Western Blotting

Also contains info on antibodies in common use in the lab
Also a protocol for India Ink staining the membrane

NOTE 1: Prepare protein samples (Whole Cell Extracts (WCEs), immuno-precipitates, recombinant proteins, etc) as desired and determine protein concentration if possible. Mix sample in an ependorff 1:1 with 2x reducing loading buffer. Heat to 85ºC, 5 min and put on ice. Spin microfuge, 12000rpm, 5 min, RTº and load as desired.

NOTE 2: For >90% of experiments small gels are used.

SDS-PAGE:

1. For a small gel do not load >75µg protein per well or gel has tendency to smile. Avoiding the outer lanes can help. **ALWAYS** use a marker lane. In case of emergency it is possible to load sample and markers in the same well if immunological detection is planned (although some crappy Abs could cross-react).
NOTE 3: 1.5mm spacers usually used. Comfortable volume: 10 well comb ≈ 25µl, 15 well comb ≈ 15µl
NOTE 4: Acrylamide 10 - 15%. For general WCE resolution I use 12%, if histones are under test, 15%. Choose depending on size of protein you're interested in. Consider loading controls for Westerns - you'd like to use a protein far away from the protein under test (that way you can slice the same membrane).

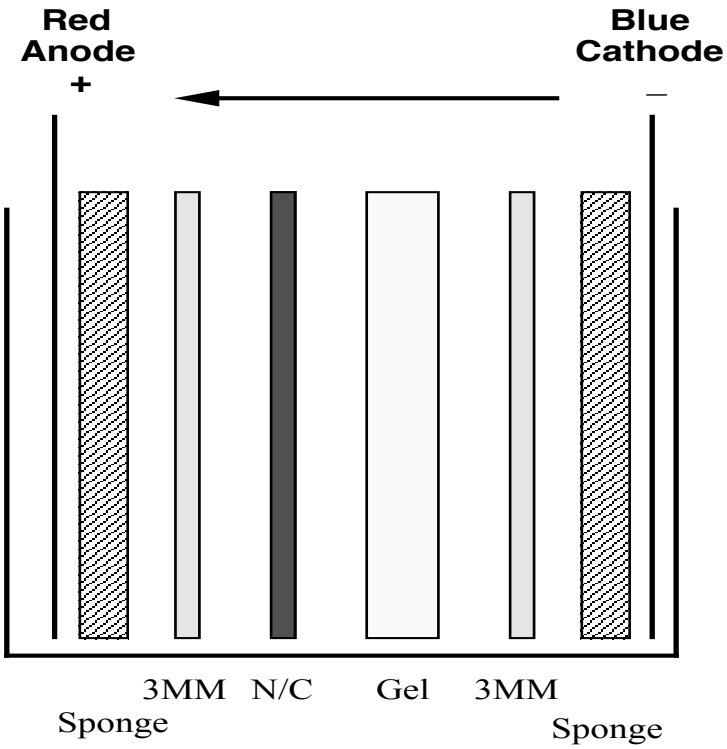
2. Running buffer: 1x TGE-SDS
Small Gel: Stack 100V, 20 min. Resolve 150V until desired (12% gel will take ≈ 60min to run dye front off bottom).
Large Gel: Stack 100V, 60 min. Resolve 170V until dye front off bottom or 10V O/N (for a 12% gel a good window is 800V hours)

Gel Transfer:

3. After desired separation, transfer the gel onto supported nitrocellulose or PVDF membranes.
NOTE 5: Soak PVDF in MeOH for 10 minutes before moving to transfer buffer. For supported nitrocellulose prewet in transfer buffer.

4. Prewet everything in transfer buffer before assembly. Apply membrane to gel on wet 3MM. Cover with wet 3MM and roll out air bubbles. It is important to remove all air bubbles as otherwise they will interfere with your Ab probing. Assembly order for sandwich are overleaf. Ensure the membrane sandwich is completely covered in transfer buffer when immersed in the tank and no air-bubbles are present.
NOTE 7: Have a stack of pre-cut 3MM and nitrocellulose available.
NOTE 8: If worried about incomplete transfer of some proteins, modifying the MeOH and / or SDS concentration can affect transfer efficiency (**eg**. if you're interested in proteins ≈ 200kD (eg. RNApII large subunit), drop the MeOH to 10%).

Transfer assembly:



5. **Small gel**: Transfer 60V 4 hours, 4°C. Check periodically to ensure no over-current alarm. Alternatively transfer 25V O/N 4°C. Ensure gel is cooled throughout the run with ice pack inserts. Once gel has transferred, disassemble, mark to distinguish protein from non-protein side, and transfer to blocking buffer (**blotto**). Block with gentle shaking O/N 4°C, or 60 min RT°.

Western:

6. Probe with appropriate Ab at RT° for 1hr with gentle shaking. Use smallest volume possible to conserve the Ab stocks. Optimal dilution as determined; Ab usually diluted in blotto + 0.001% Thimerosal (Dilute 1/1000 from 1% stock in ddH_2O at –20°C).
NOTE 9: For some phospho-antibodies it is recommended to use TBST / 1% BSA instead of blotto.

7. Remove Ab and store at -20°C. Note the # of uses on the Falcon. For many Abs it is possible to reuse the mix. This is Ab dependent.

NOTE 10: Some common Abs used in the lab, along with info for Westerns (and ChIP if available).

(i) TBP, ≈ 27kD. Rabbit polyclonal, 1/2000 blotto. Reuse up to 5 times. Not recommended as a loading control: signal too strong. **ChIPs:** (3µl Ab per 10µl protein A Sepharose)

(ii) eIF-5a, ≈ 17kD (Hyp2, YEL034W: Rabbit polyclonal from Richard S. Zitomer, SUNY Albany). 1/5000 blotto. Reuse up to 5 times. Clean, bck @ ≈ 40kD. *Great loading control*. **Not Tested in ChIP**

(iii) Rpn5, ≈ 52kD (YDL174W: Rabbit polyclonal (**#4798**) from Dan Finley, HMS). 1/5000 Blotto. Reuse up to 5 times, very clean blot. *Great loading control*. **Not Tested in ChIP**

(iii) Rpn8, ≈ 38kD (YOR261C: Rabbit polyclonal (**#4797**) from Dan Finley, HMS). 1/5000 Blotto. Reuse up to 5 times, very clean blot. *Great loading control*. **Not Tested in ChIP**

(iv) Rpt1 ≈ 52kD (YKL154W: Rabbit polyclonal from Dan Finley, HMS). 1/2000 Blotto. Reuse up to 5 times, very clean blot (looks better 2nd blot on). *Great loading control*. **Not Tested in ChIP**

(v) Htz1 (C-terminus) ≈ 14kD (YOL012C: Affinity purified Rabbit Polyclonal, Upstate 07-718) 1/400 blotto. Weak signal; strong background band about 40kD. **Crap in ChIPs**

(vi) Htz1-K14^{Ac} (Affinity purified Rabbit Polyclonal, Upstate 07-719) 1/2000 blotto. Great signal; strong background band about 40kD. **Crap in ChIPs**

(vii) HA (***mouse*** ascites, clone 12CA5 (clone HA.11 is not as good)). Use 1/2000. Great Ab although some yeast WCEs give a strong background band ≈ 40kD, cleans up on reuse. **ChIPs:** 3µl Ab per 10µl protein A Sepharose.

(viii) FLAG (***mouse***: α-FLAG-M2, Sigma). Use 1/2000. Yeast WCEs give a horrible background on first use or two, much better on later uses. **ChIPs:** Struhl lab uses, no personal experience.

8. Wash membrane TBST 5 x 5 min RT^{o} with gentle shaking. It is important to remove all the non-specifically bound Ab.

9. If 2nd layer is required (and it generally is), add (as appropriate) goat α-rabbit*HRP or goat α-mouse*HRP (dilute 1/10,000 in blotto). Incubate RT^{o} for 1hr with gentle shaking. Wash membrane TBST 5 x 5 min RT^{o} with gentle shaking.

10. Generally use ECL-detection system: Read and follow the instructions for the specific kit carefully. For the Pierce SuperSignal West Pico kit (Prod # 34080) mix detection reagents 1:1. **USE FRESH TIPS EVERY TIME YOU ENTER THE BOTTLES.** For a Small membrane use 2mls of each. Ensure membrane is fully wetted in the mix and shake 5 min at RT^{o}. Drain the excess buffer from the membrane and place **Protein side up** on a piece of Saran-Wrap. Wrap and gently smooth out any air-bubbles. Expose protein side to blue-film for multiple exposures in the dark room: bracket 10s, 1min, 5 min, etc (optimal will be antibody / sample dependent).

11. India-Ink Staining: Useful if you want to check your transfer, and usually done after the Western. Remove membrane from saran-wrap and wash with PBS / 0.4% Tween-20. Add ink solution: 100µl India-ink (Pelikan Black 17, 30ml #221143) in PBS / 0.3% Tween-20. Stain RT° with gentle shaking 15 min – overnight. Destain with numerous changes of PBS. Photograph for records.

BUFFERS:

2x reducing loading buffer: 60mM Tris pH 6.8
2% SDS
10% Glycerol
0.2% Bromophenol Blue
100mM DTT

TBST: 20mM Tris pH 7.5, 150mM NaCl, 0.1% Tween 20

Blotto: TBST + 5% skimmed milk powder + 0.001% Thimerosal (see **NOTE 9**).
NOTE 11: Do not add Azide

SDS-PAGE Gels: A good gel to start with is 10% : good for proteins 20 - 60kD (Maniatis), or essentially everything (me) (see **NOTE 4**).

10% resolving gel (15mls):	10% SDS	150µl
	H_2O	5.9mls
	30% Acryl	5mls
	10% APS	150µl
	TEMED	6µl
	1.5M Tris pH8.8	3.8mls

Allow the gel to polymerise - layer 80% IPA over the mix to remove bubbles.

5% Stacking Gel (5mls):	10% SDS	50µl
	H_2O	3.4mls
	30% Acryl	830µl
	10% APS	50µl
	TEMED	5µl
	1 M Tris pH6.8	630µl

10x TGE-SDS buffer: 1.92M Glycine
0.25M Tris base
10mM EDTA
(1.0% SDS)

Western Transfer Buffer: 1x TGE buffer
add before use: 10 - 20% MeOH (see **Note 8**).