WESTERN BLOT

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PROTOCOL

Pre-work:

- Turn heat block to 100°C (If making lysates)
- Allow protease inhibitor (Hopper, upper shelf) to defrost at RT (If making lysates)
- Monday: Seed
- Tuesday: Transfect
- Thursday: Collect & Start Western

Making gels:

1. Clean an appropriate number of 1mm glass plates & front cover plates. Assemble the plates into the green holder. Place a grey rubber piece on the white 2-gel holder and add the plates. Test the seal by adding water between the glass plates and wait 2-5 minutes. Dump water and dry with a kimwipe if seal is ok.
2. For a single 10ml 12% **separating gel** , mix:
    - 2.3ml H_20
    - 3.5ml 30% acrylamide mix
    - 2.0ml 1.5M Tris (pH **8.8**) (w/ SDS)
    - 60uL 10% APS (Bowdish chemical cabinet; lasts 1 week at -20°C)
      → Needs to be made fresh; 100mg APS in 1mL H_2O.
    - 3uL TEMED
    - Notes:
        - Add APS and TEMED immediately before pouring the gel into the mold
        - Can make extra mix as a means to check for gel polymerization
        - Can be done the day before if required. Store at 4°C.

	2 Gels	**3 Gels**	**4 Gels**
H_2O	5.18 mL	7.48 mL	10.35 mL
30% Acrylamide	7.88 mL	11.38 mL	15.75 mL
Tris (pH 8.8)	4.5 mL	6.5 mL	9 mL
APS	135 uL	195 uL	270 uL
TEMED	6.75 uL	9.75 uL	13.5 uL

3. Pour gel into mold and top up with 70% EtOH. Wait ~40min for gel to polymerize.

→ I suck up 1mL into a blue pipette tip and let it rest on the bench. When it is not possible to expel the liquid (ie it has polymerized) from the tip, the gel is set.

4. After polymerization, pour out EtOH and wash with MilliQ water.
5. For a single 5ml **stacking gel**, mix:
    - For a single gel:
    - 1.3ml H20
    - 320uL 30% acrylamide mix
    - 234uL 1.0M Tris (pH **6.8**) (w/ SDS)
    - 20uL 10% APS
    - 1.5uL TEMED
    - Notes:
        - Add APS and TEMED immediately before pouring the gel into the mold
        - Can make extra mix as a means to check for gel polymerization

	2 Gels	**3 Gels**	**4 Gels**
H_2O	2.93 mL	4.26 mL	5.85 mL
30% Acrylamide	720 uL	1040 uL	1.44 mL
Tris (pH 6.8)	530 uL	761 uL	1053 uL
APS	45 uL	65 uL	90 uL
TEMED	3.38 uL	4.86 uL	6.75 uL

6. Pour gel into mold and insert comb. Wait ~10min for gel to polymerize.
7. After polymerization, remove comb and wash with MilliQ water to remove bubbles.

Preparing samples:

1. Aspirate media off cells. Wash with 2.5ml PBS. Remove PBS.
2. Mix 25µl protease inhibitor per 5ml of **NP40** (or **RIPA**) **lysis buffer**.
3. Add 250µl lysis buffer solution to each well.
4. Incubate plates at -80°C for 10min. Grab ice bucket.
5. Scrape all cells into solution.
6. Transfer solution to Eppendorf tubes and spin at 4°C and 13,000rpm for 10min. Discard pellet. Keep supernatants on ice.
    - ➔ **Can freeze at -80°C and stop at this point**
8. Add 250µl DTT per 750µl **3× sample buffer**.
9. Add 50µl 3× sample buffer to 100µl cell solution.
    - Extra 900µl of samples can be stored at -80°C.
10. Boil sample for 10 min.
    - ➔ **Can freeze at -80°C and stop at this point**

Running and transferring samples:

1. Assemble the MiniPROTEAN cassette according to manufacturer's instructions.
   ➔ Use dam on far end if there is an odd number of samples
2. Pour 1.2L **1× running buffer** into the MiniPROTEAN cassette tank.
   ➔ Fill tank to top mark
   ➔ 5x stock, make to 1x by diluting 240mLs of 5x stock to 1.2L with MilliQ water.
3. Load maximum **30µl** of samples into the wells in the MiniPROTEAN cassette.
   - Use 7.5µl protein ladder where necessary (Precision Plus Kalidescope – Common Reagents -20°C)
   - Use long tips to load samples
4. Run for 40min at 200V and RT. Can use 110V for 75 minutes.
   ➔ Check @ 20 minutes; the gel may require longer. Run until the dye is roughly at the green part near the bottom of the MiniPROTEAN cassette.
5. Make transfer buffer (1x)
   ➔ 100mL 10x transfer buffer, 100mL methanol, 800mL MilliQ water.

OPTIONAL: Soak the gel in transfer buffer for 15 min to 1 hour.

6. Soak 2 pieces of Whatman paper (per gel) in transfer buffer along with the sponges (RIGHT BEFORE)
   ➔ Assemble in transfer buffer inside the pyrex dish
7. Soak PVDF membrane in methanol until wet (in little box)
   - Mark PVDF membranes to differentiate them → use PEN
   - Needs to be cut to correct size (use sizer piece of paper)
   - Remember to remove the wells (stacking gel portion) from the gel using a green cutter.
8. Assemble "sandwich": Black plastic -> Sponge -> Whatman paper -> Gel -> PVDF membrane (upside down) -> Whatman paper -> Sponge -> White plastic
   - Make sure there are no bubbles between the gel and PVDF membrane; roll with a 15 mL tube.
9. Place "sandwich" in MiniPROTEAN cassette. Pour 1L **transfer buffer** into tank.
   →Easy way to remember: "Runs to the light", so the black part of the sandwich should face the black part of the cassette, the protein will travel towards the light side of the sandwich (into the PVDF).
   ➔ Add small ice pack to tank
10. Run for 70min at 70V and 4°C.

Antibody staining:

1. Incubate PVDF membrane rocking in 20ml **blocking solution** for 1hr at RT (or 4°C overnight).
2. Add 1° Ab to blocking solution. Incubate 1hr at RT (or 4°C overnight).
3. Discard blocking solution. Wash PVDF membrane 3× with 10ml TBST for 10min per wash.
   ➔ Just eyeball it, make sure its fully covered.
   ➔ Can leave for 1hour if needed
4. Add 2° Ab to a fresh portion of blocking solution. Incubate rocking for 30min at RT. **MAX 1 HOUR.**
   - For anti-mouse and anti-rabbit use at 1:100,000 to 1:200,000 (if using the ones from Jackson that we have).
   - Fiona has a working solution of anti-mouse (Franklin, Fiona's shelf). Use at 1:10,000 dilution (2µl per 20ml).
   - Anti-rat (ED31) working solution is available in the HRP box (Curie, top shelf). Use at 1:20,000 dilution (1µl per 20ml)

5. Discard blocking solution. Wash the PVDF membrane 3× with 10ml TBST for 10min per wash.

Visualizing samples:

1. Place a piece of Parafilm on a flat surface and place the membrane protein side up on top.
   ➔ If more than 1 membrane, overlap around 0.5cm.
2. Mix together solutions in the **ECL kit** according to the manufacturer's instructions. Use ~2ml per gel.
   ➔ **1mL of each solution**
   ➔ **ECL Kit is GE Healthcare White/Orange box in Fridge**
   ➔ **DO NOT MIX TWO SOLUTIONS UNTIL RIGHT BEFORE** (ie when gel is on parafilm)
3. Place membrane in cassette and expose to film. Start with 2min and expose for longer periods afterwards as necessary.
   ➔ Fold film in the top right corner
   ➔ Don't swish it around, just place properly the first time

Antibody stripping (optional):

1. Incubate membrane in MeOH for 10min.
2. Remove MeOH. Wash 3× with TBST for 5min per wash.
3. Apply 20ml stripping buffer. Incubate rocking for 1hr. Do this twice, changing buffer in between.
4. Remove stripping buffer. Wash 3× with TBST for 5min per wash.
5. Incubate membrane in 8ml of blocking solution for 1hr.
6. Apply 1° Ab. Incubate overnight.
7. Continue at **Step 3** of **Antibody staining**.

Protocols for making Western blotting solutions:

Running buffer

- Made from a 5× stock. Add 960ml MilliQ H_2O to 240ml stock solution (5x).
- Making 5× stock:
    - 15.1g Tris base (25mM)
    - 94g glycine (250mM, pH = 8.3)
    - Add 500ml MilliQ H_2O
    - 50ml 10% SDS (0.1%)
    - Top up to 1L with MilliQ H_2O.

Transfer buffer

- Made from a 10× stock. Add 800ml MilliQ H_2O and 100ml MeOH to 100ml stock solution.
- Making 10× stock:

Blocking solution

- 5% skim milk powder dissolved in TBST
    - 100ml solution = 5g milk powder + 100ml TBST
    - Stir for 20min
- Making 1L TBST:
    - ➔ **In large flasks by computer**
        - 100ml 10× TBS
        - 900ml MilliQ H_2O
        - 1ml Tween 20
        - Stir 5min
- Making 10× TBS:
    - 500mM pH 7.4 Tris
    - 1500mM NaCl