IMMUNOBLOTTING

I. Preparing the cell lysates

1. Using a mouth pipette, transfer the cells into a 0.6 mL microtube. Place them on the side near the bottom. Watch through the microscope as you expel the cells to make sure that they all come out of the pipette and that you do not transfer excess medium. Do a quick centrifugation at max speed to pellet the sample at the bottom. Remove excess medium from tube.

2. Add required volume of Laemmli buffer and β-Mercaepthanol mix solution (1:1 ratio). Centrifuge briefly so that the drop with the cells slides into the buffer.

3. Denature by heating to 95-100^{o}C for 5 minutes (the MJ Cycler). Freeze at -20^{o}C until use. Samples may also be frozen prior to denaturation in -80^{o}C.

Note: It is important to always be wearing **gloves**, in particular for all the remaining steps that follow.

II. Preparing the polyacrylamide gel for electrophoresis

1. Prepare a polyacrylamide gel using the tables below. Before starting, test glass plate mold for leaks by filling it with water. Make sure the water level has not changed after 5 min before emptying apparatus. Start with the preparation of separation gel.

2. Add ingredients in the same order listed below in 15mL tube. Mix well with pipette before pouring in mold.

12%	10 mL	15 mL
Water	3.3	5.0
30% Acrylamide mix	4.0	6.0
1.5M Tris (pH 8.8)	2.5	3.8
10% SDS	0.1	0.15
10% APS	0.1	0.15
TEMED	0.004	0.006

Notes:

- 1.5-mm spacers will produce more concentrated and denser protein bands, but 1-mm spacers may produce a more even transfer onto the PVDF membrane and thus 'nicer' bands.
- Use fresh APS (ammonium persulfate) (prepared the day of)
- Remember to leave enough room for the stacking gel, but leave no more than ~1 inch. After pouring the gel, add enough water to the top to smoothen the edge of the separation gel (add it to the edge of the glass plate as to avoid splashing it onto the gel).

1. Let the separating gel sit for at least 30 min before layering the stacking gel on top. Make sure gel has set and that you can see a clear line between gel and water.

2. Remove the water from the top of the separating gel immediately before you are ready to pour the stacking gel. Add ingredients in the same order listed below in 15 mL tube. Mix well with pipette before pouring in mold.

Stacking	6 mL
Water	4.1
30% Acrylamide mix	1.0
1.0M Tris (pH 6.8)	0.75
10%SDS	0.06
10% APS	0.06
TEMED	0.006

Note: Set the comb between the glass plates so that the lanes are exactly perpendicular to the top of the separating gel. In other words, so that each sample has to migrate the same distance before entering the separating gel.

3. Let the stacking gel sit for at least 20 min. Make sure the gel has set and that the wells are ready before proceeding. Do not remove comb!

III. Electrophoresis

1. Assemble the cassette with gel(s). If you are only running one gel, use a dummy-mold. Make sure cassette is properly assembled to avoid leaks. Place in the bath, and fill with Running (electrophoresis) buffer (**RB**). Be sure that the buffer level is higher than the top of the wells. Pour **RB** into the box (outside of cassette) up to suggested lines.

Notes:
- Use fresh electrophoresis buffer each time.
- Make sure **RB** is not leaking from cassette.

2. Slowly remove the comb by pulling it straight up. Buffer should flow into the empty wells.

3. Load each well, including marker lanes. Expel each sample slowly so that it sinks to the bottom (the LB contains glycerol) and does not splash or flow into a neighbouring well.

4. Ensure that the leads are properly connected (**red to red, black to black**) and start electrophoresis at 80V. After the samples have entered the separating gel, increase at your discretion up to 150V.

Note: Ensure that buffer continues to cover the top of the wells throughout run. Running may stop if buffer inside cassette has reached too low of a level. Stop apparatus and fill it up again.

5. Run the gel until the proteins of interest have migrated ~2/3 of the way to the bottom. If you do shorter runs, you waste the resolving power of the gel.

IV. Transfer

1. Pre-chill Transfer buffer (TB) at -20^{o}C. Use fresh buffer for each transfer.

2. Minutes before the end of the electrophoresis run cut off a piece of PVDF membrane of the appropriate size. Cut a small notch in one corner. Wet the membrane quickly (10 sec) in methanol.

3. Place the sponges (x2), blotting paper (x4) and membrane in a dish containing ice-cold transfer buffer for 10 min with gentle agitation before starting the transfer.

4. When the electrophoresis is complete, disassemble the apparatus and remove one of the glass plates holding the gel (it easier to manipulate the gel later if the glass plate with the spacers is removed first). Keep track of which is lane 1. Cut off unnecessary parts of the gel (but don't make it too small) and slide it off the remaining glass plate onto the membrane - oriented so that lane 1 is to the LEFT and the top of the gel next to the notch of the membrane.

5. Fill a shallow rectangular dish with ice-cold transfer buffer and place an open transfer cassette, clear side down, in the dish.

6. Assemble the transfer cassette as shown below. Press gently on the gel and PVDF membrane to ensure that there are no air bubbles.

Notes: It is easier to assemble the transfer cassette starting from the clear side (clear side down)

Black
Sponge
Filter Paper
Filter Paper
Gel
Membrane
Filter Paper
Filter Paper
Sponge
Clear

7. Fold over the black side of the transfer cassette, clamp it, and place in the cassette holder in the bath (**clear side of the cassette should face red side of the holder and the black, the black side**). The bath should contain ice-cold transfer buffer, a frozen ice-pack, and a small stir bar. Ensure that the level of transfer buffer is almost to the top of the cassette.

8. Carry the whole assembly to the cold-room, place on the stirrer/hot plate, and start stirring.

9. Connect the electrodes (**red to red, black to black**) Double-check. If you get it wrong, your sample will transfer out of the gel into the buffer. Transfer for 90 min at 100V. If you need to visualize larger proteins (e.g. > 150 kDa), extend the transfer time to 2 hours.

10. Disassemble the apparatus and remove the PVDF membrane. If you see the marker proteins, the transfer worked. If you don't, it didn't. Lane 1 will be on the left side of the membrane. Allow the membrane to air dry protected from dust.

V. Antibodies

1. Rewet the membrane in methanol. Ensure that the membrane does not become folded, broken or smudged at any point during the rest of the detection procedure.

2. Block for at least 45 min in blocking buffer at room temperature on a shaker. 30 mL in a small dish such as the base of a pipette-tip box is convenient. **FROM THIS POINT ON, THE MEMBRANE CANNOT BE ALLOWED TO DRY AT ANY STEP OF THE PROCEDURE**.

3. Prepare a suitable plastic bag using the bag sealer. Add the appropriate volume of primary antibody solution. 5 mL is usually sufficient. Using forceps put membrane in bag and seal. Check that it is sealed and free of bubbles, and incubate overnight in the cold-room with gentle shaking.

4. The next day, cut open the bag at the top, remove the membrane using forceps and place in a dish containing PBST. Remove the primary antibody solution from the bag and into a 15 mL tube - return to the fridge.

Note: Most primary antibody solution can be stored and re-used. Time-frame for storage and re-usability will vary depending on antibody.

5. Repeat wash 3 x 10 minutes in PBST with agitation.

7. Prepare a plastic bag containing the membrane exactly as above this time with the secondary antibody solution (add 5 µL of HRP-conjugated secondary antibody in 5 mL of blocking buffer - 1:5000).

8. Incubate for 1 hour at room temp with gentle agitation.

9. Remove the membrane from the bag, and wash 3 x 20 (6 x 10) minutes in PBST.

VI. Signal detection

1. During the washes after the secondary antibody, prepare the ECL solution. Allow 0.1 ml per cm^2 of membrane. Store protected from light.

2. Also check the film processor – turn on and run the cleaning film if necessary.

3. Cut a piece of saran wrap that is comfortably larger than the membrane. Place it flat on a lab bench.

3. Using forceps, remove the membrane from the last PBST wash, touch it to a Kim-wipe to drain excess PBST and lay it flat **PROTEIN SIDE UP** (membrane notch on then top-left corner) on the saran wrap. Using a P1000, add the ECL detection reagent. Ensure that the whole membrane is covered. Surface tension should keep the solution on the membrane. Cover with something non-transparent and let it sit for 5 minutes.

4. Cut a new piece of saran wrap and lay it flat on a bench top. Using forceps, pull the membrane out of the ECL solution, touch it to a Kim-wipe to drain excess solution, and lay it **PROTEIN SIDE DOWN** (membrane notch on the top-left corner) on the saran wrap. Fold over the sides of the saran wrap to seal the membrane. Allowing the membrane to dry at this point is a common source of background.

5. Turn the membrane over, so the protein side is now up, and put it in a film cassette. Tape one corner to the cassette to keep it immobile (small piece of tape is fine).

Note: Put membrane away from ambient light in cassette as quickly as possible.

6. Expose to film in the dark room. Make several exposures of different durations so that both strong and weak signals can be seen.

Note: Do not expose film to any source of light (e.g. cell phone screens) until is has been processed.

7. Alternatively use the phosphorimager. For scanning, remove the membrane from the saran wrap and place protein side down on the glass plate. It may help to wet the back of the membrane after you place it on the glass. Set the sensitivity to 100 (more sensitive) not 200 (less sensitive).

VII. Quantification

1. Quantify using Image J. Draw a rectangle around first band (where height > width) and press (Ctrl + 1).

2. Drag rectangle to second band and press (Ctrl + 2). Repeat for all other bands while still pressing (Ctrl + 2) every time. When all bands have a rectangle around them press (Ctrl + 3).

3. A mean intensity graph for each band will appear. Draw a bar at the base of the peaks for each band. Click on the 'Wand' of the Image J menu. Drag it to, click and select the area under each peak one by one.

Note: Make sure that the bar crosses the graph twice to clearly define an area under each peak.

4. A table will appear giving you the area of each peak representing the intensity of each band.

Solutions
If any buffer is cloudy or has particles in it, it is no good and must be replaced.

10X TG
30.3g Tris Base
144.0g Glycine
1000mL Water
(Do not adjust pH)

Transfer Buffer
700mL Water
100mL 10X TG
200mL Methanol
Keep chilled at -20^{o}C

Running Buffer
850mL Water
100mL 10X TG
5mL 20% SDS

PBS

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Water	800 ml

Adjust pH to 7.4
Add water to bring final volume to 1000 ml

PBST
PBS, 0.1% Tween-20

Blocking buffer
PBST, 5% skim milk

Buffer for primary antibodies
PBST, 5% BSA (fraction V)

10% APS (prepared fresh)
0.1g APS
1mL Water
(Prepared in Eppendorf; vortex tube)