SDS-PAGE & Western Blotting Protocols
Adapted from existing protocols by Vinh Pham

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 5x10^8.
2. Pellet at RT for 1-2’ and remove s/n.
3. Extract protein by SDS/Urea Method, TCA-precipitation, or sonication.

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.

   \[
   \begin{align*}
   4\text{ml} & \quad 10\% \text{ resolving gel mix (dH}_2\text{O)} \\
   48\text{ml} & \quad 40\% \text{ acrylamide mix} \\
   25\text{ml} & \quad 1.5\text{M Tris, pH 8.8} \\
   25\text{ml} & \quad 10\% \text{ SDS} \\
   1\text{ml} & \quad 2\% \text{ APS (ammonium persulfate)} \\
   75\text{ul} & \quad \text{TEMED}
   \end{align*}
   \]

   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.

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2ml 5% stacking gel mix</td>
<td>2ml</td>
</tr>
<tr>
<td>(dH$_2$O) 73ml</td>
<td></td>
</tr>
<tr>
<td>40% acrylamide mix</td>
<td>12.5ml</td>
</tr>
<tr>
<td>1.0M Tris, pH 6.8</td>
<td>12.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1ml</td>
</tr>
<tr>
<td>25ul 2% APS</td>
<td></td>
</tr>
<tr>
<td>2.5ul TEMED</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X SDS Gel Loading Buffer</td>
<td>2ml</td>
</tr>
<tr>
<td>-20% SDS</td>
<td>2.5ml</td>
</tr>
<tr>
<td>-500 mM Tris, pH 7.6</td>
<td>3ml</td>
</tr>
<tr>
<td>-1% Bromophenol Blue</td>
<td></td>
</tr>
<tr>
<td>50% Glycerol</td>
<td>4ml</td>
</tr>
<tr>
<td>Protein sample</td>
<td>5ml</td>
</tr>
<tr>
<td>1M DTT</td>
<td>6ml</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Running Buffer: -4X SDS-PAGE Running Buffer</td>
<td>250ml</td>
</tr>
<tr>
<td>-Tris Base</td>
<td>12g</td>
</tr>
<tr>
<td>-Glycine</td>
<td>58g</td>
</tr>
<tr>
<td>-dH$_2$O</td>
<td>1L</td>
</tr>
<tr>
<td>-10% SDS</td>
<td>750ml</td>
</tr>
</tbody>
</table>

**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 30ml/well. Use 20ml for the protein standards ladder (heat BIO-RAD ladder to 37°C 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 50°C in a covered box. The longer the incubation, the better.

   Coomassie Blue Stain R-250 2.5g
   Methanol 1.0L
   Acetic Acid 0.2L
dH₂O 0.8L

   2. Wash with Destaining Solution 1 (50% MeOH, 7% HAc) 2X, 30’each, at 50°C.
   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 65°C.

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
   -dH₂O 700ml

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

   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.
   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 dH₂O 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 dH₂O). 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 Ab 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 Ab 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 dH₂O 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 Alphalmager 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/Chemiluminescence mode following exposure to ECL-Plus reagents (blot can be dry).