**Western Blot - Electrophoresis**

### Introduction:

This is the standard lab protocol for setting up and running a Western. It was adapted from the Invitrogen protocol and is designed for use with Invitrogen products.

### Electrophoresis Materials:

* Protein samples
* XCell SureLock Mini-Cell
* Gel: NuPAGE Bis\_Tris gel
* Loading buffer: NuPAGE LDS Sample Buffer (4x)
* Running buffer: NuPAGE MOPS SDS Running Buffer (20X)
* ddH2O
* NuPAGE Sample Reducing Agent (10X)
* NuPAGE Antioxidant
* Protein ladder
* 70 degree water bath

### Procedure – Sample Preparation:

1. Heat water bath or heat block to 70 degrees.
2. Thaw protein samples (if not isolating fresh) and protein ladder in ice.
3. Prepare running buffer (900 ml):
* 45 ml SDS Running Buffer + 855 ml ddH2O
	+ 600 ml for lower buffer chamber
	+ 200 ml for upper buffer chamber (add 500 ul Antioxidant within 30 minutes of loading)
1. Prepare protein samples:
* Protein: 10 pairs of ovaries
* Sample buffer (4x): 12.5 ul
* Reducing agent (10x): 5 ul
* ddH2O: 32.5 ul
* total: 50 ul 🡪 5 ul/pair (which is generally a good amount to load)
1. Heat protein samples at 70 degrees for 10 minutes. Remove from bath and place on ice immediately.

### Notes:

* The concentration of the protein sample can change based upon the gel’s well size and the number of ovaries you want to run per lane. Keep the ratio of buffer to reducing agent the same, and be sure to note what concentration you are using.

### Procedure – Electrophoresis:

1. Cut open the gel cassette pouch with scissors and remove cassette.
2. Drain away the gel packaging buffer.
3. Remove the gel cassette from the pouch and rinse with ddH2O.

\*Note: Always handle the cassette by its edges only.

1. Peel off the tape covering the slot on the back of the gel cassette.
2. In one fluid motion, pull the comb out of the cassette. This exposes the gel loading wells.
3. Use a pipette to gently wash the cassette wells with 1X running buffer. Invert the gel and shake to remove buffer. Repeat twice, for a total of three rinses. Fill the sample wells with running buffer. Note: Be sure to displace all air bubbles from the cassette wells as they will affect sample running.
4. Lower the Buffer Core into the Lower Buffer Chamber so that the negative electrode fits into the opening in the gold plate on the Lower Buffer Chamber as shown in the figure.



1. Insert the Gel Tension Wedge into the XCell SureLock™ behind the buffer core. Make sure the Gel Tension Wedge is in its unlocked position, allowing the wedge to slip easily into the XCell SureLock™ unit. The Gel Tension Wedge should rest on the bottom of the Lower Buffer Chamber.
2. Insert gel cassettes into the lower buffer chamber. Place one cassette behind the core and one cassette in front of the core. For each cassette, the shorter “well” side of the cassette faces in towards the buffer core. The slot on the back of the cassette must face out towards the lower buffer chamber (see figure below).



1. If you are running only one gel, replace the rear gel cassette with the Buffer Dam.
2. Pull forward on the Gel Tension Lever in a direction towards the front of the XCell SureLock™ unit until lever comes to a firm stop and the gels or gel/buffer dam appear snug against the buffer core (see figure below).



1. The Upper Buffer Chamber (cathode) is the void formed between the two gel cassettes (or one cassette and the buffer dam) on each side of the buffer core. Fill the Upper Buffer Chamber with 200 mL of running buffer. Use enough running buffer to completely cover the sample wells. Ensure that the Upper Buffer Chamber is not leaking. If the level of running buffer drops, the electrophoresis core and cassettes are not properly seated.

\*\* If running under reducing conditions, add 500 μL of antioxidant to the 200 mL Upper Buffer Chamber within 30 mins of gel loading. \*\*
2. Use pipette to underlay the samples into the gel wells (see figure below). Lower the tip to the bottom of the sample well and slowly pipet sample into well without contaminating another well with the sample. To promote uniform running of the stacking front, load sample buffer in all the wells, whether or not they contain samples.
3. Use Fill the Lower Buffer Chamber (anode) by pouring 600 mL of running buffer through the gap between the Gel Tension Wedge and the back of the Lower Buffer Chamber as shown in the figure below. \*Fill the lower buffer chamber completely (600 mL) as this will help dissipate heat during the run.
4. Align the lid on the Buffer Core. The lid can only be firmly seated if the (-) electrode is aligned over the banana plug on the right. If the lid is not properly seated, no power will go through the mini-cell.
5. With the power off, connect the electrode cords to power supply {red to (+) jack, black to (-) jack}. Turn on the power. Run at ~200V for ~1 hour.

\*Check gel after 5 minutes to ensure it is running straight, if not, reduce voltage or prep a fresh gel

\*While gel is running, prep for transfer.

**Western Blot - Transfer**

### Transfer Materials:

* PVDF
* Wattman paper
* Methanol
* ddH2O
* Transfer buffer (see below)
* Shallow plastic tray for equilibration of membranes, filter paper, blotting pads

### Procedure – Transfer:

1. Prepare transfer buffer:
* Transfer buffer (20X): 20 ml
* Antioxidant: .4 ml
* Methanol: 40 ml
* ddH2O: ~340 ml
* total: 400 ml
1. Use ~200 mL of transfer buffer to soak the blotting pads until saturated. Remove air bubbles by squeezing the blotting pads while they are submerged in buffer. Removing air bubbles is essential as they can block the transfer of biomolecules.
2. 7x10 cm Wattman Blotting paper is slightly too wide for the transfer sandwich apparatus. Trim ~0.5 cm from one edge of each Wattman paper you will need to use.
3. Pre-wet the PVDF membrane for 30 seconds in methanol. Briefly rinse in deionized water and then place the membrane in a shallow dish containing 50–100 mL transfer buffer for several minutes.
4. Soak the watttman paper briefly in transfer buffer immediately before using, paper must be rather saturated to conduct transfer, but don’t soak it so long it loses all rigidity.
5. After electrophoresis, separate each of the 3 bonded sides of the gel cassette by inserting the gel knife into the gap between the cassette’s 2 plates. The notched (“well”) side of the cassette should face up. Push up and down on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated. The gel may adhere to either side of plates upon opening the cassette. Carefully remove and discard the plate without the gel The gel remains on the other plate
6. Remove wells on the gel with the gel knife.
7. Place a piece of pre-soaked filter paper on top of two pre-soaked sponges to begin your transfer sandwich. Keep the filter paper saturated with transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a glass pipette.
8. If necessary, trim the foot of the gel.
9. Wet a smooth spatula with transfer buffer, then lift the corner / edge of the gel up off the gel casing. Gently, pick up the gel with your hands. Briefly rinse it in water, then lay it flat atop the wet filter paper + sponge sandwich.
10. Wet the surface of the gel with the transfer buffer and place pre-soaked transfer membrane on the gel. Remove air bubbles by rolling a glass pipette over the membrane surface.
11. Place the pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles.
12. Place 2 soaked blotting pads into the cathode (–) core of the blot module. The cathode core is the deeper of the 2 cores and the corresponding electrode plate is a darker shade of gray.
13. Carefully pick up the gel membrane assembly with your gloved hand and place on the pad in the same sequence, such that the gel is closest to the cathode plate (see figure below).



1. Add another presoaked blotting pad on top of the first membrane assembly. Position the second gel/membrane sandwich on top of blotting pad in the correct orientation so that the gel is closest to the cathode side. **Note:** To blot two gels at same time, arrange gels as follows:



1. Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components. **Note**: To ensure a snug fit, use an additional pad since pads lose their resiliency after many uses. Replace pads when they begin to lose resiliency and are discolored.
2. Position the gel membrane sandwich and blotting pads in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit.
3. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module fits into the unit in only one way, such that the (+) sign is seen in the upper left hand corner of the blot module. The inverted gold post on the right hand side of the blot module fits into the hole next to the upright gold post on the right side of the lower buffer chamber. Place the front wedge (without the screw hole) such that the vertical face of the wedge is against the blot module. Slide in the rear wedge and push it down firmly. **Note**: When properly placed, the rear wedge will not be flush with the top of the lower buffer chamber. There will be a gap between the rear wedge and lower chamber.
4. Fill the blot module with transfer buffer until the gel/membrane sandwich is covered in transfer buffer. **Do not fill all the way to the top as this will generate extra conductivity and heat.**
5. Fill the outer buffer chamber with ~ 650 mL ddH2O by pouring in the gap between the front of the blot module and front of the lower buffer chamber. The water level should reach approximately 2 cm from the top of the lower buffer chamber. This serves to dissipate heat produced during the run. **Note**: If you accidentally fill the outer buffer chamber with the transfer buffer, it will not adversely affect the transfer. The liquid in the outer buffer chamber serves as a coolant. We recommend adding deionized water to the outer buffer chamber to avoid any exposure of the mini-cell to methanol as the mini-cell is susceptible to methanol.
6. Place the lid on top of the unit. With the power turned off, plug the red and black leads into the power supply. Run at 30V for 1 hour for one gel, possibly longer for two gels. \*Prep milk block while transfer takes place.

**Western Blot - Probe**

### Probe Materials:

* Milk block (5% milk)
* 1X TBST (0.05% tween)
* Antibodies
* ddH2O
* Plastic dish with lid

### Procedure – Probe:

1. Prepare milk blocking solution by vortexing the following in a conical tube:
* TBST: 50 ml
* Milk block: 2.5 g

**Note:** It may be useful to make up all block for procedure at this time, however, keep in mind that milk block spoils over time and spoiled block can alter results.

~ 100 mL TBST + Milk used for one membrane and nine independent antibody incubations

1. Place block in plastic dish with lid, large enough to fit membrane.
2. Turn power supply off, and disassemble sandwich to expose PVDF. Remove PVDF membrane from wattman paper with flat forceps. Quickly, rinse PVDF with ddH2O to remove excess methanol. **Do not allow PVDF to dry out.** Place PVDF immediately into Ponceau stain (see Ponceau protocol) or milk block, a**lways keeping the transfer side up**.Once in milk, snip membrane corner with razor blade to mark top from bottom, left from right.
3. Block for 1 hour at room temperature.
4. Leaving membrane in the dish, pour block off membrane, immediately replacing it with enough TBST to cover the entire membrane. Rinse on orbital for 10 minutes. Repeat 2 times.
5. Mix enough block to cover entire membrane (usually ~15 ml needed depending on size of plastic) with desired concentration of primary antibody. Leaving the membrane in the dish, remove rinse from membrane, and add primary antibody mixture. Shake overnight on orbital at 4 degrees. **Note: Milk will spoil overnight if not refrigerated, which may affect results.**
6. Wash membrane with TBST 3x for 10 minutes each.
7. Mix enough block to cover entire membrane with desired concentration of secondary antibody.
8. Remove rinse and incubate with secondary on orbital for 1 hour at room temperature.
9. Rinse with TBST on orbital at room temperature 3x, for 10 minutes each.

### Notes:

* Never allow the membrane to dry out – have solutions ready so that when one comes off, the next can be immediately added.
* Be sure to keep stay oriented as to which side of the membrane is up and which is top versus bottom, or it can become very tricky to interpret results.

**Western Blot - Detection**

### Detection Materials:

* Detection Reagents
* 15 ml conical tube
* SaranWrap
* Film Cassette
* Film
* Timer
* Paper towels

### Procedure – Detection:

1. During first final TBST wash, turn on developer (in Conti lab dark room) and hit green “run” button.
2. Get out SaranWrap, cartridge and detection kit (stored in big refrigerator). Allow reagents to come to room temperature. Place two clean pieces of SaranWrap flat on bench. Have paper towels on bench.
3. During final TBST wash, combine Reagent A and Reagent B in the ratios specified on their box. Make enough detection to cover membrane entirely (~3-4 ml/membrane)
4. Remove membrane from final wash with forceps, touch corner of membrane to paper towel to remove excess buffer. Then place protein side up on fresh SaranWrap. Immediately pipette detection solution onto membrane. Let sit for 1-5 minutes (time specified by specific detection reagents protocol) **Do not let the membrane dry out.**
5. After incubation, remove excess detection reagents from membrane by lifting membrane with forceps and touching corner to a paper towel.
6. Move membrane to other piece of SaranWrap **protein side up.** Enfold membrane in wrap, so membrane is laminated and remove as many air bubbles as possible.
7. Transfer membrane, cassette, and film to dark room. Bring timer. With white lights off (red light is alright), expose film to membrane in cassette for desired time (30 seconds is a good amount of time to begin optimization).
8. Place film into developer, and let developer process.
9. Repeat 7-8, optimizing exposure time as needed.

### Notes:

* Membranes with excessive signal can be developed with normal ECL reagents (not ECL Plus), rinsed with TBST and then redeveloped, or can be stripped with Restore Buffer to reduce intensity.
* If signal is too weak to develop, rinse with TBST, and redevelop with ECL Plus Reagents.
* If all else fails, run a new gel, transfer, and optimize antibody concentrations.