THE GOODELL LABORATORY

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Title	**Western Blotting**	
Introduction	This protocol describes sample preparation, electrophoresis, transfer and immunoblotting procedures during Western Blotting.	
Materials	1. Lysis buffer – 2% SDS in water 2. Immunoprecipitation buffer – 2% IGEPAL (NP-40) in PBS + protease inhibitor cocktail (Sigma P8340) 3. TBS – 50mM Tris, 150mM NaCl, pH 7.5-7.6 4. TBS-T – TBS + 0.05% Tween 20 5. Hypotonic buffer - 10-20mM Tris, pH7.5 + protease inhibitors 6. Laemmli Sample Buffer (2X) – Bio-Rad cat.# 161-0737 7. 2-mercaptoethanol 8. 10X Tris/Glycine/SDS buffer – Bio-Rad cat#161-0732 9. 10X Tris/Glycine buffer – Bio-Rad cat#161-0734 10. Protein assay reagent – Bradford, BCA (Pierce(Thermo)) 11. SDS acrylamide gel – Bio-Rad 12. Nitrocellulose or PVDF membranes 13. Non-fat dry milk powder 14. Dish for membrane incubations (pipette tip box lid) 15. Sealable plastic pouches (Kapak SealPAK pouches) 16. GelCode Blue Stain Reagent (PIERCE/Thermo) 17. Blocking solution (5% non-fat dry milk in TBS-T) 18. Primary and secondary antibodies 19. ECL/ECLplus detection reagent (GE Healthcare) 20. Chemiluminescence film or phosphorimager	
Protocol	**A. Preparation of cell lysates**	*Notes*
1.	Collect cells by trypsinization, followed by washing with PBS. Pellet cells by centrifugation: max 1000g for 10 min.	
2.	Lyse the pellet with denaturing lysis buffer (2% SDS in water) or with native lysis buffer (immunoprecipitation buffer (2% IGEPAL (NP-40) in PBS + protease inhibitor cocktail), or hypotonic buffer (10-20mM Tris pH7.5 + protease inhibitors). If native is the preferred method for lysis, keep the lysates on ice at all times.	*If the cells are grown in plates, the wells/plates could be washed with PBS and the cells can be lysed directly in the well. Following the addition of the lysis buffer, use a cell scraper to ensure complete lysis and to collect the entire lysate. The amount of the lysis buffer should depend on the desired final protein concentration. A good rule: lysing of a well of a six-well plate with confluent 293T cells, grown for*

		total of 3 days, with 100-200µl buffer should result in a lysate with total protein concentration 3-5 mg/ml.
3.	Pass the lysate 10-15 times through a 25G needle using a 1ml syringe to break the remaining nuclei or to liquefy the viscous lysate when SDS buffer is used.	*If the lysate is larger than 1ml, sonication should be a method of choice. For smaller lysates, less than 0.3ml, the Bioruptor (sonicator with a water bath) could be an alternative of the needle passing (30 sec ON and OFF cycles for 15 min. should be enough).*
4.	Spin at 14,000 rpm (16,000 g) or maximum speed in an Eppendorf microfuge for 10 min at 4°C.	*This step is unnecessary when SDS buffer is used.*
5.	Transfer the supernatant to a new tube and discard the pellet.	*Thing to consider: detergents interfere with the protein assays, especially with the Bradford assay. Check online for more information or you can test the lysis buffer compared to water - add 10µl of lysis buffer or water to 1ml of Bradford reagent and measure on spectrophotometer. BCA also have limitations but it works without problems with the specified here buffers.*
6.	Mix equal volumes of lysate and 2x sample buffer (Bio-Rad) and boil for 5-10 min.	
7.	Cool and flash spin to bring down condensation prior to loading gel.	
8.	Load on a gel or store in +4°C for up to 2-3 days, or in the freezer for long term storage.	
	B. SDS-PAGE	

We use Mini-Protean II electrophoresis apparatus and the transferring module from Bio-Rad. Along with that, we use Bio-Rad brand ready gels, sample, running and transfer buffers. For larger number of samples and/or for longer separation we also have available Criterion electrophoresis apparatus and transfer module also from Bio-Rad. In addition, we have in possession an Invitrogen electrophoresis apparatus, which could be used if desired. Make sure the gel(s) are placed properly. If only one gel, use the dam breaker (Bio-Rad) (not a large gel plate). Make sure to remove the plastic from the bottom of the gel. After the assembly, pour buffer first in the inner chamber and make sure that there isn't any leaking. Then, fill the outer chamber. Next, remove the combs and using a transfer pipette wash the wells (this step is optional).

1.	Bring samples to room temperature to make sure SDS is in solution.	*A quick way is to incubate the samples in 37°C water bath for 5-10 min.*
2.	After flash spinning and mixing, load the samples into the wells.	*The amount of the sample loaded would depend on what is being detected. If this is an exploratory western, load two wells, one with the maximum volume and the*

		other with half. If SDS lysis buffer was used, loading of large volumes, more than half of the well-volume would decrease the resolution quality of the gel. Use loading tips or Hamilton syringe to ensure better sample packing and maximum load into the well. Regular tips could also be used, however exercise care not to contaminate neighboring wells especially when large volumes are loaded. Bio-Rad mini gels mas volume/well: 10well gell – 30µl; 15well gel – 15µl (up to 20µl)
3.	Be sure to use markers.	*We use 5 µl Bio-Rad Kaleidoscope Prestained Standards #161-0324.*
4.	Run.	*Use 20 mA PER GEL, not per apparatus, for the mini gels and 200V, regardless of the number of gels, for the Criterion). Usual running time is about 50 – 90 min.*
	C. Preparation of membrane	
Do not touch the membranes with bare hand it would leave prints. Always wear gloves when handling the membranes and/or use clean tweezers - hold edges or corners. Do not fold, scratch or damage the membranes, especially the transfer surface. After transfer, do not let the membrane dry at any time until done with detection.		
1.	Cut a piece of nitrocellulose membrane (Bio-Rad) or PVDF membrane (Millipore).	*3.5 by 2.5in is sufficient for the mini gels or 3.5 by 5.5in for the Criterion*
2.	Pre-wet the membranes.	*NITROCELLULOSE - place in transfer buffer for at least 10 min. prior to the assembly. Make sure the membrane is completely covered by the buffer. PVDF membranes - place for about 10-30 min in methanol on a rocker at room temp. After that, remove methanol and add 1x Blotting buffer. The membrane is ready for use.*
3.		
	D. Protein transfer	
After transfer, do not let the membrane dry at any time until done with detection.		
1.	Pre-wet the sponges, filter papers (slightly bigger than the gel) in 1x Blotting buffer.	
2.	Assemble transfer "sandwich" for Bio-Rad's Transblot : Assemble on the black side of the plastic blotting cassette in the following order: (everything should be pre-wet) Sponge - filter paper - gel -	*Important: note the orientation of the gel - use the protein marker as a reference. When you are placing the membrane over the gel make sure that there are no bubbles between them. To help avoiding that, use*

	membrane - filter paper – sponge.	*the plastic roller or something else plastic, to press and role, thus pushing out any remaining air bubbles. In general, air bubbles should not exist anywhere between the filter papers, gel and membrane. That would interfere with the protein transfer.*
3.	Place the assembled sandwich in the transfer cell. Make sure you place the gel-side of the sandwich on the negative electrode (black side) and the membrane-side on the positive (red) side. If you have followed the order of assembly described above you should place the black side of the plastic cassette on the black side of the transfer cell. Place the lid matching the colour of the electrodes.	
4.	Transfer for 1 hr at 100V at 4°C on a stir plate. To prevent overheating a cool pack could be placed in the container.	*These conditions are for optimal performance. However, transfers could be done in room temperature, without cool pack and without a stir plate. Warning: do not run over an hour at 100V. Buffer will get very hot.*
5.	When finished, incubate membrane in Blocking solution for at least an hour at room temperature or overnight in +4^{o}C.	*In general, this and all consequent incubations (unless otherwise specified) are done in a clean dish (usually a lid from a pipette tip box) big enough for the membrane.*
6.	Optional: Staining of the transferred gel.	*The transferred gel can be stained using variety of stains. The easiest, I have used, is GelCode (Pierce/Thermo). Simply insert the gel in 25-50ml of stain and incubate for minimum of 30-60min (you can also leave it overnight). Then, wash with water until distained (this can also be done overnight). The stained gel can serve as a control for loading. Also, to see if the proteins were resolved well.*
	E. Immunoblotting and detection	
1.	Incubate with primary antibody diluted in Blocking solution overnight at +4^{o}C with mixing. Primary antibody incubation could also be done for 1-3 hrs at room temp.	*The membrane is placed in a sealed plastic bag (to save antibody; 8-10ml of antibody solution is sufficient for one mini blot), which is then placed on a turning wheel. Alternatively, the membrane could be incubated in a plastic container on a rocking plate – Note: use enough antibody solution to cover the membrane, and cover the container.*

2.	Wash 3 x 10 min with TBS-T.	
3.	Incubate with enzyme conjugated (usually HRP (horse radish peroxidase)) secondary antibody diluted in TBS-T for 1 hr at room temp.	
4.	Wash 3 x 10 min with TBS-T.	
5.	Detection.	*Use ECL or ECL+ reagents from GE Healthcare. Detect with a film (Kodak BIOMAX Light Film Chemiluminescence (Sigma # Z370401-50EA) or if ECL+ is used, also by scanning of the membrane on a phosphorimager (Storm 860 or similar).*