Sarah's Western Blot Protocol

The following protocol is optimised for use with the Invitrogen XCell *SureLock* Mini-cell and the XCell II Blot modules, and Invitrogen pre- cast 4- 12 % Bis- Tris gels. I also use the ECL detection system from Amersham Biosciences (ECL DualVue western blotting markers, Hybond- ECL membrane, ECL detection reagents and Hyperfilm- ECL). A list of all the buffers used can be found on the last page.

Day 1

1. Set up O/N cultures in 5 ml 1x LB (with antibiotic if necessary) or whatever media you are using (minimal media etc)

Day 2

1. Take the OD_{650} of the O/N culture and spin down 500 µl culture (from the flask) for 90 seconds. Remove as much of the supernatant as possible and store the pellet in the freezer until ready to be used.
2. I then inoculate 500 µl O/N culture into 50 ml 1x LB (with antibiotic if necessary) and leave to grow at 37 °C. At various time points throughout growth I take an OD_{650} reading and spin down 2 ml, 1 ml or 500 µl (depending on the OD_{650}). Again, remove as much of the supernatant as possible and store the pellets in the freezer.

Day 3

1. When you are ready to run the protein gel, calculate how much cracking buffer to add to your pelleted samples by multiplying the actual OD_{650} of the sample by 200 (2 ml), 100 (1 ml), or 50 (500 µl), depending on how much you spun down at that time point.
2. After adding cracking buffer, heat the samples at 90 °C for 5 minutes, vortex for 10 seconds then spin down the samples for 15 seconds. If the sample is still 'globular' and hard to pipette, then repeat step 4. Sometimes it takes a very long time to resuspend the pellet
3. Load 15 µl or 20 µl of your samples onto an SDS- PAGE gel with an appropriate marker (e.g. ECL DualVue marker).
4. Run the gel at ~ 150 V until the blue dye front reaches the bottom of the gel
5. Remove the gel and soak in transfer buffer for 10 minutes.
6. Cut a piece of nitrocellulose membrane (e.g. Hybond- ECL) so as it is the exact same size as the gel and soak it in distilled water for 5 minutes and then in transfer

11. Wash 3 times for 5 minutes each with ~ 25 ml of TBS/T (with vigorous shaking)
12. Incubate the membrane with the primary antibody (at the appropriate dilution) in 10 ml blocking buffer with gentle agitation overnight at room temperature (in a sealed bag).

1:10,000

Day 4

1. Rinse the membrane quickly with TBS/T, then wash 3 times for 5 minutes each with ~ 25 ml of TBS/T (with vigorous shaking)
2. Incubate the membrane with HRP- conjugated secondary antibody at the appropriate dilution (I usually use 1:2500) in 10 ml blocking buffer with gentle agitation for 1 hr at room temperature (in a sealed bag). If you previously used the ECL DualVue marker then also add the S- protein- HRP- conjugate (1:10000 dilution) with the secondary antibody (Although the S- protein-HRP- conjugate can be added during either the primary or secondary antibody incubations).
3. Wash the membrane 3 times for 5 minutes each with ~ 25 ml TBS/T (with vigorous shaking)

The following steps are for if you are using the ECL detection system

4. Remove the detection reagents from storage at 2-8 °C and allow to equilibrate to room temperature. Mix detection reagents A and B in a ratio of 40:1 (For two 100 cm^3 gels I use 6 ml solution A and 150 µl solution B).
5. Drain the excess TBS/T from the membrane and place protein side up on a sheet of cling film. Pipette the mixed detection reagent onto the surface of the membrane (Should be held on by surface tension) and leave for 5 minutes at room temperature.
6. Drain off the excess detection reagent by holding the membrane with a pair of tweezers and touching the edge against a tissue. Place the membrane, protein side down, onto a fresh sheet of cling film. Wrap up the membrane in the cling film and ensure that all air bubbles are removed.
7. I then use the syngene machine on the 8th floor for exposure. ALTERNATIVELY you can use the x-ograph machine in the basement. After wrapping up the membrane in cling film, place it in an x- ray film cassette and place a sheet of autoradiography film (I use Hyperfilm ECL) on top (under the safe red light). Close the cassette and expose the film. The film is then removed and developed immediately using the x-ograph machine. Based on the results, the next film may need exposing for a longer/ shorter time.

buffer for another 5 minutes. Make sure that you only touch the membrane around the edges with clean tweezers. Also cut 8 pieces of filter paper (for each gel) so as they are the same size as the gel and the membrane aswell. Soak the filter paper with the blotting pads in transfer buffer for 5 minutes.

7. Set up the western transfer as shown on the following page, making sure that the gel is closest to the cathode plate. Remove any air bubbles by rolling a glass pipette over the surface of each layer. Make sure that you add enough blotting pads to ensure a snug fit into your blot module.
8. Transfer conditions will vary according to the system being used, but I transfer at 30 V for ~ 3 ½ hours in transfer buffer (at room temperature). You can do the transfer at a higher voltage for a shorter time (1 ½ hours), but I prefer to do it this way.

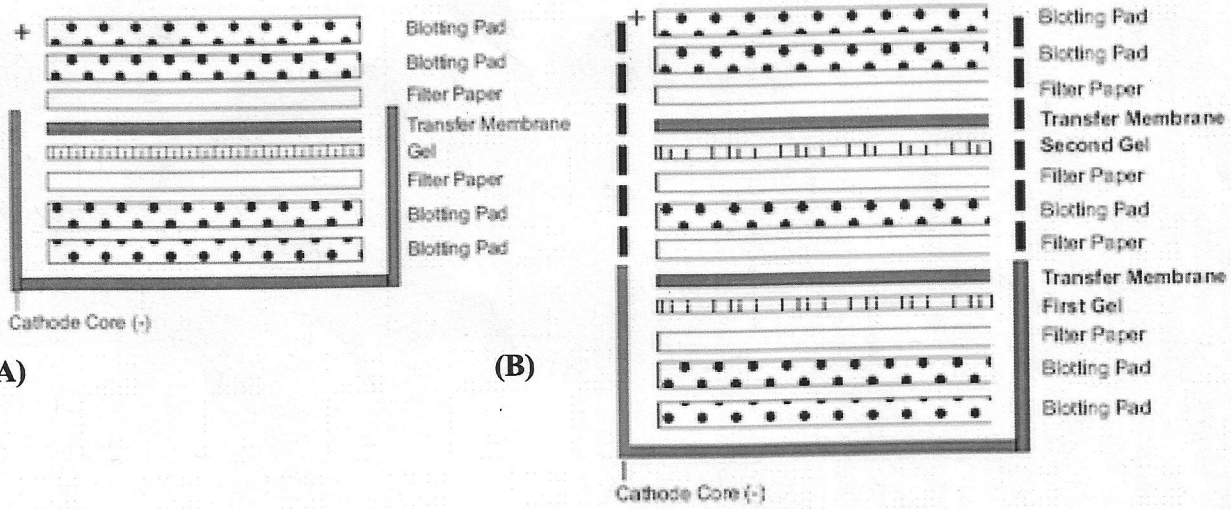

Figure: Western transfer set- up for either one (A) or two (B) gels: Every time is says to add one piece of filter paper, I use two.

The following volumes are for 10 cm x 10 cm (100 cm^2) of membrane: for different sized membranes, adjust the volumes accordingly.

9. Following transfer, wash the nitrocellulose membrane with 25 ml 1 x TBS for 2 x 5 minutes at room temperature, with vigorous shaking.
10. Incubate the membrane in 25 ml of blocking buffer for 1 hour at room temperature

Any questions, see Sarah Piper (SEP154@bham.ac.uk)

Cracking Buffer (SDS- PAGE loading buffer)

2 g SDS, 20 ml glycerol, 5 mg bromophenol, made up to 92 ml with a 1:10 dilution of the following buffer (15.15 g Tris, 10 ml 10 % SDS, 500 µl Temed- make up to 100 ml with distilled water then pH to 6.8).

When you come to use the cracking buffer, take a 1 ml aliquot and add 87 µl β-mercaptoethanol.

Transfer Buffer

For 2 l add 5.8 g Glycine, 11.6 g Tris base, 100 ml methanol and make up to 2 l with distilled water- adjust to pH 8.3 with HCl

10 x TBS (Tris- buffered saline)

To prepare 1 l of 10 x TBS: Add 24.2 g Tris base and 80 g NaCl to 800 ml distilled water. Adjust pH to 7.6 with HCl and make up to 1 l with distilled water (Use at 1 x)

Blocking Buffer

1 x TBS, 0.1 % Tween- 20 with 5 % w/v nonfatdry milk. For 150 ml, add 15 ml 10 x TBS to 135 ml water and mix. Add 7.5 g nonfat dry milk and 0.15 ml Tween- 20 (100 %)

TBS/T (Wash Buffer)

1 x TBS, 0.1 % Tween- 20: For 400 ml I use 40 ml 10 x TBS, 4 ml 10 % Tween, and make it up to 400 ml with distilled water.