

SDS-PAGE and Western blotting for *Ashbya*

- Grow at least 10 mls of cells to a density comparable to use in transformation.
- Pellet cells at 3000 rpm and remove media. Tubes can be put at -70 C for storage before making lysates. If frozen, thaw lysates quickly by warming in the hand and then immediately put on ice. Always keep samples on ice from here to limit protease activity.
- Make Lysis Buffer and add protease inhibitors just prior to use:
 - NP-40 lysis buffer:
 - 50 mM Tris-HCl (pH 7.5)
 - 150 mM NaCl
 - 5 mM EDTA
 - 1% NP-40
 - 1 mM sodium pyrophosphate
- Store buffer in 10 ml aliquots at -20 C. Have chilled before adding to cells.
- Resuspend pellet in an equal volume lysis buffer (ex 500 μ l cells/500 μ l LB) and transfer to an eppendorf tube. Add half the total volume in small glass beads (ex. 500 μ l). Vortex in cold room for 4 X 15 minutes, pausing between each 15 minute period for one minute to allow lysate to cool.
- Clarify lysates by spinning at full speed-14,000 rpm- for 10 minutes at 4 C. Remove supernatant and transfer to a new tube.
- Determine protein concentration by Bradford assay. Dilute Bradford Dye 1/5. Use BSA for a standard curve and use 1-2 μ l lysate to measure concentration. Be sure to use lysis buffer + protease inhibitors alone as a blank. Measure OD at 595 nm.
- Load 50 μ g protein/lane.
 - SDS running buffer 4X:
 - 12 g Tris
 - 57.6 glycine
 - in 1L H₂O
 - Add 10 ml, 10% SDS just before use.
- Transfer proteins from gel to nitrocellulose:
 - Transfer buffer:
 - 1X:
 - 48 mM Tris base

- 39 mM glycine
 - 20% methanol
 - Make a 10X stock without the Methanol and add the methanol just before use.
- After transfer, block blot in 2% milk in PBS-T (1X PBS+0.05% Tween20). Incubate with antibodies 2 hours and wash after both primary and secondary 3X10 minutes in about 100 ml PBST, shaking quickly.