or 15V overnight.
    i. When done make sure to note that the gel did transfer completely. If not, it should be run longer.
    j. Take apart sandwich and keep membrane.
    k. Trim side of membrane if needed.
7) Make enough 1xTBST (1x TBS & 0.1% Tween-20) for preblock, primary antibody incubation, washes and secondary fluorescent antibody incubation.
8) Preblock membrane with 15mL of 1xTBST with 5% milk for 1 hour at room temp.
9) Make up antibody dilution in 1xTBST with 5% milk on ice. Antibody dilution is usually 1:1000, but can range from 1:500 to 1:15,000 (the larger dilution is for really good antibodies only). If target primary antibody is not mouse, you may also do Nucleoporin

loading control antibody at this point in order to save time later. To save precious antibody, you can use sealed bag with 5-7mL (less volume for membranes smaller than entire gel size).

10) Incubate in cold room overnight on rocker.
11) Next day wash 3 times for 15 minutes with 1xTBST.
12) Perform Secondary Ab for 1 hour at room temp. For secondary fluorescent antibodies, dilution is 1:15,000 (1µL of each in 15mL of 1XTBST with 5% milk). Two secondary antibodies with different fluorescence can be done on the same membrane at the same time. We currently have anti-mouse (680nm in the red channel), anti-rabbit (800nm in the green channel) and anti-goat (800nm in the green channel). The protein ladder shows up in the red channel.
13) Perform Secondary Antibody incubation for 1 hour on rocker at room temp protected from light.
14) If secondary antibodies will be needed again, put secondary antibodies in 1xTBST +5% milk in 15mL tube in fridge protected from light for use later.
15) Wash 3 times for 15 minutes with 1xTBST protected from light.
16) Go to the 7th floor and use the imaging instrument as follows:
17) Wipe glass imaging surface.
18) Pour a few mL of 1xTBST solution from tray with membrane on glass surface.
19) Place membrane facing down and upside down.
20) Open Odyssey Software.
21) Go to File → New → Scan → Browse to Folder
22) You will be prompted to enter the password kast.
23) In the option window perform a preview to verify placement and determine best options (doing the lowest resolution and quality is necessary to obtain an image that can be viewed since "preview" button produces an image that is very limiting in what can be seen and manipulated).
24) Once you've determined the membrane(s) are placed correctly, choose the following settings:
    a. Preset: membrane
    b. Res: 84 (84 may be overkill)
    c. Medium quality
    d. Check the 700 and 800 channel boxes
25) Scan
26) Once image is completed, adjust the image color intensities as needed and save.
27) Export images.
28) If Nucleoporin control was not done, repeat steps 10 through 29 with Nucleoporin control antibody, but incubate this primary antibody for one hour only at room temp.