SDS-PAGE & Western Blotting Protocols

Adapted from existing protocols by Vinh Pham
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MATERIALS
All materials required are mentioned within the respective sections below.

PROCEDURE
Part I – Sample Preparation

1. Collect cells or fruiting bodies. Klett 100 cells are at a density of 5E8.
2. Pellet at RT for 1-2' and remove s/n.
3. Extract protein by SDS/Urea Method, TCA-precipitation, or sonication.
4. Measure [protein] by Bradford (use 1-2 ul of each sample).

Part II – SDS-Polyacrylamide Gel Electrophoresis

1. For each SDS-PAGE gel, sandwich one small and one large glass plate, separated by spacers (smeared with silicone lubricant) and an alignment card.

   Optional: The inside surface of the small glass plate can be treated with Rain-X to facilitate later removal of the gel.

2. Slide into holder without tightening the screws.
3. Place on pouring stand, making sure that the glass plates are pushed all the way to the bottom before tightening the screws. If the assembly is correct, then the whole set-up should snap into place when placed above the gray gasket.
4. Remove alignment card.
5. Slide in comb and mark a line at 2-3cm from the bottom of the comb. Make sure that the comb thickness is the same as the that of the spacers'.
6. Remove comb.
7. Pour resolving gel to the mark in step 5.

4ml	10% resolving gel mix (dH_2O		48ml
		40% acrylamide mix	25ml
		1.5M Tris, pH 8.8	25ml
		10% SDS	1ml)
75ul	2% APS (ammonium persulfate)		
7.5ul	TEMED		

   Optional: Create an agarose plug with newly liquified 1% agarose (i.e., the agarose needs to be hot when used). The agarose does not affect the running of the SDS-PAGE, although it does reduce the effective size of the resolving gel.

8. Add upper layer of water-saturated *n*-butanol to the top of the gel.
9. Let polymerize (this takes ~10-15').

10. Invert gel to remove *n*-butanol. Touch with filter paper to wick off residual liquid.
11. Pour stacking gel to fill the remaining space between the glass plates.

2ml	5% stacking gel mix	(dH_2O	73ml
		40% acrylamide mix	12.5ml
		1.0M Tris, pH 6.8	12.5ml
		10% SDS	1ml)
25ul	2% APS		
2.5ul	TEMED		

12. Insert comb and let polymerize (this takes ~10-15').
13. Prepare 20-30μl of sample, as follows:

10X SDS Gel Loading Buffer	2μl	2.5μl	3μl
-20% SDS			
-500mM Tris, pH 7.6			
-1% Bromophenol Blue			
50% Glycerol	4μl	5μl	6μl
Protein sample	12μl	15μl	18μl
1M DTT	2μl	2.5μl	3μl

14. Boil samples for at least 3'.
15. Place gel in holder/electrode, then transfer to running tank.
16. Fill with 1X Running Buffer (keep inside and outside buffer chambers separated).

1X Running Buffer:	-4X SDS-PAGE Running Buffer		250ml
	-Tris Base	12g	
	-Glycine	58g	
	-dH_2O	1L	
	-dH_2O		750ml
	-10% SDS		10ml

Note: Mix by inversion in a Parafilm-covered graduated cylinder. Remove excess bubbles and foam with wads of paper towels.

17. Add a little bit of 1X SDS Gel Loading to each well to help define well space. This will also help with detecting stray pieces of polyacrylamide that might be plugging up any wells. In general, these stray pieces will not affect the separation of the samples, although they will reduce the volume that can be loaded.
18. Load samples. The max volume for a 10-well comb is 30μl/well. Use 20μl for the protein standards ladder (heat BIO-RAD ladder to 37ºC for 1' to dissolve solids that may have precipitated during freezing). Use two different kinds of ladder for orientation (e.g., NEB broad range (6-175 kDa) and BIO-RAD low range (21.2-108 kDa) prestained marker).
19. Run at 150V through the stacking gel.
20. Run at 100V-200V until the dye front reaches the bottom of the gel.

Part III – Coomassie Blue ("Brilliant Blue") Staining (Optional)

1. Stain the gel with Coomassie Blue for at least 1h at 50ºC in a covered box. The longer the incubation, the better.

Coomassie Blue Stain R-250	2.5g
Methanol	1.0L
Acetic Acid	0.2L
dH_2O	0.8L

2. Wash with Destaining Solution 1 (50% MeOH, 7% HAc) 2X, 30'each, at 50ºC.
3. Wash with Destaining Solution 2 (5% MeOH, 7% HAc) a few times, with the final wash being done overnight. All washes are at RT.
4. Place gel over 2 pieces of 3mm paper, cover with Saranwrap, and dry in gel dryer for 1-2h at 65ºC.

Part IV – Western Blot

1. Prepare fresh 1X Transfer Buffer (TGM). Approx. 1,250ml will be needed.

1X TGM:	-10X TG salts	100ml
	-1.92M glycine	
	-0.25M Tris Base (no need to pH)	
	-MeOH	200ml
	$-dH_2O$	700ml

Note: Prepare TGM while the SDS-PAGE is running. Chill buffer at 4ºC.

2. Cut off stacking gel and nick top left-hand corner of resolving gel for orientation.
3. Measure the dimensions of the gel and note the positions of the ladder bands.
4. Transfer gel, while still attached to glass plate, to box containing TGM and peel off gently with a spatula.
5. Agitate 15-20' at RT to remove salts and SDS.
6. Cut a piece of nitrocellulose membrane to the size of the gel and mark and/or clip one corner as the top left-hand corner. Handle only with flat forceps.
7. Immerse membrane in TGM for 10-15'.
8. Cut 2 pieces of ≥3mm filter paper to the dimensions of the gel (or slightly bigger).
9. Open a gel holder cassette in a casserole dish, black side down and hinges to the left and below the black side.
10. Soak a fiber pad with TGM and place in the center of the black side.
11. Soak one piece of filter paper with TGM and place on top of the fiber pad.
12. Roll out bubbles with glass tube and add 3ml of TGM onto the top.
13. Pour out old TGM (contains SDS+salts from gel) and add fresh TGM to gel.
14. Fish gel out with a glass plate.
15. Place gel on top of filter paper.
16. Roll out bubbles with glass tube and add 3ml of TGM onto the top.
17. Place membrane on top of gel, with the gel's top left mark facing the membrane's top left mark.

18. Roll out bubbles with glass tube and add 3ml of TGM onto the top.
19. Soak a second piece of filter paper with TGM and place on top of the membrane.
20. Roll out bubbles with glass tube and add 3ml of TGM onto the top.
21. Soak a second piece of fiber pad and place on top of stack.
22. Roll out bubbles with glass tube and add 3ml of TGM onto the top.
23. Close the gel holder cassette.
24. Place in a transfer tank (orient the white and black sides of the cassette with the white and black panels of the electrode) and fill with TGM (~800ml).
25. Place the tank in a styrofoam box containing ice.
26. Run 1h at 100V, or o/n at 30V (in 4ºC room).

Part V – Immunodetection

1. (Optional) Rinse blot with dH_2O several times to remove MeOH and salts, and stain all protein bands with 0.5% Ponceau S (0.5g Ponceau S, 1ml Glacial HAc, 99ml dH_2O). Take a picture and rinse off the Ponceau S with TBS or PBS.
2. Following Western Blot transfer, place membrane in a box containing 3% gelatin in TBST for 1h to o/n at RT. Other blocking reagents that can be used are 1-5% nonfat milk and 1-10% BSA.

Optional: The blot can be air dried on filter paper and stored at RT for later detection.

3. Wash 2X briefly with TBST (1X TBS with 0.1% Tween-20).
4. Wash again 2X for 5’ each with TBST.
5. Place blots in box containing 10ml 1º αB in TBST and incubate 1h to o/n. Use 1:500 to 1:5000 dilutions.
6. Wash 15’ with TBST, then 2X for 5’ each.
7. Replace blot in box containing 10ml 2º αB in TBST and incubate 1h to o/n. Use 1:1000 to 1:5000 dilutions.
8. Wash as in step 5.
9. Wash with TBS for 5’ to remove Tween-20.
10. Rinse twice with dH_2O for 1-2’. This removes the Tween-20.
11. Place membrane on Saranwrap.
12. Add ECL-Plus detection solution (40:1 ratio for soln A:B). Warm mix to RT prior to use. For a small blot, add 50ul soln B to 2ml soln A.
13. Incubate for 5’ at RT. This is best done in the dark. Ensure that the entire membrane is covered by detection solution.
14. Wick off excess detection fluid and place membrane inside a page protector or a low-fluorescence plastic hybridization bag. Do not use plastic wrap.
15. Roll out bubbles.
16. Scan on AlphaImager using the chemiluminescence filter. Also take a reflecting light image to record the ladder bands. Adjust aperture, zoom, and focus, as needed.

Alternatively, scan on STORM machine using Blue Fluorescence/Chemi-luminescence mode following exposure to ECL-Plus reagents (blot can be dry).