Protein protocols – Western blotting

A. Protein sample prep

Grow cells in 10 ml LB and appropriate antibiotic overnight (12-16 hrs)
Spin down cells in 15 ml sterile tubes
Resuspend in 0.5 - 1 ml PBS (keep samples on ice)
Sonicate –
Clean sonicater tip 2-3 times with DI H2O
Sonicate 2x for 30 sec – 1 min at 50% duty cycle, output control – microtip limit (5)
Keep samples on wet ice! Samples will heat up.
Transfer cell lysates to a sterile 1.5 ml microcentrifuge
Spin at 4°C and max rpm for 30 minutes to separate soluble and insoluble fractions
Transfer supernatant to clean 1.5 ml tube and label as soluble fraction (keep on ice or store in 4°C)
Optional: resuspend insoluble fraction in 0.5 - 1 ml 8 M urea (keep volume the same as soluble fraction). Incubate at 100°C for 5-10 min and recentrifuge 10 minutes at room temp and max rpm. Save supernatant as insoluble fraction. Store in 4°C

B. DC protein assay (BioRad) – From Microplate protocol

Prepare 5 dilutions of a protein standard (BSA – BioRad Protein Assay Standard II) containing from 0.2 mg/ml to about 1.5 mg/ml (or concentration of standard). Standard curve should be prepared for every assay
Pipet 5 µl of standards and samples into clean dry microtiter plate. Usually do samples in a 1:10 dilution
Add 25 µl reagent A into well
Add 200 µl reagent B into well. Mix (pop any bubbles) and let sit for 15 min to 1 hr.
Read absorbances at 750nm on Spectramax plus UV platereader (Molecular Devices)
Fit standard curve (A vs Conc) and determine protein concentration

C. Protein Gel

Prepare Samples (~15 µl in a well) – add to protein -10 X Nupage antioxidant and 4X Nupage LDS sample buffer (to 1X concentration) Make sure same amount of total protein is run (if running insoluble same volume fraction as soluble)
Heat samples at 100°C for 5-10 min
Warm up standards to room temperature – Magic Mark Western standard (Invitrogen) or BioRad Precision Plus Protein all blue standard
Set up protein gel – Use Nupage 4-12% BisTris Gel from Invitrogen (stored at 4°C)
Open package (pour out any excess buffer) and remove gel cartridge
Remove white strip to expose foot (needed to expose gel to buffer when running)
Remove comb carefully (press straight up)
Rinse wells with DI H2O to remove residual buffer
Place in XCell sure lock cell (see diagram)
Fill inside compartment with 1X MES to top, then outside partway to ensure foot is exposed
Fix any lanes that are deformed
Load Gel with protein samples
Run @ 190V for 45 minutes. Dye to run off gel and stop in foot.

After run gel is removed by "cracking" casing around gel, push off of casing at foot – wet fingers with DI water before handling gel
After running gel, place never before used 1X MES buffer in once used 1XMES or discard if second use. Clean cell with DI water and store dry.
D. Western Blot – Transfer proteins to membrane
Soak ImmunBlot PVDF Membrane (0.2µm) in MeOH for 30sec to 2 min
Soak 2x extra thick blot paper (criterion size) in 1X Nupage transfer buffer
Soak membrane in transfer buffer between blot paper
Remove TransBlot SD semidry transfer cell (words facing you) and remove lid and inner top
On transfer cell lay blot paper then PVDF membrane
Carefully place gel on PVDF membrane with foot facing up and no bubbles! – be careful not to move around on membrane
Cut off the raised foot of gel and place blot paper on top
Use pipet to remove excess buffer by rolling over blot paper – don't move gel around!
Check membrane to make sure it is still lined up
Reassemble apparatus, Plug into machine
run @ 15V for 30 minutes (proteins will travel down onto membrane) – be careful removing membrane at end.
Dry surface, rinse with DI well, then 70% EtOH and let dry before storing

E. Western blot – Blotting
Make blotting buffer – 50 mL 1X TBST w/ 2.5 g milk protein Buffer is used to coat surface of membrane with proteins
Carefully remove gel from membrane. Make sure protein transfer occurred.
Incubate in 50ml blocking buffer for at least 1 hr, continuous rocking
Remove blocking buffer. Add 10ml blocking buffer to membrane.
Add primary antibody 1:5000 to 1:20000 dilution
Incubate 2 hrs at room temperature, Optional: add more 1X TBST to prevent membrane from drying out and incubate overnight
Quick wash 2x with 10 ml 1X TBST
Wash 3x with 10 ml 1X TBST for 5 minutes (rocking)
Incubate 1 hr in 50 ml 1X TBST with 1 µl secondary antibody (goat anti-rabbit)
2x quick wash with 25 ml 1X TBST
3x wash with 50 ml 1x TBST for 10-20 min
Mix Immun-Star westernC Chemiluminescent kit reagents – 1 ml of luminol/enhancer and 1ml peroxide buffer
Remove wash buffer from membrane and add 2ml of kit reagents. Make sure no air bubbles so surface is completely covered with substrate
Drain membrane by touching one corner of membrane to kim wipe and let solution wick off. Do not let it dry completely
Develop using Bio-Rad molecular imager ChemiDoc to analyze