1. Thaw **samples** on ice. Turn on hot plate to 70° C. Take the **NuPAGE LDS Sample Buffer (4x)** out of the fridge and allow to get to room temperature. Find the ladder in the fridge too, and put it on ice (**Precision Plus Protein All Blue Standards**).
2. Make a stock bottle of each: **1x TBS**; **1x TBS-T with 0.1% Tween**; and **1x MES SDS NuPAGE Running Buffer**
3. Ensure that your samples are diluted properly in the same diluent.
4. To prepare samples for gel loading, mix **samples**:**NuPAGE LDS Sample Buffer (4x)**:**DI H_2O** in a ratio of 1:1:2. Make a **ladder**:**DI H_20** solution in a ratio of 3:2. This is now the **ladder solution**.
    a. To determine how much to load in each well, remember to use 12ul of **samples** in the sample wells, and use 5ul of **ladder** in the ladder wells.
5. Heat the sample mixtures on the hot plate for 10 minutes.
6. While sample heat, get the **pre-cast gel** out of the packaging, remove the white tape, and place it in the gel rig with the tape side on the outside. Be sure to lock the gel in place by snapping the white lever to the center location. Fill the rig with the **MES SDS NuPAGE Running Buffer**. Ensure the center chamber is watertight and fill it to the top, and fill the side compartment up to where the tape used to be on that side. Gently remove the **gel comb**.
7. Take samples off the hot plate, and begin loading the gel. If there are fewer samples than wells, use the center wells first instead of starting on one end and only going halfway across the gel.
8. Load 12ul of **sample**/well and 5ul of **ladder solution**/well. Place the cover on the rig and start the power source.
9. Wait for the dye front to move all the way down the gel before unplugging the electrodes and turning off the power source. Use the **protein spatula** to separate the two pieces of the gel plastic, cutting off the fringe pieces and removing the bottom strip of the gel.
10. Gently, slowly, and carefully let the gel fall off the plastic piece into the **20% ethanol solution** in a pipette lid tray.
11. Follow transfer directions on the iBlot2 tutorial for the next few steps. Familiarize yourself with the process before reaching this point, ideally.
12. Following transfer to the membrane via the iBlot2 process, remove the **top stack**, **filter paper**, and the **pre-run gel**. Pick up the **membrane** very softly with the available **forceps**. Label the side of the membrane that was <u>not</u> in contact with the gel. Use the **Li-Cor pen** to reduce background fluorescence later, **not Sharpie**.
13. After labeling with the **Li-Cor pen**, flip the **membrane** over again with the forceps, so the side that was touching the **gel** is facing up. Use a new **razor blade** to cut the **membrane** into sections and place each section in the appropriate **pipette box lid tray** on the **rocker,** with the gel-facing side **UP**.
14. **BLOCKING:** Pour some **5% Non-Fat Dry Milk (NFDM)** on each membrane at **room temperature** for **30 minutes**.
15. **WASH:** Pour some **0.1% TBS-T** on each membrane, **3 x 5 minutes** at **room temperature**.
16. **PRIMARY AB INCUBATION:** Include these reagents in a 15mL conical tube for each antibody. After adding NFDM, be sure to keep the tubes on ice.

0.25%	**Non-Fat Dry Milk (NFDM)**
0.02%	**Sodium Azide**
0.01%	**Sodium Dodecyl Sulfate**
10 mL	**TBS-T**
Check websites	**Primary Antibody**

OVER →

17. **PRIMARY INCUBATION CONTINUED:** Wrap each tray in Saran Wrap or a Ziploc with the antibody on it and leave in the **4° C cold room** overnight.
18. **WASH: 3 x 10 minutes** at **room temperature** with **TBS-T**.
19. **SECONDARY INCUBATION:** Include these reagents in a 50mL conical tube. Be sure you make enough secondary antibody for each protein of interest. <u>These solutions are light sensitive after addition of secondary antibodies. Keep the tubes under foil or in the dark before adding to the membranes, and after adding to the membranes, wrap the rocker in the foil.</u>

0.25%	**NFDM**
0.02%	**Sodium Dodecyl Sulfate**
30 mL	**TBS-T**
Check websites (680RD and 800CW are 1:15,000)	**Secondary Antibody**

20. **WASH**: **3 x 10 minutes** at **room temperature** with **TBS-T**. <u>Cover with foil.</u>
21. After washing 3x, dry the membrane in a sandwich of **Whatman Paper**. The buns of the sandwich are each 2 pieces of **Whatman Paper**, with the **membranes** in the middle of the stack of 4 **Whatman Papers**.
22. Image the membrane(s) at the **Li-Cor CLx machine** in the Division.
23. Spray rubber pad and imaging surface with 70% EtOH, follow directions written on the cover, and open the Imaging software. Never use metal tweezers with the machine. Ask someone for help with questions. It is often used and many people know how to use it. Remember to place the membranes on the imaging surface face down, so the side that was originally touching the gel in the iBlot2 is on the glass. We are still working out the analysis settings we want to see for our proteins and to get a useable WB. This protocol will be updated when we are ready.