Protocol
Bradford Protein Assay and Western Blot
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1. Bradford Protein Assay

1.1 Introduction

Bradford protein assay is a means to determine protein concentration in solution by spectroscopic method. The assay is based on the absorbance shift of dye Coomassie Brilliant Blue G-250. Three forms of the dye exist: cationic (red), neutral (green), and anionic (blue) (Compton and Jones 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (Amax = 470 nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (Amax = 595 nm) (Reisner et al. 1975, Fazekes de St. Groth et al. 1963, Sedmack and Grossberg 1977). Because the extinction coefficient of dye remains constant over a 10-fold concentration range in dye-albumin complex, measuring solution absorbance at 595nm and applying Beer's law can be used to accurately determine protein concentration.

1.2 Reagents and Equipment

Bio-Rad Protein Assay Dye Reagent Concentrate (Cat. # 500-0006) (Also sold in store room, Cat. # 6-4007)
BSA [Conc] > 600μg/ml
Milli-Q H_2O
Greiner 96 Flat Transparent plate
Tecan infinite F200

1.3 Standard Protocol

1.3.1 Tecan Setup

1. Make sure the 595nm filter is inserted into Tecan. (If not, slide filter out by pressing the "Slide Filter Out" button on the menu bar, and insert 595nm filter. The 595nm filter is in the filter box on the shelf directly above the machine)
2. On the lower left of the screen, click "Standard". A "Measurements" menu will show up on the left. Double click to select "Absorbance".
3. Select plate Greiner 96 Flat Transparent plate in "Plate Definition".
4. Roll to the bottom of page. Make sure the wavelength measured is 595nm. To increase accuracy, select "Multiple reads per well".
5. Select "2*2" in "size", with a border of 1000μm. (Avoid selecting more than 4 reads per well, increased reading number will produce a timeout error.)

1.3.2 Measuring Absorbance of Std. Protein Concentration

1. Perform steps on ice to minimize protein degradation.
2. Dilute BSA to 0, 100, 200, 300, 400, 500, and 600μg/ml in Eppendorf tubes, 1 ml each.
3. Label new tubes from 0 to 6, add 790μl of H_2O into each tube. Avoid pipetting error.
4. Transfer 10μl of diluted BSA into respective tube (i.e.: 10μl of 100μg/ml BSA into tube 1). Avoid pipetting error. Vortex to generate homogeneous solution.
5. Pull tubes out of ice bucket and put into a rack, add 200μl of 5X Bradford Red Dye into each tube. Avoid pipetting error.
6. Vortex all tubes to create homogeneous solutions. Allow solution to react for 5 minutes. Tubes with protein should turn slightly blue.
7. At the end of 5 minutes, quickly transfer 200μl (per well) of solution 0 into wells A1, A2, A3. In the same manner, transfer std. solution 1 into wells B1, B2, B3. Continue until all 7 std. solutions have been transferred. Make sure no bubbles are present in any well
8. Insert the 96 well plate into Tecan, select the wells with std. solution and take absorbance readings.

1.3.3 Generating Std. Curve

1. In Excel (or other data processing software), calculate average of absorbance for each std. solution, apply Beer's Law and find best fit line. (A sample Excel document is provided with the protocol.)
2. If the best fit line has a R^2>0.99, consider redo steps 3-9. If the recreated best fit line still has a R^2>0.99 with a profile similar to the first try, consider re-dilute using new BSA.
3. After generating the Std. curve, store the rest of Std. protein at -20 degree Celsius.

1.3.4 Measuring Absorbance of Protein Samples

1. Perform steps on ice to minimize protein degradation.
2. For X samples, mark X tubes. Add 799μl of H_2O into each tube. Avoid pipetting error.
3. Add 1μl of protein sample with unknown concentration into each tube, vortex to generate homogeneous solution.
4. Pull tubes out of ice bucket and put into a rack, add 200μl of 5X Bradford Red Dye into

each tube. Avoid pipetting error.
5. Vortex all tubes to create homogeneous solutions. Allow solution to react for 5 minutes.
6. At the end of 5 minutes, quickly transfer 200μl (per well) of protein-dye solution into three wells. Repeat for all samples.
7. Insert the 96 well plate into Tecan, select the wells with protein-dye solution and take absorbance readings.

1.3.5 Calculating Protein Concentration

1. Calculate average absorbance for each protein-dye solution, make sure the absorbance is within the range of previously generated Std. curve.
2. Apply Beer's law to calculate concentration of protein-dye solution, times 10 to derive the actual concentration of protein sample. Protein samples collected from a six-well plate have a concentration of around 1mg/ml.

2. Western Blot

2.1 Introduction

Western blot is an analytical technique to separate protein by electrophoresis and detect specific ones by antibodies. After separation by peptide length, proteins are transferred to a membrane to be stained by antibodies. Proteins tagged by antibodies can be detected by chemiluminescence, fluorescence or in some cases ionizing radiation. Western blot can help determine the presence of specific protein and its relative concentration. This protocol will focus on chemiluminescence of antibodies and electronic detection. The protocol used to make polyacrylamide gel is based on that of national diagnostics'.

2.2 Reagents

5X Bromophenol Blue (Sigma-Aldrich, 114391-5G)
1X SDS Running Buffer
10X Transfer Buffer
10X TBS
Triton X-100 (Fisher BioReagents, Order #151-500)
Methanol
ProtoGel 30% (National Diagnostics, Order # EC-890)
1.5M Tris-HCl, pH 8.8
0.5M Tris-HCl, pH 6.8
10% SDS
10% (w/v) ammonium persulfate
TEMED (Fisher BioReagents, Order # BP150-20)
Isopropanol
Chemiluminescent HRP Substrate (Milioire Corporation, Immobilon™ Western, Cat. # WBKLS0100)
PageRuler Pre-stained Protein Ladder (Lot 00036958)
Milli-Q water
Whatman Paper
Nitrocellulose membrane 0.1μm pore (Whatman™, Protran BA 79)
Nitrocellulose membrane 0.2μm pore (Whatman™, Protran BA 83)
Nitrocellulose membrane 0.45μm pore (Whatman™, Protran BA 85)

2.3 Standard Protocol

2.3.1 Making Polyacrylamide Gels

1. Assemble the front and back side of casting glass and insert them into the casting stand. Clamp two pieces of glass tight. Set up the casting apparatus, make sure the bottom of the casting glass is sealed. Pour water into casting glass to check for leaks. If not leaks are present, pour out the water by flipping the apparatus upside down.
2. Use the chart below to determine volumes of reagents required for desired gel composition. Mix all reagents in a 50ml conical. (Size-Percentage table based on Abcam's guide)

Protein size (kDa)	Gel Percentage (%)
4-40	20
12-45	15
10-70	12
15-100	10
25-200	8

2 gel				4 gel		
6%	ProtoGel 30%	5ml		6%	ProtoGel 30%	10ml
	1.5M Tris-HCl, pH8.8	6.25ml			1.5M Tris-HCl, pH8.8	12.5ml
	10% SDS	0.25ml			10% SDS	0.5ml
	Milli-Q Water	13.225ml			Milli-Q Water	26.45ml
8%	ProtoGel 30%	6.675ml		8%	ProtoGel 30%	13.35ml
	1.5M Tris-HCl, pH8.8	6.25ml			1.5M Tris-HCl, pH8.8	12.5ml
	10% SDS	0.25ml			10% SDS	0.5ml
	Milli-Q Water	11.55ml			Milli-Q Water	23.1ml
10%	ProtoGel 30%	8.325ml		10%	ProtoGel 30%	16.65ml
	1.5M Tris-HCl, pH8.8	6.25ml			1.5M Tris-HCl, pH8.8	12.5ml
	10% SDS	0.25ml			10% SDS	0.5ml
	Milli-Q Water	9.9ml			Milli-Q Water	19.8ml
12%	ProtoGel 30%	10ml		12%	ProtoGel 30%	20ml
	1.5M Tris-HCl, pH8.8	6.25ml			1.5M Tris-HCl, pH8.8	12.5ml
	10% SDS	0.25ml			10% SDS	0.5ml
	Milli-Q Water	8.225ml			Milli-Q Water	16.45ml
15%	ProtoGel 30%	12.5ml		15%	ProtoGel 30%	25ml
	1.5M Tris-HCl, pH8.8	6.25ml			1.5M Tris-HCl, pH8.8	12.5ml
	10% SDS	0.25ml			10% SDS	0.5ml
	Milli-Q Water	5.75ml			Milli-Q Water	11.5ml

3. Pour the gel solution into a vacuum flask. Degas gel solution for 10 minutes under vacuum.
4. Pour the solution back into the original 50ml conical, avoid producing air bubbles. Add 250µl of 10% (w/v) ammonium persulfate for the 2 gel solution (500µl for 4 gel), swirl gently to mix without introducing bubbles.
5. Add 25µl of TEMED to the 2 gel solution (50µl for 4 gel). Polymerization will start in 5 minutes.
6. Use a serological pipette to transfer gel solution into casting glass. Stop when the solution level is 2cm below the lower glass (3cm below the higher glass).
7. To generate a sharp interface, overlay the gel with 0.5 ml of isopropanol.

8. Make 10ml of a 4% stacking gel in a 15ml conical, follow the chart below:

ProtoGel 30%	1.3ml
0.5M Tris-HCl, pH6.8	2.5ml
10% SDS	0.1ml
Milli-Q Water	6ml

9. Add 50µl of 10% Ammonium Persulfate and 10µl of TEMED. Without degassing, the gel will polymerize in 20 minutes.
10. Pour off the isopropanol after the running gel has polymerized. The unused gel solution in the 50ml conical can serve as a reference for whether the gel has polymerized.
11. Use a P-1000 to transfer the stacking gel solution into casting glass. Fully fill the casting glass.
12. Select appropriate comb, do not use if the comb has broken edges. Insert plastic comb slowly into the casting glass, the smooth side should face the higher glass. The comb should make a complete seal without any air bubbles trapped under the comb.
13. Let the solution polymerize for 30 minutes. The unused gel solution in the 15ml conical can serve as a reference.
14. If not using the gel immediately: do not take out the comb or open the casting glass. Wrap the casting glass with wet paper towel. Store in a plastic bag in 4 degree Celsius freezer.

2.3.2 Preparation of Protein Samples

1. Perform all steps on ice.
2. Calculate how much of the protein samples is needed. A typical well in a ten well gel can hold 40µl, while a typical well in a fifteen well gel can hold 25µl.
3. If required, dilute all protein samples to the same concentration. 32µl of each sample if using a ten well gel, 20µl if using a fifteen well.
4. Add 8µl of 5X bromophenol blue to 32µl of protein sample (or 5µl into 20µl protein sample).
5. Heat the sample and dye to 95 degrees Celsius for 5 minutes, then rapidly cool down the samples using ice bath.

2.3.3 Electrophoresis

1. Insert gel with casting glass into the running cassette, with the lower side of the glass on the inside of the cassette. Fill the running cassette with 1X Running Buffer and check for leaks. Make sure the liquid level is above the top of the inside glass. Slowly remove the plastic comb.
2. Fill the container with 1X Running Buffer to the 2 gel or 4 gel mark. Move the container next to the power source if not already done so. Avoid moving the container after loading the samples.
3. Load pre stained protein ladder (5µl) and samples. Write down the loading sequence and location of ladder. Fill any empty well with 1X bromophenol blue.
4. Connect the cassette to power supply, run at 50V until the dye exits the stacking gel and reaches the running gel. The dye should look like one straight line. Increase voltage to 150V, stop running when the lowest band of the ladder (green) is 1cm away from the bottom of the gel.

2.3.4 Transfer

1. Select the correct size of nitrocellulose membrane. Use 0.45μm pore sized membrane for proteins greater than 20kDa. For smaller proteins, try using membranes with 0.2μm pore. If protein of interest has low expression, try membrane with 0.1μm pore.
2. For each gel, prepare 6 pieces of whatman paper and 1 nitrocellulose membrane. Cut whatman paper and membrane to appropriate size (slightly smaller than the holding clamp).
3. Prepare 2L of Transfer Buffer. Fill a 2L graduated cylinder with 400ml of methanol, 1400ml of Milli-Q water, and 200ml of 10X Transfer Buffer. Add 10X Transfer Buffer last, since the salt will precipitate in methanol.
4. Fill 2 glass pans with 1X Transfer Buffer. Use forceps to pick up dry nitrocellulose membrane and submerge it in 1X Transfer Buffer. Methanol will activate the nitrocellulose membrane. Let sit for more than 5 minutes.
5. Disassemble the running cassette. Do not open the casting glass. Leave gel and casting glass in either the container or the glass pan.
6. Open the holding clamp in a glass pan with 1X Transfer Buffer. Start making the transfer stack by laying 1 sponge, 3 pieces of whatman paper and then 1 nitrocellulose membrane (with forceps) on the clear side of the clamp. Avoid bubbles.
7. Open the casting glass by inserting a razor blade between two pieces of glass. Cut off the stacking gel on the top. On the side of first protein sample, cut a corner off the top of the running gel. This will help distinguish the direction of loading.
8. Gently put the gel on nitrocellulose membrane, avoid bubbles between the gel and the membrane. Lay 3 pieces of whatman paper on top of the gel, and then a sponge on top of the whatman paper.
9. Roll fingers on top of the sponge from left to right to expel any air bubbles. Close the holding clamp. Leave the transfer stack in transfer buffer while setting up other transfer stacks. The transfer stack, from top to bottom, should be: clear side of clamp, sponge, 3 whatman paper, nitrocellulose membrane, gel, 3 whatman paper, sponge, black side of clamp.
10. Put transfer cassette into the container and fill 3/4 of container with transfer buffer. Insert the holding clamps into the transfer cassette. Make sure the black side of the holding clamp faces the black side of the transfer cassette. Protein is negatively charged after exposure to SDS and flows to the anode. If inserted backwards, protein will flow from the gel to whatman paper instead of to the membrane.
11. The power source for transfer is located in the 4 degree cold room. Link the transfer cassette to power source, make sure the current direction is correct. Select constant current at 90mA on the power source, transfer overnight.

2.3.5 Blocking, Primary Antibody Incubation

1. Make 2L of 1X TBST by adding 1800ml of Milli-Q water to 200ml of 10X TBS. Add 2ml of Triton to solution, stir vigorously by shaking the solution and by magnetic stir bar.
2. Make 5% milk by adding 2.5g of non-fat milk powder to 50ml of 1X TBST. Stir vigorously with magnetic stir bar on a heated surface to decrease dissolving time.
3. Spin down primary antibody at 5min max RPM. Take the top layer of antibody and dilute to desired concentration with 5% milk. Commercial antibodies usually specify desired

concentration in the product information page.

4. Pull Transfer stacks out of transfer cassette, open the holding clamp on table with the clear side facing down. Use razor blade to trim the nitrocellulose membrane to the shape and size of the gel. Do not forget to cut off the corner on the side where loading starts.
5. Peel off the gel. Ladder will show on the membrane if transfer is successful. Use pencil to mark the membrane. Write on the side where the membrane was in contact with the gel, but not in the middle of the membrane where protein binds.
6. Use forceps to transfer membranes into their respective plastic container. Make sure the protein binding side is faced up. Wash with 1X TBST for 10 min on a rocking stand. At the end of 10min, pour out the liquid in container and wash again with 1X TBST in the same manner for 10 minutes.
7. At the end of 10min, pour out liquid in container. Add 5-10ml of fully dissolved 5% milk to each container, make sure the membrane is fully covered. Incubate at room temp on a rocking stand for 1 hour. Protein in milk will block the membrane.
8. At the end of 1 hour, collect milk from the containers if not enough is left to make secondary antibody solutions.
9. If using more than one antibody on one membrane (for example using Beta-tubulin antibody to visualize Beta-tubulin as control and another antibody to visualize the protein of interest), cut the membrane so that the two or more proteins are on different pieces of membrane. Each pieces of membrane should have its own container. This is to make sure that one piece of membrane will only come in contact with one type of primary antibody.
10. Add 5-10ml of primary antibody to each membrane. The membrane should be fully covered by primary antibody solution. Incubate at 4 degree Celsius on a rocking stand overnight. Incubation time can be extended if protein expression is known to be low.
11. If less than 5ml of primary antibody is available, membrane in container may not be fully covered. In this case, put the membrane in a clean plastic paper holder and trim the plastic paper holder to 2cm wider and longer than the membrane. Use heat seal machine to seal 3 sides of the plastic. Add primary antibody to the membrane inside the plastic pocket, expel any bubbles, and seal the opening. Incubate at 4 degree Celsius on a rocking stand overnight. Incubation time can be extended if protein expression is known to be low.

2.3.6 Secondary Antibody Incubation and Imaging

1. Choose the correct secondary antibody. Spin down secondary antibody at 5min max RPM. Take the top layer of antibody and dilute to desired concentration with 5% milk. Commercial antibodies usually specify desired concentration in the product information page.
2. If needed, collect primary antibody from the container and store in 4 degree Celsius freezer. If the primary antibody need to be stored for more than 2 weeks, add 1% sodium azide to primary antibody solution to reach a final concentration of 0.02% sodium azide.
3. Wash the membranes in separate containers with 1X TBST for 15 minutes on a rocking stand. Make sure the protein binding side is faced up. At the end of 15 minutes, drain the liquid and wash again (2 washes in total). If primary antibody solution contains sodium azide, wash another time (3 times in total) and increase the volume of 1X TBST used for each wash.

4. Drain liquid in container, add 5-10 ml of secondary antibody solution to the membrane. Make sure the membrane is covered by solution. Incubate for 1 hour at room temp on rocking stand. Do not extend incubation time or there will be increased background noise during imaging.
5. At the end of 1 hour, wash the membrane 2-3 times with 1X TBST, each for 15 minutes. After the last wash, add a few ml of 1X TBST to the container so that the membrane does not dry out.
6. Carefully transfer 750µl of HRP substrate Peroxide solution and Luminol reagent to separate Eppendorf tubes. Avoid cross contamination.
7. Move all container with membranes and two Eppendorf tubes to the imager, located in Tjian lab, 475 LKS. Also bring a P-1000, tips and a pair of forceps.
8. Image membranes one by one. Lay the membrane (protein binding side faced up) on a white backboard and slide into the imager. If the membrane was cut before primary antibody incubation, assemble the pieces to resemble the uncut membrane. Open new protocol and press “select>custom>epi white” under “application”. Set exposure to manual, 0.07 seconds.
9. Position the membrane. Mix 150µl each of Peroxide solution and Luminol reagent together and pipette up and down several times to create a homogeneous mixture. Drip 300µl of the mixture evenly on the membrane. Close the imager door without moving the position of the membrane.
10. Take an image. This image will record the position and shape of membrane and also the ladder in grey scale. Do not forget to save the image.
11. Do not open the imager door or disturb the membrane. On the protocol page, click “select>blots>chemi high sensitivity”. Under exposure mode, select signal accumulation mode. Click setup, select total accumulation length and interval of image. 10 minutes of accumulation should reveal protein with the lowest concentration. 10 seconds between each image is a commonly used interval.
12. Click run. A window will pop up and will show an image at selected interval. If desired exposure is reached before selected accumulation time is reached, stop run. Right click on image and save all. If desired exposure is not reached, wait until the imaging stops and save all images.
13. Open the epiwhite image and also the image with desired exposure. On the tools menu on the left, select “merge”. Merge two images so that the ladder, membrane and luminescence are integrated in one image.
14. Repeat steps 8-13 to image the rest of the membranes. Remake peroxide solution and Luminol reagent mixture for every membrane before exposure.
15. Print the merged image and save all images on flash drive. Delete local files.

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