Protocol for General Western Blot Troubleshooting

Note: Before beginning, homogenize tissue and run protein assay for ALL samples!

Day One

Preparing Samples

- Turn on hot water bath, get ice.
- Follow protein assay spreadsheet to pipet precalculated uL of protein into tiny Eppendorf tubes.(A total of 50ug of protein
- Follow spreadsheet and dilute with dH2O to obtain 10 uL total.
- Add 10 uL of 2x SDS blue buffer (see blue) to each tube.
- Add 2 uL of DTT (the smelly stuff) to each tube. Total of 22 uL prepared sample
- Vortex samples
- Spin samples
- Place in hot water bath for 7 Min
- Return to ice

Preparing Electrophoresis

- Open gel package and remove strip and comb. Place in electrobox.
- Pour 50 mL SDS Tris-glycine solution and 450 mL dH2O into center of box
    - 500 mL total
- Pipet standard into furthest well. (12 uL) USE THE LONG TIPS!
- Pipet in each sample with pipettor set to 23 uL.
- Run at 125 mvolts and 50mAmps (the mV is most important) for 150 min (or until the last ladder arrives at the bottom of the gel. Check every 10 min until all samples have reached the bottom of the gel.

Transfer to Membrane -- iBlot

- Get out gel transfer stacks (red and white box)
- Take out iBlot machine, top, bottom, sponge and 1 piece of filter paper.
- Get 1 large weigh boat of ddH2O.
- Cut filter paper in half and soak in the ddH2O.
- Open top and bottom of transfer stack.
- Sponge goes to top of iBlot with the metal touching the corner metal piece.
- The membrane is in the bottom (the clear plate). Label it with initials, date, blot number and test on BOTH halves using alcohol-impermeable marker.
- Open gel cassette, cut off wells.
- Place filter paper on gel, press spatula through the gap in the cassette to put the gel onto your palm. Cut off extra.
- Lay gel flat on membrane with ladder to the left. Roll.
- Repeat with other cassette.
- Cover with gel and copper mesh top, roll. Discard red tray.
- Place this combine assembly into machine and close. Press start.
- When done, wipe electrodes with 70% ethanol and labwipe.

Blocking buffer

- Place membranes in ~**10 mL of (1% BSA -0.1%Tween) blocking buffer and** rock in the refrigerator overnight

DAY 2

- Next day, wash twice while rocking at room temperature, each time with enough volume of PBS-tween (about 10-15ml) shake for 15 minutes.

- Primary antibody
- Place membranes in 10 mL of diluted mu-Opioid receptor primary antibody overnight.
    - Dilution: **1:1000**
    - This antibody is **Anti-Rabbit**

Day 3

- Next day, return primary antibody to container in the refrigerator
- Wash 3 times for 15 min each in 1x PBS-Tween (0.1%)

Secondary antibody

- Put on secondary for 1 hour
    - Anti-Rabbit 1:10,000 (1 µL in 10 mL blocking buffer)
    - Antibody: Goat- Anti-Rabbit

- Wash 3 times for 20 min each in 1x PBS-Tween (0.1%)
    - Before 3rd wash starts, go turn on the developer (it takes 20 min to be ready)
- Pour out 3 mL (4 mL for 2 blots) of each of western blotting detection reagents (GE HealthCare #RPN2209)
    - Combine reagents and gently shake for one minute

Developing

- Dump both solutions on one blot and start time for 30 sec. When the timer stops, dump solution onto next blot. Pour down the sink when finished.
- Wrap in saran wrap, taking care to ensure no wrinkles or bubbles are trapped in the plastic
- Tape blot into developing cassette. Turn off lights.
- Remove film (cut off only what you need) and place on blot. Set timer for desired time and close the cassette when ready.
- After time is up, remove film and feed into developer. Lights can go back on.