

2.3.8 DNA Agarose Gel Electrophoresis

Agarose (350 mg) was dissolved in Tris/Borate EDTA buffer (50 ml; TBE) by heating in a microwave until boiling, this solution was allowed to cool and ethidium bromide was added (0.05 µg/ml). Gels (0.7 %) were then cast in a 10 x 20 cm gel tank, any bubbles were removed with a pipette tip and then gel comb was added and the gel allowed to set. Gel electrophoresis was carried out in a horizontal tank containing TBE buffer and was run at 100 V for 1.5 h. Gels were imaged using the Typhoon 9410 Variable Mode Imager. This technique was used for the analysis of polyplexes (Chapters 4 and 6) and in the confirmation of plasmid preparation and purification (Fig. 2.6).

2.3.9 Western blotting

2.3.9.1 Preparation of a Cell Lysate

First a cell lysis buffer was prepared: 1 % (v/v) triton-X-100, 0.5 mM EDTA, 15 mM NaCl, 2 mM Tris base. This solution was aliquoted and stored at -20 °C. Before use the following protease inhibitors were added: leupeptin (2 µg/ml), pepstatin A (1 µg/ml), aprotinin (2 µg/ml) and phenylmethylsulphonyl fluoride (100 µg/ml).

In the case of the adherent cell lines: Caco-2; COS-1; COS-7; DU145; MCF-7 and PC3 cells, the cells were grown to 70-90 % confluence in a 75 cm² flask, washed with ice cold PBS on ice and 1 ml of the lysis buffer was added to cells. They were incubated on ice with rocking for 5 min before transfer to a -80 °C freezer. The flasks were removed from -80 °C and defrosted on ice, cells were scraped from the flask and the contents transferred to 1.5 ml eppendorfs. These were vortexed for 30 s then centrifuged at 13000 RCF at 4 °C for 10 min. The supernatant was removed and analysed for protein content using the BCA assay as described in Section 2.3.5. This solution was aliquoted and the samples stored at -80 °C until use in western blot experiments. In the case of the suspension cell line: U937, 20 ml of culture (at 70-90 % maximum cell density) was centrifuged at 200 RCF and resuspended in 1 ml of lysis buffer. This was incubated on ice for 5 min then frozen at -80 °C. This was removed from the freezer and defrosted on ice before a 30 s vortex. The lysate was then cleared by centrifugation at 13000 RCF for 10 min at 4 °C then the supernatant was removed. This solution was analysed for protein content using the BCA assay as described in Section 2.3.5. The remaining solution was aliquoted and stored at -80 °C until use in western blot experiments.

In the case of the differentiated U937 cells, 1×10^6 cells were re-suspended in cell culture medium (10 ml) containing PMA (150 nM) and transferred to a 75 cm² flask. After 24 h the cells were washed with ice cold PBS on ice and 1 ml of the lysis buffer added. They were incubated on ice with rocking for 5 min before transfer to a -80 °C freezer. The flasks were removed from -80 °C and defrosted on ice, cells were scraped from the flask and the contents transferred to 1.5 ml eppendorfs. These were vortexed for 30 s then centrifuged at 13000 RCF at 4 °C for 10 min. The supernatant was removed and analysed for protein content using the BCA assay as described in Section 2.3.5. This solution was aliquoted and the samples stored at -80 °C until use in western blot experiments.

2.3.9.2 SDS-PAGE Gel Electrophoresis

The gel cast was assembled with 1.5 mm spacers and tested with ddH₂O for leaks. Once the cast was assembled and tested for leaks, 12.5 % gels were prepared for separation. These were prepared with 25 % (v/v) separating buffer (0.55 M Tris base, 0.4 % (w/v) SDS pH adjusted to 8.8 with HCl) 31.25 % (v/v) acrylamide / bis-acrylamide solution, 42.6 % (v/v) ddH₂O, 0.1 % (v/v) TEMED with 1 % (w/v in ddH₂O). Ammonium persulphate (APS) was added to start the crosslinking reaction (1 % v/v). This solution was immediately transferred into the cast with a plastic Pasteur pipette and overlaid with isopropanol, to stop air interfering with the crosslinking reaction. Once set (approximately 15 min, confirmed by remaining solution having set in the universal) the isopropanol was removed using blotting paper.

The stacking gel (5 %) was composed of: 25 % (v/v) stacking buffer (1.64 M Tris base, 0.4 % (w/v) SDS pH adjusted to 6.8 with HCl), 12.5 % (v/v) acrylamide / bis-acrylamide, 61.5 % (v/v) ddH₂O, 0.1 % (v/v) TEMED and 1 % APS (w/v in ddH₂O). After addition of APS this was immediately overlaid on the separating gel and the gel comb inserted making sure no bubbles were in the wells. This was allowed to set for 15 min (confirmed by the remaining gel setting in the universal tube) then the comb was removed.

The gel assembly was taken out of the casting apparatus and put into the gel tank, running buffer (0.025 M Tris base, 0.192 M Glycine, 0.1 % SDS) was then poured into the tank so that the buffer level was above the top of the gel (~800 ml). Cell lysate aliquots were removed from -80 °C, appropriate volumes transferred to eppendorfs and non-reducing solubilising buffer (0.12 M Tris base, 4 % (w/v) SDS, 20 % (w/v)

glycerol, 0.004 % (w/v) bromophenol blue pH adjusted to 6.8 with HCl) added at 1/6th of the volume of lysate and then boiled (100 °C) for 5 min. Lysates were loaded into wells using gel loading tips and ran against protein standards. Gels were electrophoresed at 150 V for 45 min.

Transfer of protein from SDS-PAGE gel onto PVDF membrane was made. PVDF membrane was cut to the correct size for the gel and prepared for transfer by soaking in methanol. Sponges and blotting paper were soaked in cold transfer buffer (0.025 M Tris base, 0.192 M Glycine, 20 % methanol (v/v)). Gels were removed from the electrophoresis tank and carefully removed from the glass plates. They were sandwiched in a transfer cassette as shown in Fig. 2.7. The transfer cassette was loaded into the gel tank and an ice pack added before cold transfer buffer addition; electrophoresis was performed at 150 mA overnight in the cold room (4 °C).

Western blotting (Fig. 2.8) of the PVDF membrane was performed after protein transfer. The MW marker lane was cut off the PVDF membrane and stored at 4 °C. First, the membrane was blocked with blocking buffer (40 ml; 0.01 M Tris base, 0.2 M NaCl pH adjusted to 7.4 with HCl 0.05 % Tween 20, 5 % (w/v) milk protein) for 30 min on a rocker followed by three washes of 5 min with blotting buffer (10 ml; 0.01 M Tris base, 0.2 M NaCl pH adjusted to 7.4 with HCl 0.05 % Tween 20).

Following this, the primary antibody was incubated on the membrane (polyclonal = 1 h, monoclonal = 2 h). Three washes of 10 min were then made with blotting buffer before addition of the secondary antibody for 45 min. Finally 3 washes of 10 min were made before chemiluminescent detection was made. The secondary antibodies used were horse radish peroxidase conjugates. Detection was made in a dark room using enzyme linked chemiluminescence (ECL) detection reagents (A and B) from Amersham (UK) (Fig. 2.8). This works on the basis of the horse radish peroxidase antibody conjugate catalysis of luminol in alkaline conditions. The luminol is then in an excited state which decays in a chemiluminescent reaction. ECL reagent A was mixed with ECL reagent B in a 1:1 ratio to give sufficient solution to cover the membrane at 0.125 ml/cm². The ECL reagent was then incubated on the membrane for 1 min before transfer of the membrane to be sandwiched between clingfilm then placement in an x-ray cassette containing film. Films were scanned using a Canoscan8000F scanner.

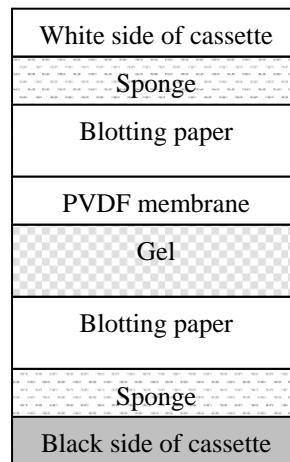


Figure 2.7 - Assembly of SDS-PAGE transfer cassette.

After electrophoresis of cell lysates they were transferred electrophoretically in a transfer cassette. The gel was placed in the cassette as shown and protein was transferred and retained by the PVDF membrane.

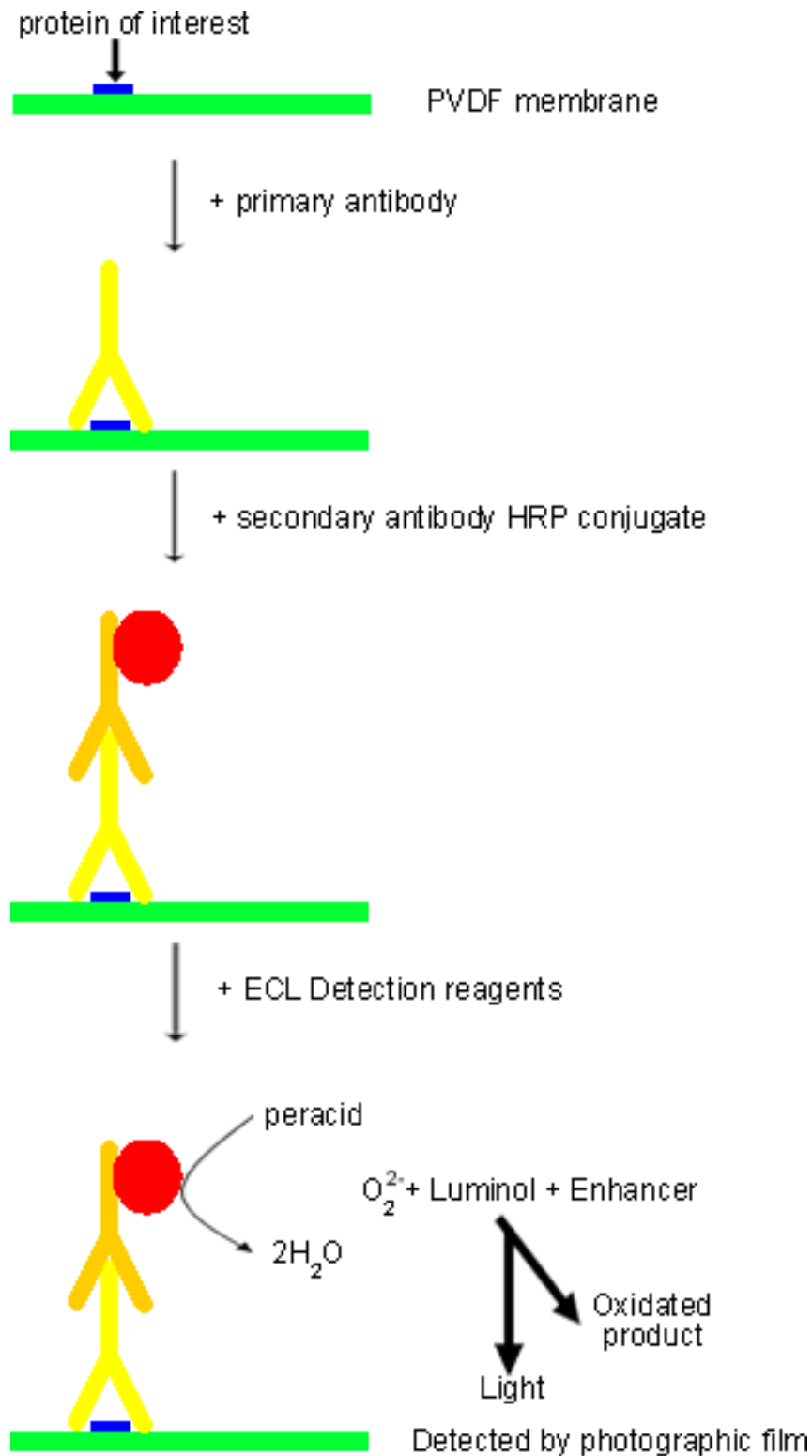


Figure 2.8 - Western blotting and detection

Figure showing the western blot process after transfer of protein to PVDF membrane has been performed.