OTTO LAB WESTERN PROTOCOL

Western Blot: Live De-Cap Checklist

Things You Will Need: Sample Containment		
1- Styrofoam box of ice	1- 100ul pipette w/ tips	4 (per rodent subject)- 50ml tubes- labeled for each specific brain tissue region
1- Homogenizer with two beakers of distilled water	1- 10ul pipette w/ tips	3ml (per rodent subject)- protease inhibitor solution mixed at last minute in lysis buffer (chilled)
Liquid Nitrogen	1- Bottle of Triton X-100	1- Agitating Plate (placed in fridge)
4 (per specific region of brain)- Micro Aliquots (labeled)		
Things You Will Need: Tissue Extraction		
1- Small Rongeurs	1- Large Rongeurs	1- Spatula
1- Flat Scapel Blade (#12)	1- Ice Micro-Dissection Tray (chilled)	1- Curved Forceps
1- Guillotine	1- Body Bag with Plastic	1- Sharp Scissors
Parafilm		

Detailed Protocol:

Pre-Live De-Cap:

1. Fill Styrofoam box with ice- make sure that the box is big enough to hold all **the 50ml tubes (4 per rodent)** you will be using to hold each rodent subject's individual brain regions (both hemispheres of each anatomical region go in one 50ml tube).
2. Make sure you have some liquid nitrogen (you don't need a lot, but I would guess around 200ml).
3. Label all the necessary 50ml tissue tubes with rodent subject number and precise tissue to be held in said tube (ex. "#50 D" means subject number 50, with both hemispheres of dorsal hippocampus). Place in ice to chill.
    a. Do the same for all the micro-aliquots **(4 per individual piece of tissue)** (ex. For both hemispheres of dorsal hippocampus extracted from a subject, you will need a total 4 micro-aliquots for that specific tissue). You will need **16 micro-aliquots per subject assuming you wish to take 4 different regions of their brain**.
4. **Set centrifuge machine to 4^0 C**
5. Make sure micro-dissection tray is in freezer with parafilm across black disk. Replace ice in tray if necessary.
6. Prepare protease buffer at the last minute before live de-cap. **Mix 10ul of protease inhibitor cocktail and 10ul of EDTA solution per 1ml of lysis buffer**. Keep **very cold** in ice. You will be using about ~**.6-.7ml of protease buffer per tissue sample** (.6ml for dorsal hippocampus, .6ml for ventral hippocampus, ect.- it really depends on the size of the extracted tissue; basically cover enough to have a 1cm headspace of buffer above the top of the sample). **IF MAKING LYSIS BUFFER FROM SCRATCH- DO NOT ADD TRITON X-100**. We will be adding it back in later.

Live De-Cap:

1. Guillotine rodent; discard body into body bag and proceed with brain extraction. **BE VERY GENTLE**.
2. Place brain on freshly chilled Micro-Dissection Tray and let it sit for about 10 seconds to firm up a little bit
    a. Flip brain over so you can see the optic chiasm; make a flat cut across the optic chiasm with a #11 razor blade
    b. Make another 2mm cut behind the optic chiasm; flatten slice on tray and extract amygdala; place into chilled 50ml tube.
    c. Flip brain over and use blade to cut across the corpus callosum (do not cut down too deep).
    d. Use spatula to gently roll out the hippocampus; remember the dorsal hippocampus is always to most anterior segment; place both ventral and dorsal hippocampus into their prospective 50ml tissue tubes.
    e. Cut a chunk of cortex as a control; place in 50ml tube.
3. Immediately after micro-dissections, replace parafilm wrap and place tray back into the freezer.
4. **Add 1ml of protease buffer into each 50ml tissue tube**; be sure to push down the tissue fragment into the bottom of the tube.
5. Homogenize the tissue until the liquid becomes light tan. After each tissue sample, run homogenizer in 1 beaker of clean distilled water for 5 seconds and than run homogenizer in another beaker of distilled water to remove tissue contaminants between samples.
    a. After each sample homogenization, **add 10ul of Triton X-100 into each 50ml tissue tube**.
    b. Triton X-100 is very viscous; warm bottle in hand before pipetting and while pipetting, allow the fluid time to crawl up the pipette tip.
    c. When pipetting Triton X-100 into 50ml tissue tube, hold tip just above the protease buffer and let the Triton X-100 drop into the solution; if you attempt to dispense Triton X-100 in the buffer solution, it will be difficult for it to detach from the pipette tip.
6. Once all samples have been homogenized with Triton X-100 dispensed (AFTER homogenization), place all 50ml tubes onto agitating plate in the fridge **at 4^0 C for 2 hours**. Agitate briskly.
7. After agitation, remove tubes from fridge and place back into box of ice; Pipette solution from each 50ml tube into 1 corresponding micro-aliquot.
8. Place micro-aliquots into centrifuge machine; **Centrifuge for 20 min at 12000 rpm at 4°C.**
9. After 20 minutes, remove aliquots from machine and pipette all supernatant (NOT THE PELLETS) into corresponding clean micro-aliquot. Place in ice for the time being.
10. Vortex all aliquots briefly. Place back in the ice.
11. **Split solution in main aliquots into 3** (take $2/3^{rd}$ of supernatant out of these main aliquots-pipette into remaining 2 corresponding micro-aliquots). The goal is to create three micro-aliquots per tissue sample. This will prevent the need to freeze/thaw a sample.
12. Immerse all the aliquots into liquid nitrogen to "freeze snap" the supernatant in it. Store in -80^0 C fridge.
13. Wash 50ml tissue tubes to remove remaining detergent out of the tubes; use acetone to wash sharpie marker off top of tubes.

Nonident-P40 (NP40) buffer 1%: Lysis Buffer		
	Sodium chloride (150mM)	8.8 g/L
	1% NP-40 (Triton X-100 can be substituted for NP-40)	10 g/L Or 9.35ml/L Or 9.35ul/ml

	Tris, pH 8.0 (50mM)	6.1g/L
Halt Protease Inhibitor Single-Use Cocktail (100X)		
*Inhibitors are added fresh to the lysis buffer.	Each 100µl microtube contains sufficient cocktail to treat 10ml of lysate Mix with NP40 buffer before adding to tissue sample Mix EDTA if desired (same concentration as cocktail)	

Bradford Assay (optional)

Things You Will Need:		
Lysis buffer with protease-inhibitors	BSA protein standards	15 micro aliquots
Bradford Reagent	1- 10ul pipette w/ tips	Distilled water
Samples thawed in ice	1- 100ul pipette w/ tips	

Detailed Protocol:

Bradford Assay:

1. Equilibrate all reagents, unknowns, and protein standards and mix thoroughly: prepare the same buffer as used during homogenization

1 ml Standard Assay

Tube #	Standard Volume (µl)	Source of Standard	Diluent Volume (µl)	Final [Protein] (µg/ml)
1	70	2 mg/ml stock	0	2,000
2	75	2 mg/ml stock	25	1,500
3	70	2 mg/ml stock	70	1,000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8 (blank)	–	–	70	0

2. Prepare **7 standard aliquot dilutions (standard)** following the chart above: (Dilute with exact same lysis buffer **without any samples inside**)
3. Remember to vortex each aliquot before moving to the next aliquot- or else the concentrations will be off.
4. **Next we have to make the standard-Bradford assays. Use a 1:30 sample to Bradford reagent ratio**: add 10ul of diluted standards made above to 300ul of working reagent. Do this for each protein concentration.
5. Mix well via gentle vortexing and allow **incubating for at least 5 minutes, no more than 1 hour at room temperature**
6. While the standards are incubating, prepare the unknown lysates dilutions. Normally 10ul of the unknown homogenate mixed with 300ul of the Bradford reagent will lead to a signal that is far

greater than the machine can read (the absorption is way too high so any protein concentration estimate will be off).
    a. As a result, you will need to prepare dilutions of the sample for analysis
    b. For example, a 1:5 dilution can be prepared by combining 2ul of the unknown lysates, 8ul of pure (sample-free) lysis buffer, and 300 ul of Bradford reagent.
    c. A 1:10 can be prepared by combining 1ul of unknown lysates, 9ul of pure lysis buffer, and 300ul of Bradford reagent.
    d. Normally one should make 1:5, 1:7, 1:10, and 1:20 dilutions to cover the possible ranges of protein concentrations.
    e. **Remember**- when given the concentration of any of the dilutions, you must multiply that protein concentration by the amount diluted. A 1:5 sample reading of 100 ug/ml will actually be 500ug/ml.
7. **Using BSA standards halves the actual protein concentrations**- so when using BSA standards, remember to also double any concentration given to you before factoring the dilutions.
8. **Distilled water** is to be used as the "blank"
9. **10ul of pure lysis buffer mixed with 300ul of Bradford reagent** will be your "reference sample".
10. On the nanodrop machine- select "Protein Bradford" from the home screen. If the wavelength verification window appears, ensure that the arm is down and select "okay"
11. Enter the values of each standard concentration in the right panel- all standards can be measured in replicates.
12. When all standards have been measured, measure the samples. Record and input into sample excel sheet.
13. Once you have determined the concentration of each sample, **you can freeze them at -20°C or -80°C** for later use or prepare for immunoprecipitation or for loading onto a gel.

Theory Behind Procedure:

1. The purpose of the Bradford assay is to determine HOW MUCH protein concentration is in the supernatant that you obtained from the live-decap procedure. Basically, the first step is to create a standard curve with each of the 7-control aliquots. The Bradford assay turns different shades of blue depending on how much protein is in the solution and a nano-drop machine reads the change in light absorption for each aliquot. Since for the standards we KNOW the protein concentration (since we made them from scratch), we can make a standard curve telling us what absorption correlates to how much protein in the supernatant.
2. After we make the standard curve, we than measure the actual unknown sample concentration- the reason we dilute the unknown is because frequently, the concentration of pure unknown supernatant is way beyond the standard curve we created. This is bad because the model we created can only accurately predict protein levels within a certain range (max 1,500ug/ml). After that, the model can't tell the difference between say 2,000 ug/ml to 10,000 ug/ml.

Preparation of Samples for Loading into Gel/ Electrophoresis

Things You Will Need:		
1- Laemmli Sample Buffer (chem. cabinet)	1- hot water bath (set to 70^0 C)	1 micro-aliquot per protein lane (labeled)
1- B-mercaptoethanol (western room)	1- 10ul pipette w/ tips	Distilled water
Samples thawed in ice	1- 100ul pipette w/ tips	1- Agitating Plate (placed in fridge)
Things You Will Need: RIGHT AFTER for Electrophoresis		
1L Running Buffer	TGX Pre-Cast Gels	BioRad Precision Plus Western C standards (thawed)
1- 10ul pipette w/ tips	1- 100ul pipette w/ tips	Electrophoresis Tank

Detailed Protocol:

Pre-Sample Preparation:

14. Set hot water bath to **70^0 C FIRST**
15. Retrieve supernatant samples from -80^0 C fridge and thaw in ice; boxes are labeled with "OTTO" in fridge.
16. Label 1 micro-aliquot per lane to be run down the gel; each lane will consist of 1 specific supernatant sample.
17. Mix up Laemmli Sample Buffer/ B-mercaptoethanol solution. You want a **5% B-mercaptoethanol solution** (you will use 20ul of this buffer solution per lane you plan on running). **For 8 wells, I usually mix 10ul of B-mercaptoethanol with 190ul of Laemmli Sample Buffer**. Vortex well.
18. Fill 1 separate micro-aliquot with distilled water.

Sample Preparation:

1. In each lane micro-aliquot, pipette in 20ul of Laemmli Sample/ B-mercaptoethanol solution.
2. Next, pipette 15ul of distilled water into each micro-aliquot
3. Pipette 5ul of the corresponding protein supernatant into each micro-aliquot
4. Vortex all the aliquots, than place in water bath for 10 minutes. After the 10 minutes, vortex all aliquots briefly.
5. During those 10 minutes, make sure the running buffer, protein markers, and gels are all ready to go.
    a. Take protein markers out of freezer to thaw
    b. Take gels out of fridge and remove plastic covering; rinse wells briefly with distilled water.

Theory Behind Procedure:

1. The purpose of the sample preparation step is to prepare the proteins for resolution in the gel. B-mercaptoethanol is critical to breaking the disulfide bonds in native protein structures, while SDS coats the proteins in a constant negative charge. The end result is a flattened protein strand that can run evenly down the gel towards the POSITIVE charge.

Electrophoresis:

1. Use Mini-PROTEAN ® TGX™ Precast Gels (8.6 x 6.7 x 0.1 cm) and place into electrophoresis tank.
    a. Remove the Ready Gel from the storage pouch. Gently remove the comb and rinse the wells thoroughly with distilled water or running buffer.

   b. **Pull the clear tape at the bottom of the Ready Gel Cassette to expose the bottom edge of the gel**.
   c. Load into the Inner Chamber Assembly- short plate facing inwards.
   d. Note: If only one gel is to be run, use the mini cell buffer dam.
2. Lower the Inner Chamber Assembly into the Mini Tank. **Fill the inner chamber with ~125 ml of running buffer until the level reaches halfway between the tops of the taller and shorter glass plates of the Gel Cassettes.**
3. **Add running buffer to the Mini Tank till it hits the marked line.**
4. Use special gel loading tips or a micro-syringe to load the complete sample in a narrow well. Take care not to poke the well bottom with the tip as this will create a distorted band.
5. Never overfill wells. This could lead to poor data if samples spill into adjacent wells, and poorly resolved bands.
6. Load **20ul from each aliquot per mini-gel well**. On the left most well, add 5µl of full range rainbow marker to follow the protein resolution as well as indicate a successful transfer.
7. Place the lid on the Mini Tank. Make sure to align the color coded banana plugs and jacks. **The correct orientation is made by matching the jacks on the lid with the banana plugs on the electrode assembly**. A stop on the lid prevents incorrect orientation.
   a. Apply power to the Mini-PROTEAN 3 cell and begin electrophoresis: set to constant **AMPS: .03 (~2 hours or until blue line eludes out of the gel)**

Theory Behind Procedure:

1. Due to the negative SDS coat on every protein strand, all the proteins will migrate DOWN the gel towards the positive charge. The gel serves as a resistor- slowing down the migration of proteins down the gel. Proteins of larger molecular weight will have a harder time migrating down the gel; as a result, proteins will be separated by size as they go down the gel.
2. However, one must use a fairly low current/amp when running these proteins because if you use too much power, than you will drag all the proteins down regardless of weight- leading to terrible resolution. The rule is: slower run = better resolution aka separation of proteins based on size.

Running Buffer		
	Tris Base	30.3g
	Glycine	144g
	SDS	10g
	Dissolve and bring total volume up to 1,000 ml with deionized water	
	store at 4° C. If precipitation occurs, warm to room temperature before use	
	use: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run. Mix thoroughly before use.	

Gel Removal/ Transfer

Things You Will Need: Gel Removal		
Transfer Buffer (1L per gel, chilled in fridge)	Flat razor blade	Glass Plates (1 per gel)
1- Gel Releaser	1- 10ul pipette w/ tips	Paper towels/ 4 plastic trays
Things You Will Need: Transfer		
Filter pads (3 per gel)	Nitrocellulose Membrane (1 per gel)	Mesh pads (2 per gel)
Transfer Buffer (1L per gel, chilled in fridge)	Cassette (1 per gel)	Transfer Tank with a box of ice; agitating plate

Detailed Protocol:

Gel Removal:

1. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
2. Remove the tank lid and carefully lift out the Inner Chamber Assembly. Pour off and discard the running buffer.
3. Open the cams of the Clamping Frame. Pull the Electrode Assembly out of the Clamping Frame and remove the Gel Cassette Sandwiches.
4. Remove the gels from the Gel Cassette Sandwich by gently separating the two plates of the gel cassette. The green, wedge shaped, plastic Gel Releaser may be used to help pry the glass plates apart.
    a. To remove the gel from a Ready Gel Cassette, first slice the tape along the sides of the Ready Gel Cassette where the inner glass plate meets the outer plastic plate.
5. **Remove the gel by floating it off the glass plate by inverting the gel and plate under fixative or transfer solution**, agitating gently until the gel separates from the plate. Let the gel float onto a glass plate placed in the plastic tray- this will make retrieval of the gel easier later on.
6. **Move straight to some transfer buffer- don't let it dry out**. Let the gel incubate in transfer buffer for 15 minutes.
    a. During the 15 minutes, cut the membranes and the filter paper to the dimensions of the gel. Always wear gloves when handling membranes to prevent contamination.
    b. **Incubate all the filter pads, mesh pads, and nitrocellulose membrane in separate plastic trays in transfer buffer as well**.
7. Rinse the Mini-PROTEAN 3 cell electrode assembly, Clamping Frame and Mini Tank with distilled water after use.

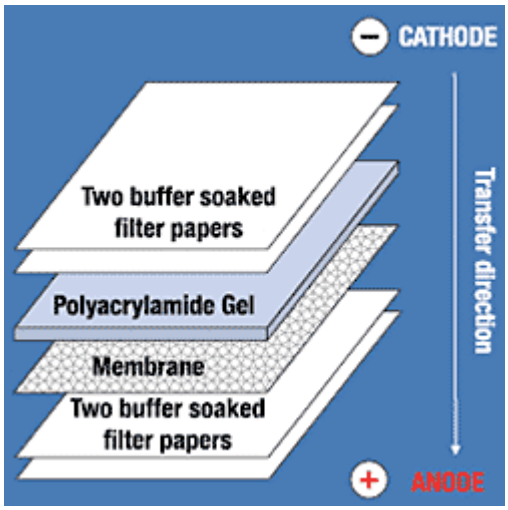
Theory Behind Procedure:

1. It is important to equilibrate the gel, the filter pads, and the membranes in transfer buffer for some time before actual transfer because the methanol will cause things to shrink and warp a little bit. If we tried to assemble the transfer cassette before allowing all the layers to equilibrate, everything will warp and shrink DURING the transfer process which will lead to poor transfer and possibly ripped gels (the gel is under a lot of pressure when clamped into the cassette).

Transfer:

1. Prepare the gel sandwich- assemble in transfer buffer.
    a. Place the cassette, with the gray side down, on a clean surface.
    b. Place one pre-wetted fiber pad on the gray side of the cassette.
    c. Place a sheet of filter paper on the fiber pad.
    d. Place the equilibrated gel on the filter paper.*

e. Place both pre-wetted membrane on the gel.*
f. Complete the sandwich by placing a piece of filter paper on the membrane.*
g. Add the last fiber pad.



2. Removing any air bubbles that may have formed is very important for good results. Use a glass tube to gently roll air bubbles out.
3. REMEMBER: **All proteins are NEGATIVE after the SDS treatment- make sure that the cassette is positioned so that the membrane is between the gel and the positive charge (red).**
4. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.
5. Place the cassette in module.
6. Place in ice-pack.
7. Fill transfer tank completely with the transfer buffer- place lid on top of tank and place in icebox.
8. Place icebox on top of agitator to make sure ions remain evenly dispersed during transfer. Back ice all over the transfer tanks. **Agitate SLOWLY**.
9. **Several Run times can be used**:
    a. I used 500mAmps for 1 hour.

Theory Behind Procedure:

1. We transfer the proteins from the gel to a piece of membrane because this allows for more stable storage of our separated protein strands and further experimental analysis. However, the membrane is not perfect in catching flowing proteins, if you run the power too long or at too high of a setting, you can pull the proteins through the membrane.
2. You must roll out the bubbles during cassette assembly because the electric current CAN'T pass through bubbles- so anywhere there are bubbles there will be no protein transfer.
3. It should be noted that after the transfer and brief rinse with TBST, once can store the membranes with the protein strands in the fridge with enough TBST to cover the membrane for 1-2 weeks with little ill-effect.

10X Transfer Buffer Stock		
	Tris base	30.3 g
	glycine	144.1 g
	Distilled Water to 1 liter	
	Store at 4° C	

1X Transfer Buffer		
	10X stock	100ml
	Methanol	200ml
	Distilled water	500ml
	Bring to 1 L with distilled water	
	Store at 4° C/ Chill to 4° C before use	

Visualization of Proteins in Membrane: Ponceau Red Staining (optional)

Things You Will Need: Ponceau Stain		
Ponceau Stain (10ml per membrane)	Agitating plate	TBST
Membrane box (1 per membrane)	1-forcep	

Detailed Protocol:

Ponceau Staining:

1. To check for success of transfer, wash the membrane briefly in TBST (for a TBST recipe, see below).
2. **Ponceau stain recipe: 1g Ponceau S, 50ml of glacial acetic acid, than make up to 1L with distilled water. Store at 4°C.**
3. Pour enough to cover membrane
4. Incubate on an agitator for 5 min.
5. Wash extensively with TBST until the water is clear and the protein bands are well-defined. The membrane may be destained completely by repeated washing in TBST.

Theory Behind Procedure:

1. The ponceau stain is a general, non-specific stain the bonds to all protein molecules. This allows you to see, generally, how the transfer turned out, where each lane is, and whether or not there were any bubbles left in the transfer cassette.
2. It also lets you see if the membrane/gel shifted at all during transfer cassette assembly.

Blocking

Things You Will Need: Blocking		
5% BSA solution	Agitating plate	TBST

Detailed Protocol:

Blocking:

1. Make sure membrane is completely clean (wash with TBST)
2. To prepare a 2-5% milk or BSA solution, **weigh 2-5 g per 100 ml of Tris Buffer Saline Tween20 (TBST) 1x buffer.** Use enough volume to cover the transfer membrane. Use gloves/forceps when handling membrane to prevent artifacts.

3. **Incubate for at least 1.5-2 hour at room temperature under agitation**. Don't incubate too long or else the BSA/milk solution will cover the antigens and lead to a reduced antibody signal (questionable- some people say blocking forever will have no ill-effect). After blocking, wash membrane for 5 seconds with TBST

Theory Behind Procedure:

1. It is necessary to block the membrane before further analysis because as of right now, the membrane still has plenty of open spaces where it could potentially "grab" hold of antibodies that we will be using later (remember, the transfer only moved bands of proteins to the membrane- so right now, the membrane has a banded protein pattern, all the space between these protein bands are susceptible to non-specific binding that could lead to high background).

Primary Antibody Incubation

Things You Will Need: Primary Incubation		
Primary Antibody (control and experimental)	Agitating plate	TBST
5% BSA solution (optional)	Membrane Incubation Tray	1- 10ul pipette w/ tips

Detailed Protocol:

Primary Incubation:

1. Depending on the experimental and control proteins of interest, one will cover the membranes with 10ml of either TBST or 5% BSA solution; for ARC, use 10ml of TBST and for ACTIN, you can use either.
2. **Don't let the membranes dry out after blocking!**
3. Remove primary antibody aliquots from fridge- vortex briefly.
4. **For ARC, we want a 1/10,000 dilution**- so we will pipette 1ul of primary ARC antibody into the 10ml incubation tray.
5. **For ACTIN, we want a 1/20,000 dilution**- so we will pipette .5ul of primary ACTIN antibody into the 10ml incubation tray.
6. When pipetting antibody into the tray with the membrane and TBST/5% BSA solution, **do NOT eject the fluid directly over the membrane**. Eject in a corner and immediately place on agitating plate.
7. Place plate at **at 4°C. Let it run for 12-16 hours.**

Theory Behind Procedure:

1. The purpose of the primary antibody is to bond directly to the protein of choice. It helps specifically target individual bands of proteins for further processing later on down the line.
2. This is the reason that we had to block the membrane earlier on- to make sure that the primary antibodies ONLY bind to their specific protein; however, even with blocking, if you incubate with primary antibodies for too long, it can bind non-specifically to other proteins.
3. The purpose of incubating it overnight in the fridge is to enhance the specificity of the primary antibodies- as with everything else, slower equals better results.
4. We do not want to eject the antibodies directly onto the membrane because that can lead to smudging.

5. For all new proteins in the future, one will have to troubleshoot the perfect amount of antibodies to use (at what dilution).

Washing Step

Things You Will Need: Washing		
Membrane Incubation Tray	Agitating plate	TBST

Detailed Protocol:

Washing:

1. After the primary incubation, dump the 10ml TBST/ 5% BSA solution down the drain and replace with enough clean TBST to cover the membrane.
2. Agitate membrane for 5 minutes at room temperature
3. Dump TBST and replace with more clean TBST
4. Repeat for 5-6 more times.

Theory Behind Procedure:

1. We have to wash off any excess non-bound primary antibody away from the membrane and incubation tray, this process will accomplish that goal as well as clean the image up.

Secondary Antibody Incubation

Things You Will Need: Primary Incubation		
Secondary Antibody (control and experimental)	Agitating plate	TBST
5% BSA solution (optional)	Membrane Incubation Tray	1- 10ul pipette w/ tips

Detailed Protocol:

Secondary Incubation:

1. Depending on the experimental and control proteins of interest, one will cover the membranes with 10ml of either TBST or 5% BSA solution; for ARC, use 10ml of TBST and for ACTIN, you can use either.
2. **Don't let the membranes dry out!**
3. Remove secondary antibody aliquots from fridge- vortex briefly.
4. **For ARC, we want a 1/10,000 dilution**- so we will pipette 1ul of primary ARC antibody into the 10ml incubation tray.
5. **For ACTIN, we want a 1/20,000 dilution**- so we will pipette .5ul of primary ACTIN antibody into the 10ml incubation tray.
6. When pipetting antibody into the tray with the membrane and TBST/5% BSA solution, **do NOT eject the fluid directly over the membrane**. Eject in a corner and immediately place on agitating plate.
7. Place plate **at room temperature. Let it run for 1 hour.**

Theory Behind Procedure:

1. The secondary antibodies serve as the light source for analysis- it binds only to the primary antibody and after exposed to imaging reagent, will glow for a few hours. The intensity of the light band has a direct correlation with how much experimental/control protein there is in the sample.

Washing Step

Things You Will Need: Washing		
Membrane Incubation Tray	Agitating plate	TBST

Detailed Protocol:

Washing:

1. After the primary incubation, dump the 10ml TBST/ 5% BSA solution down the drain and replace with enough clean TBST to cover the membrane.
2. Agitate membrane for 5 minutes at room temperature
3. Dump TBST and replace with more clean TBST
4. Repeat for 5-6 more times.

Imaging Step

Things You Will Need: Imaging		
ECL regent	Agitating plate	Plastic covering
Curved forceps		

Detailed Protocol:

ECL:

1. Prepare the substrate working solution by mixing equal parts of Detection reagents 1 and 2. **Use .125ml of the combined solution per cm^2 of membrane.**
2. Incubate blot with working solution for 1 minute at room temperature.
3. Remove blot from working solution and place it in plastic sheet protector- remove excess liquid with an absorbant tissue and carefully press out all the bubbles between the blot and the membrane protector.
4. Place the protected membrane in a film cassette with proteins facing up for exposure.
5. Light emission is most intense during the first 5-30 minutes after development incubation. However, light emission will still continue for several hours- you will just have to expose longer.
6. For ARC bands I image at F.stop 1.4 for 200 seconds, while for ACTIN, I image at F.stop 1.4 for 5 seconds.
7. ALWAYS USE GLOVES

Stripping Membrane

Things You Will Need: Stripping Membrane		
Restore Western Blot Stripping Buffer	Agitating plate	TBST
Membrane Incubation Tray		

Detailed Protocol

Stripping Membrane:

1. Warm Restore Western Blot Stripping Buffer to room temperature
2. After imaging blot with ECL reagent in CCD Imager, remove blot from plastic wrap and place in plastic cassette
3. Cover blot with ~20-30mLs of stripping buffer
4. Incubate on agitating plate at room temperature for 40 minutes to 1 hour. Longer incubation times will be required for more strongly bound antibodies.
5. Remove blots from stripping buffer. Wash in TBST 3 times for 5 minutes each time to remove residual stripping buffer (do not want any to be stuck on blot).
6. Store in TBST or incubate with primary antibodies when ready.

Theory Behind Procedure:

1. The stripping protocol is critical to saving time and resources when planning to image protein bands. Frequently, one hopes to analyze the band densities of two proteins (experimental and house-keeping); however, one cannot incubate multiple primary or secondary antibodies at the same time- doing so will lead to cross-reactions that impair the quality of the image. As a result, the stripping protocol allows one to incubate the primary and secondary of a particular protein of interest, image those specific bands, and then remove those antibodies before reusing the nitrocellulose membrane for incubations with a different set of antibodies.
2. The process helps one to reuse a single blot over and over again.
3. The stripping buffer is designed to only remove primary and secondary antibodies; as a result, a second incubation in blocking solution is not necessary. Also, lighter signals are removed easier than denser signals. Thus, one should image an experimental protein first because its signal can be removed easily.