Running protein gels and detection of proteins

1. Protein concentration determination using the BIO RAD reagent

This assay uses a colour change reaction to give a direct measurement of protein concentration. The protein sample to be measured is added (typically 2 or 5 µl) to distilled water to give a final volume of 800µl. After vortexing to ensure a uniform protein concentration, 200 µl of BIO RAD reagent is added to the protein solution, again with thorough mixing, and the samples are left for 5-10 minutes. A spectrophotometer is then used to measure absorbance at 595 nm against a blank containing no protein. The optical density (OD) readings obtained in this way are then converted to protein concentrations using a calibration curve previously constructed using a range of BSA standards (1-15µg/ml). Extraction buffer is used to dilute the samples tested so that all will end up with an equal protein concentration. This is important for all cases where the protein is located intracellularly. If protein transport is assessed, the protein concentration is irrelevant and everything is related to the amount of original cell suspension. Determining the protein concentration can then merely establish if material was lost during the procedures.
Alternatively the assay can be carried out on the microtitre plate scale. 1-5 µl extract diluted to 160 µl with dH_2O (mixed thoroughly), 40 µl of BIO RAD reagent are added to the protein solution and mixed thoroughly. The absorbance at 595 nm is then measured using a microtitre plate reader. This small scale assay is ideal for the analysis of multiple samples for instance from transgenic plants. A BSA standard curve can be prepared using 1-15µg to allow calculation of protein concentration.

2. Protein gel blot analysis

2.1. Reagents for SDS-Polyacrylamide gels

Sample buffer

0.1 %	Bromophenol blue
5 mM	EDTA
200 mM	Tris pH 8.8
1 M	Sucrose

store at 4 ^{o}C

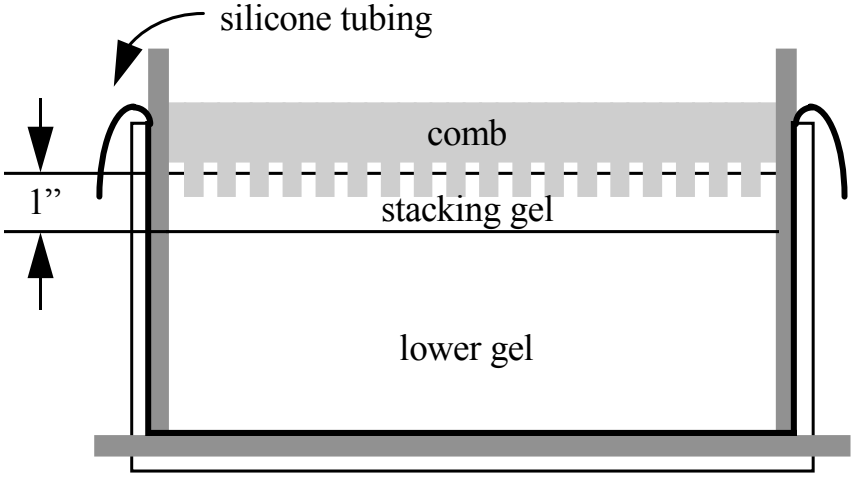
EDTA: ethylenediaminotetraacetate

Sample buffer mix

900 µl	Sample buffer (4 ^{o}C)
300 µl	10 % SDS
18 µl	1 M DTT (aliquots at –20 ^{o}C)

make up fresh

SDS: sodium dodecyl sulphate



DTT: dithiothreitol

Protogel

30 %	w/v acrylamide
0.8 %	w/v bisacrylamide

5× Running buffer

30 g	Tris
144 g	Glycine
5 g	SDS (or 50ml of 10 % SDS)

make up to 1 litre volume with distilled water

2.2. Preparation of the gel

The exact procedure depends on the size and the type of gel used. The following procedure is used for the system avaliable (home-made).

Preparation of SDS-polyacrylamide gels

Glass plates are cleaned with ethanol and assembled using 1mm spacers and silicone tubing which provide an effective seal (see above). The assembled plates are clamped together with bulldog clips and the separating gel can be prepared. This is added to a level 1 inch below the top of the wells (comb inserted to two-thirds of its length). A layer of water-saturated butanol is then carefully applied to give the gel a smooth surface and to exclude oxygen which would inhibit polymerisation (some people used distilled water, others distilled water with 1% SDS... the list is endless). When the running gel has set (1 hour later) and two distinct lines are visible, the butanol is poured off and the surface of the gel is rinsed with distilled water. After pouring the stacking gel, the comb is inserted to two-thirds of its length and the gel is allowed to

polymerise for one hour. Often gels can be prepared in advance and stored for up to 2 days in the fridge. Dehydration is prevented by covering the exposed surface of the stacking gel with damp paper towels and cling film.

For 2 Medium (30 well) gels:

Separating Gel (%)	6	8	10	12	14	16
dH_20	29.1 ml	26.1 ml	23.1 ml	20.1 ml	17.1 ml	14.1 ml
3 M Tris-HCl pH8.8	6.3 ml	6.3 ml	6.3 ml	6.3 ml	6.3 ml	6.3 ml
Protogel	9 ml	12 ml	15 ml	18 ml	21 ml	24 ml
10 % SDS	450 µl	450 µl	450 µl	450 µl	450 µl	450 µl
TEMED	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
10 % APS	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl
Final Volume	45 ml	45 ml	45 ml	45 ml	45 ml	45 ml

Stacking Gel	
20 % Sucrose	11.3 ml
1 M Tris-HCl pH6.8	1 ml
Protogel	2.5 ml
10 % SDS	150 µl
TEMED	30 µl
10 % APS	50 µl
Final Volume	15 ml

2.3. Sample preparation

The protein concentration of samples is equalised by addition of extraction buffer. An equal volume of freshly prepared sample buffer mix is then added, giving a final volume sufficient for repeat experiments. Sample buffer blank is prepared by addition of equal volumes of extraction buffer and sample buffer mix. All the samples are then heat denatured at 95 ^{o}C for 5 minutes.

2.4. Loading and running

The bottom spacer and silicone tubing are removed from the polymerised gel and it is clamped to the gel running apparatus. Running buffer is added to a level 1 cm above the well tops and to the lower chamber. Any bubbles trapped between the two glass plates beneath the gel have to be removed. With the samples ready to load, the comb is carefully removed and the wells are immediately washed using running buffer. The lanes are straightened and the blank lanes are marked and loaded with 15 µl of sample buffer blank using a Hamilton syringe a prestained molecular weight marker is usually added, 3-5 µl. 15 µl of the remaining samples are then loaded onto the gel, the syringe being washed with running buffer between samples.
With all the samples loaded, the gel is connected to a power supply and run at a limiting current of 40 mA for a medium gel or 50 mA for a large gel, the limiting voltage is 200 V. The gels usually run for 2-3 hours depending on the degree of separation required.

Once the gels are running the Hamilton must be cleaned with distilled water to ensure its' continued functionality.

2.5. Electroblotting

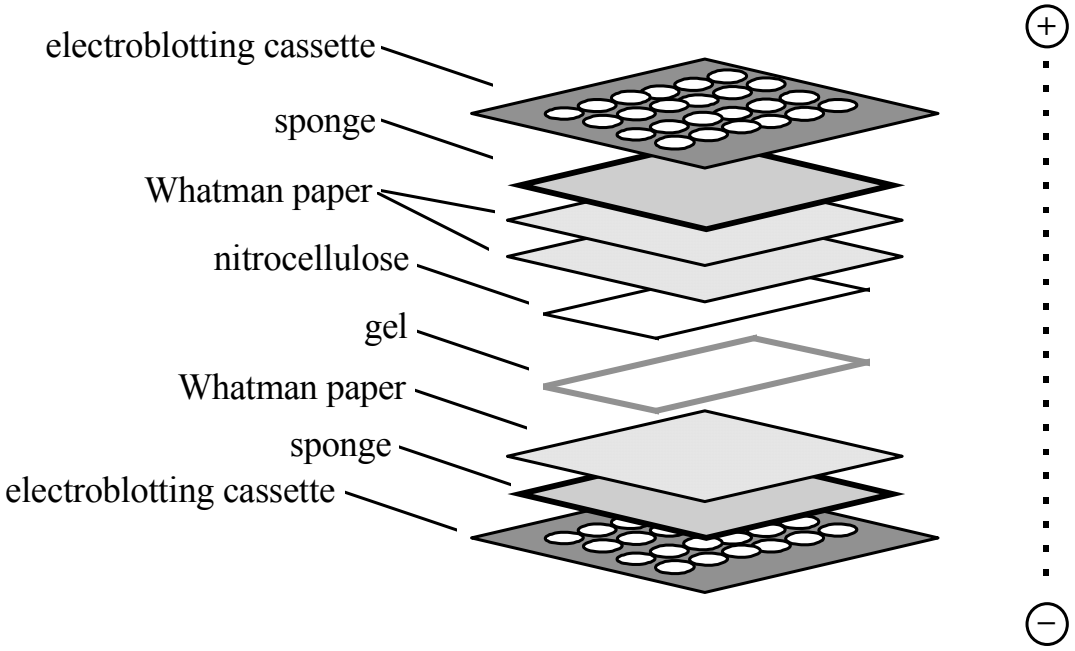
Electroblotting buffer

7.5 g	Tris
36 g	Glycine
250 ml	Methanol

make up to 2.5 litres with distilled water

Electroblotting

After running, the gel is removed from the apparatus and the glass plates are separated. The gel is cut to size using a plastic ruler and a corner is cut off to indicate the side from which it had been loaded. The blotting cassette is immersed in electroblotting buffer and the sponges massaged to ensure no air is trapped within



them. A layer of 3mm Whatman paper is then placed on the sponge. The gel is then placed on top of the paper and a piece of nitrocellulose sheet is cut to size and carefully laid on top of it. A further 2 pieces of Whatman paper are then placed on top of the nitrocellulose. The blotting cassette is closed and transferred to the blotting tank along with electroblotting buffer. Electroblotting is performed either overnight at a current of 200 mA, or for 2 hours at a current of 500 mA.

2.6. Western analysis

Solutions required:

10× PBS

87g	NaCl
22.5g	$Na_2HPO_4.2H_2O$
2g	KH_2PO_4

adjust to pH 7.4

PBS: Phosphate buffered saline

Blocking solution

to make 50 ml of blocking solution

5 %	milk powder
0.5 %	Tween 20

make up to 50 ml in PBS

Ponceau solution

0.1 % Ponceau S in 5 % acetic acid

Alternative Ponceau solution

0.2 % Ponceau S in 3 % TCA (Trichloracetic Acid)

Western blotting

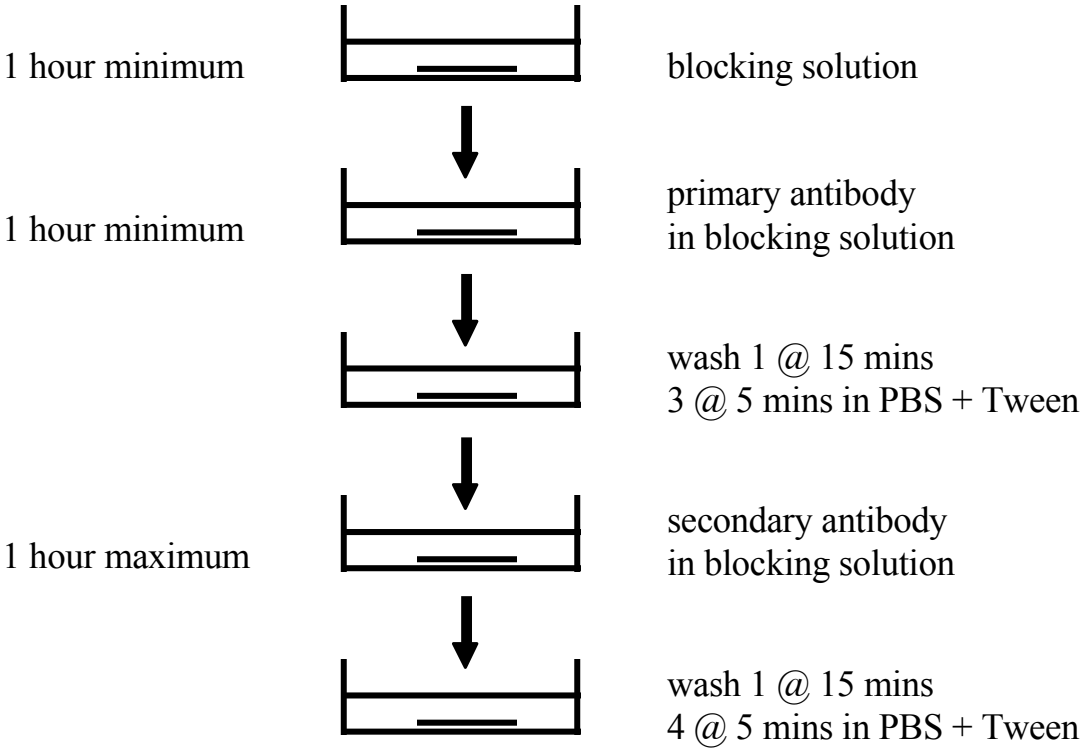
After the blotting period has been completed the cassette is removed from the tank and opened. A corner is cut off the nitrocellulose filter to indicate the direction of loading, at this point the membrane can either be rinsed in PBS and dried or the western can be carried out. If the membrane is dried to allow it will require rehydration in PBS for approximately 5 minutes before a western can be carried out. It is important to make sure that the membrane does not dry out during any of the subsequent processes as this will result in a failure to detect proteins.

2.6.1 Ponceau Stain

The membrane is placed 'proteins up' in a suitable container and covered in ponceau solution, the membrane is rocked for a minute then the ponceau solution is poured off (the ponceau solution is reusable so it is not poured down the sink but returned to its bottle). The membrane is rinsed with dH_2O two or three times, at this point it is possible to ascertain whether the blotting procedure was successful and how even the loading of the gel was. The membrane is washed several times in PBS+0.5% Tween 20 to remove any residual ponceau solution.

2.6.2 The Western

After washing away residual ponceau solution blocking solution is added to the membrane and incubated at room temperature on a rocking platform for a minimum of 1 hour. Primary antibody is either added to the blocking solution at a dilution of 1



in 5000 (5µl added to 25ml), alternatively the antibody can be stored diluted in a 1 % BSA solution with 0.02 % sodium azide in 1x PBS. If the primary antibody is diluted in BSA then the blocking solution needs to be washed away with PBS+0.5 % Tween 20 and PBS prior to addition of the diluted antibody.

- Antibodies diluted in blocking solution can be frozen at –20 °C and reused however many antibodies do not survive the freeze thaw process.

- Antibodies diluted in 1% BSA can be kept at 4 °C and reused this avoids the freeze thaw process.

After a further hour the antibody solution is stored appropriately and the filter is washed with PBS+0.5 % Tween 20. The membrane is rinsed two or three times then is washed for 15 minutes this is followed by three subsequent five minute washes. After washing, the secondary antibody (e.g. Goat anti-rabbit IgG horseradish peroxidase, stored at 4 ^{o}C) is added, diluted 1 in 5000 in blocking solution. The filter remains in this solution for a minimum of one hour. The secondary antibody solution is then discarded and the filter is again washed in PBS+0.5 % Tween 20, rinsed 2or 3 times then washed for 15 minutes and followed by four subsequent five minute washes.

Antibody visualisation using the ECL detection system

ECL Solution 1:

1 ml 1 M TRIS/HCl pH 8.5
100 µl 250 mM Luminol
44 µl 90 mM p-coumaric acid
8.85 ml dH_20

ECL Solution 2:

6 µl 30 % H_20_2
1 ml 1 M TRIS/HCl pH 8.5
9 ml dH_20

Luminol = 3-aminophthalhydrazide (Fluka): 0.44 g into 10 ml DMSO
p-coumaric acid (Sigma): 0.15 g into 10 ml DMSO

Make 105 µl aliquots of luminol, store at –20 ºC
Make 50 µl aliquots of p-coumaric acid, store at –20 ºC

After the final 5 minute wash, the membrane is washed in PBS to remove Tween which would compromise detection. Prepare the two ECL solutions, in the dark room pour off the PBS and add the two ECL solutions. Mix the solutions and swirl over the membrane for approximately a minute. The membrane is then transferred protein side down, to another piece of clingfilm. The edges of the clingfilm are folded over the membrane and it is inverted and placed in the film cassette. A piece of film is cut to size and laid on top of the filter. The cassette is then closed and the film exposed for an appropriate time (a few seconds to 1 hour). The film can then be developed and further exposures can be made if necessary. The maximum exposure time is limited to one hour by the duration of the ECL reaction. There is no point in exposing for longer times.