Protocol for *A. gossypii* Western blotting

R. Meseroll 07/30/2008 (updated 01/20/12)

Cell lysis:

1. Grow up an overnight (16h) Ashbya culture to a similar density as for Ashbya transformations (100µL-500µL dirty spores in a 200mL AFM+amp+ additional selection if necessary. Alter inoculum depending upon cell yield).

2. Harvest cells by vacuum filtration. Aim for AT LEAST 100mg cell yield. Transfer cells to a 1.6mL screw cap tube(s) with a gasket (beadbeater tubes). **Use no more than 500mg cells per tube or lysis may be hampered.** Add two times the mass (µg) in volume (µL) of lysis buffer plus protease inhibitor cocktail. Add 150-200µL 0.5mm Zirconia/Silica beads (BioSpec Products Inc.).

3. Immediately beadbeat for 1.5 minutes at top speed, rest 30 seconds. Repeat for a total of five beadbeats.

4. Spin down suds briefly in cold (4°C) microfuge. Transfer lysates to new eppendorf tubes. Spin down lysates 10min @ 13.2K rpm in cold microfuge. Transfer supernatant (soluble fraction) to new tubes and resuspend pellet (insoluble fraction) in 100-200µL lysis buffer. Store @ -20°C until ready for use.

   n.b.- You will not need the microfuge to be cold anymore at this point, so you can now raise the temperature back to RT and open the lid. If the microfuge is at a temperature other than RT, make sure the lid stays closed.

5. Do a Bradford assay to determine concentration of lysates.

Western blotting:

1. Prepare samples for loading onto gel. 10µg total protein/well is a good starting point. Too much protein loaded will lead to uneven sample separation.

2. Boil sample in sample buffer and load onto an SDS-PAGE gel. 10% acrylamide gels are appropriate for the septins. Run gels at 165V until the dye front reaches the end of the gel, or longer for greater separation (use prestained marker to judge where your proteins of interest are running). Vary acrylamide percentage and running voltage as necessary for the resolution you need.

3. While the gel is running, set up Western blot equipment. Soak PVDF membrane in methanol for approximately 10 seconds. Rinse briefly with ddH_2O then soak in ddH_2O for 5 minutes. After 5 min., soak PVDF membrane in Western transfer buffer until the gel finishes running (at least 30 min). PVDF must be presoaked or transfer will not work.

4. Fill the transfer cell most of the way up with transfer buffer. Place stir bar at the bottom of the transfer box.

6. Remove gel from running apparatus. Do not reuse protein gel running buffer. Dispose of the stacking layer and carefully place the gel on top of the PVDF membrane on the clear side of the cassette. This is easiest if the gel is wet. Make sure there are no air bubbles! Layer the other piece of filter paper and sponge from the black side of the cassette on top of the gel. Close and clamp the cassette and insert into the transfer cell, with the black half facing the black side of the cell and the clear half facing the red side of the cell.

7. Transfer at 240mA for 2 hours with moderate stirring, in the cold room.

8. When transfer is finished, disassemble the cassette and cut PVDF membrane as necessary based on expected sizes of the proteins you are blotting for. **Transfer buffer can be reused 4-5 times before the methanol is spent.** Discard buffer once it starts to turn yellow.

9. Block membrane for at least half an hour (more will give you a cleaner blot, however) in Western wash buffer with 3% dry milk. For short amounts of time, block at RT, but longer or overnight blocks can be done at 4°C.

10. Dilute primary antibody to desired concentration (2μg/mL is a standard concentration, or if concentration is not given, 1:1000 is a good rule of thumb, unless the antibody manufacturer recommends something else) in Western wash buffer with 3% milk plus 5mM sodium azide, in a 50mL Falcon tube. These antibodies can be stored at 4°C and reused. After the block is finished, place membrane strips in appropriate primary antibody. Probe the blot with primary antibody overnight on a rocker in the coldroom. This can be done directly in Falcon tubes for small membrane strips, or in the small Nalgene containers for larger membranes. Make sure all of the transfer side of membrane is coming into contact with the antibody solution.

11. Wash the blot 3x10 minutes in Western wash buffer.

12. Incubate the blot in with a secondary antibody (standard dilution 1:10,000) at room temperature for 1hour, making sure your secondary is against the species that your primary antibody was raised in. Use an AP-conjugated secondary to develop with ECF substrate (5min at RT) or an HRP-conjugated secondary to develop with ECL substrate.

13. Wash the blot 3x10 minutes in Western wash buffer. Rinse the blot with 1X PBS by swirling blot by hand.

14. Develop the blot with ECF for 5 min at room room temperature, completely dry the blot with kimwipes, place in transparent paper protector, and visualize on Storm scanner face down (core facility). Our username is gladfelter, password is 527891. Choose the fluorescence acquisition setting, check blue wavelength only (450nm), and choose voltage (800V is a good starting point, use 600V for a second pass if the first blot is overexposed). Open the blots in ImageQuant on the core facility computer and save as a tiff. Bring a flash drive so you can take your files with you, since the Storm scanner computer works very slowly on the internet.

Recipes used in this protocol:

Lysis buffer (from Doug Kellogg, UC Santa Cruz):
50mM HEPES-KOH, pH 7.6
1M KCl (high salt!)
1mM $MgCl_2$
1mM EGTA
0.1% Tween-20
5% glycerol
1X-2X Roche protease inhibitor cocktail

5X sample buffer:
1.25mL 1M Tris Hcl pH 6.8
2.5mL glycerol
4mL 10% SDS
0.5mL β-mercaptoethanol
1.75mL ddH_2O
trace of bromophenol blue (just enough to turn solution blue)
*aliquot and store at -20°C

1X PAGE running buffer:
28.8g glycine
6g Tris base
100mL 10% w/v SDS
water up to 1L

10x
288g
60g
100

1X Western blot transfer buffer:
200mL methanol
14.41g glycine
3.03g Tris base
water up to 1L

500 ml
100 ml
7.2g
1.52
upto 500

Western wash buffer with 3% dry milk:
100mL 10X PBS
14.6g NaCl
1mL Tween 20
30g dry milk (for 3% w/v)
water up to 1L
*store at 4°C
****it is best to make up a small portion of this (50-100mL) the day of use.**

Western wash buffer:
100 mL 10X PBS
14.6g NaCl
1mL Tween 20
water up to 1L

2-gel 10% acrylimide gel recipe:

Resolving layer:
2.52mL 1.5M Tris Hcl pH 8.8
3.80mL ddH_2O
3.32mL 30% acrylimide/1% bisacrylimide
250µL 10% SDS
125µL 10% APS
15µL TEMED (add last!)
*immediately overlay with 2% SDS to avoid gel drying. Pour off prior to adding stacking gel!

Stacking gel:
0.6mL 1M Tris Hcl pH 6.8
3.65mL ddH_2O
0.67mL 30% acrylimide/1% bisacrylimide
48µL 10% SDS
26µL 10% APS
4.8µL TEMED (add last!)
*immediately and carefully insert wells.

Gels can be made in advance, wrapped in a damp paper towel and plastic wrap, and stored at 4°C.