Buffers needed:

Cell Lysis Buffer: 150mM NaCl

50mM Tris (pH 7.4)

1% IGEPAL (v/v)

1:100 Protease Inhibitor Cocktail (add just before use)

Bradford Reagent: Use 4ml reagent to 16ml dH_2O (20ml total volume – enough for full 96-well plate)

[Reagent kept in fridge]

Running Buffer: 18.8g Glycine

3g Tris

10ml of 10% SDS

Make up to 1 litre with dH_2O

Transfer Buffer: 14.4g Glycine

3g Tris

200ml Methanol

Make up to 1 litre with dH_2O

PBS-Tween: PBS containing 0.1% Tween (v/v)

Blocking Buffer: PBS-Tween containing 5% non-fat milk powder

Antibody Buffer: PBS-Tween containing 1% non-fat milk powder

20% APS: 20% Ammonium Persulphate

Make up 60 mg in 300ul dH_2O.

6X SDS Loading Amount of 6X SDS Loading Buffer to add to the sample is

Buffer: (sample volume to load/6)

Bradford Assay Protocol

- Re-suspend samples in an appropriate volume of Cell Lysis Buffer (50-150ml depending on what it is) and sonicate briefly.
- Samples can then be stored at -80°C until needed.
- Before running on a gel, assay protein content of each sample using a Bradford assay:
- Prepare a 96-well plate in triplicate as below (where Red = Standards, Blue = Samples);

Standards

(use 0.5mg/ml stock of BSA)

S1 - 0ml

S2 – 1ml (0.5mg)

S3 - 2ml (1mg)

S3 - 3ml (1.5mg)

S4 – 4ml (2mg)

S5 - 6ml (3mg)

S6 - 8ml (4mg)

S7 - 10ml (5mg)

Make each up to 50ml with dH$_2$O and then add 200ml of 1x

Samples

Use 1-5ml of each sample

Do a test sample first using 2 or 3ml, make up to 50ml with dH$_2$O and add 200ml of 1x Brad

Colour of sample in each well should be around the middle range of the standards (S3-S6), if too deep,

For each sample, prepare in triplicate as below.

St1

St1

St1

S1

S1

S1

S9

S9

S9

S17

S17

S17

St2

St2

St2

S2

S2

S2

S10

S10

S10

S18

S18

S18

St3

St3

St3

S3

S3

S3

S11

S11

S11

S19

S19

S19

St4

St4

St4

S4

S4

S4

S12

S12

S12

S20

S20

S20

St5

St5

St5

S5

S5

S5

S13

S13

S13

S21

S21

S21

St6

St6

St6

S6

S6

S6

S14

S14

S14

S22

S22

S22

St7

St7

St7

S7

S7

S7

S15

S15

S15

S23

S23

S23

St8

St8

St8

S8

S8

S8

S16

S16

S16

S24

S24

S24

1. Once prepared, use plate-reader to assay protein content (wavelength 590nm)

DAY 1:

- Calculate how much of each sample to load onto gel so that there is equal loading of protein in each lane.
- Assemble plates for gel (1.0mm) and prepare desired percentage running gel according to volumes given on list (hanging up above gel tanks). Make sure to add 10% Ammonium Persulphate and TEMED last. Don't leave mixture for too long and add 1ml at a time.

- Once running gel is poured, layer a few drops of iso-propanol over the top to even the surface. Allow to set for ~30min.

- Remove iso-propanol and prepare stacking gel according to volumes on list. Add comb and allow setting for ~30min. Use so much so it doesn't overflow and to avoid bubbles, it can again be layered with iso-propanol.

- Whilst gel is setting make up 1litre of running buffer and prepare samples as follows:

1. Add ~1:20 b-mercaptoethanol to 6x SDS Loading buffer (in freezer)
2. Add appropriate amount of loading buffer to samples.
3. Make a hole on the lids with a needle and boil for ~5min before loading on gel.

Assembly 2: Green top tank which fits 2 gels opposite to each other.

- Assemble gels - thinner glass should be facing inside [or 1 gel with buffer dam on other side ('buffer dam' should face inside)] on the cassette and add to tank. Fill cassette with running buffer and fill tank ~half way.
- With a Pasteur pipette, clean the wells with Running buffer.
- Load samples alongside Protein Rainbow Marker. (Unstained marker)
- Run @50V for ~30min until samples have run through stacking gel and then run @ 100V until dye has reached bottom of gel.
- While gel is running prepare transfer buffer, 3mm Whatman paper and PVDF membrane.

Assembly 3: X Cell 11 Blot Transfer assembly

- To transfer use the X-cell Blot module, assemble as follows:
- Pour transfer buffer into a shallow dish and submerge cathode side (deep end) of blot module. Soak sponges in transfer buffer and add 2-4 to module.
- Soak two pieces of Whatman paper (cut to size) and place on top of sponges (make sure there's no bubbles)
- Remove gel from cassette and lay over the Whatman paper

- Lay nitrocellulose membrane on top of the gel (if using PVDF membrane, prepare by soaking in methanol for 30secs, then wash quickly in distilled water and soak in transfer buffer until needed)
- Soak another two pieces of Whatman paper and lay on top of the membrane
- Make sure there are no bubbles, if there are, roll a glass pipette over layers.
- Top off with the remaining sponges and place anode side on top.

Transfer Assembly Order:

3 sponges

2 filter paper

Gel

Membrane

2 filter paper

3-4 sponges

- Put assembled module in tank and clamp shut.

- Fill inside of module with transfer buffer (just use buffer that was used for soaking sponges etc) and fill outside tank about half full.

- Sit tank back in shallow dish and pack with ice.

- Run @ 50V for ~1 hour.

- Wash with PBS-Tween on shaker (15 mins.-1 hr)

- Once run, transfer membrane into ~30ml of blocking buffer. Either block for 2hr @ room temp or block overnight @ 4°C.

DAY 2:

- After blocking, wash membrane with PBS-Tween for 3x 10mins (with quick washes in between).

- Incubate in 1-5ml antibody solution containing the appropriate concentration of primary antibody (usually ~1:1000). Can either incubate overnight @ 4°C or for 2hr @ room temperature.

- Wash membrane as before.

- Incubate for 1hr in 1-5ml antibody solution containing appropriate secondary antibody (usually ~1:5000)

- Wash as before but do the final wash just in 1x PBS.

- Scan using Typhoon

OR

- Develop blot using the Amersham ECL kit (in fridge – use equal amount of each solution and make up ~2ml in total, incubate for 1min)
- Blot off excess liquid on tissue paper and wrap blot in Saran wrap.
- Stick into developing cassette and develop using the Xomat machine.