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<ul style="list-style-type: none"> • Other People: • PPE: 	<ul style="list-style-type: none"> • Refer to end of document for buffer recipes • N/A • Laboratory Coat • Safety Glasses • Gloves 								
<p>5. Instructions:</p>									
<p>Environment</p>	<ul style="list-style-type: none"> • Ensure bench area is dry and clean before starting. 								
<p>Preparation</p>	<p>Sample preparation</p> <ul style="list-style-type: none"> • Solubilise protein sample in appropriate amount of IEF rehydration buffer. (Refer table 5.1) <p>Table 5.1 : Volume of solution required per IPG strip in the IEF phrase</p> <table border="1" data-bbox="441 823 1253 961"> <thead> <tr> <th>Length of IPG strip (cm)</th> <th>Total volume per strip (□L)</th> </tr> </thead> <tbody> <tr> <td>7</td> <td>125</td> </tr> <tr> <td>13</td> <td>250</td> </tr> <tr> <td>18</td> <td>350</td> </tr> </tbody> </table> <p>Buffer preparation</p> <p>Prepare the following buffers and solutions</p> <ul style="list-style-type: none"> • Agarose sealing solution • IEF rehydration buffer • Equilibration buffers • Resolving gel solutions • 10X SDS electrophoresis buffer • 2X Laemmli sample buffer • Refer to end of document for buffer recipes 	Length of IPG strip (cm)	Total volume per strip (□L)	7	125	13	250	18	350
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<p>Step by Step Instructions</p>	<p><u>A) 1st Dimension IEF Run</u></p> <p>IPGphor™ Isoelectric focussing system setup</p> <ol style="list-style-type: none"> 1. Ensure power is connected to the IPGphor™ Isoelectric focussing system 2. Turn on the power for the IPGphor™ Isoelectric focussing system and allow it to perform self-check. 3. Upon the programme menu, key in the desired voltage programme for isoelectrical focusing. (refer to table 5.2 below for recommended voltage programming for various types of IPG strips) 								

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Table 5.2 : Recommended voltage programming for IPGphor™ Isoelectric focussing system					
A) For IPG strips with pH gradients 4–7 linear, 3–10 linear, 3-10 non-linear					
Length of IPG strip (cm)	Step	Voltage	Method	Duration Hr:Min	Volt hours (Vhr)
7	1	Rehydration ^[1] 500	- Step	12:00 0:30	- 250
	2	1000	Step	0:30	500
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	1:00	8000
13	1	Rehydration ^[1] 500	- Step	12:00 1:00	- 500
	2	1000	Step	1:00	100
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	2:00	16000
18	1	Rehydration ^[1] 500	- Step	12:00 1:00	- 500
	2	1000	Step	1:00	100
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	4:00	32000
B) For IPG strips with pH gradients 4–7 linear, 3–10 linear, 3-10 non-linear					
Length of IPG strip (cm)	Step	Voltage	Method	Duration Hr:Min	Volt hours (Vhr)
7	1	Rehydration ^[1] 500	- Step	12:00 0:30	- 250
	2	1000	Step	0:30	500
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	3:45	30000
13	1	Rehydration ^[1] 500	- Step	12:00 1:00	- 500
	2	1000	Step	1:00	100
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	9:30	75000
18	1	Rehydration ^[1] 500	- Step	12:00 1:00	- 500
	2	1000	Step	1:00	100
	3	8000 ^[2]	Gradient	0:30	2000
	4	8000 ^[2]	Step	15:00	120000

[1] The total rehydration time can be adjusted somewhat for convenience, but must be greater than 10 hours.

[2] This voltage may not be reached within the suggested step duration.

Note: Recommended current for all IEF runs is 50 µA per IPG strip.
Recommended temperature for both rehydration and IEF is 20 °C.

Preparation of IPGphor™ ceramic strip holder

1. Select the strip holder(s) corresponding to the IPG strip length chosen for the experiment. Handle the ceramic holders with care, as they are brittle.
2. Wash each holder with IPGphor strip holder cleaning solution to remove residual protein. Rinse thoroughly with double distilled water. Use a cotton swab or a lint-free tissue to dry the holder or allow it to air-dry. Handle clean

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holders with gloves to avoid contamination.

Note: The holder must be completely dry before use.

Application of rehydration solution

1. Pipette the appropriate volume of rehydration solution into each holder as indicated in Table 5.1. Deliver the solution slowly at a central point in the strip holder channel away from the sample application wells. Remove any larger bubbles. (Figure 5.3)

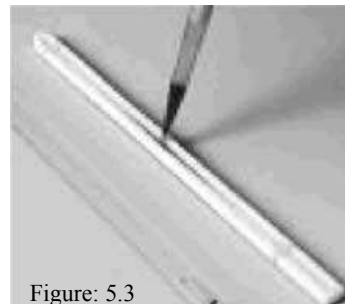


Figure: 5.3

Note: To ensure complete sample uptake, do not apply excess rehydration solution.

Placement of IPG strip

1. Remove the protective cover from the IPG strip. Position the IPG strip with the gel side down and the pointed (anodic) end of the strip directed toward the pointed end of the strip holder. Pointed end first, lower the IPG strip onto the solution. To help coat the entire strip, gently lift and lower the strip and slide it back and forth along the surface of the solution, tilting the strip holder slightly as needed to ensure complete and even wetting.
2. Finally, lower the cathodic (square) end of the IPG strip into the channel, making sure that the gel contacts the strip holder electrodes at each end. (The gel can be visually identified once the rehydration solution begins to dye the gel.) Be careful not to trap bubbles under the IPG strip.



Figure: 5.4



Figure: 5.5

Application of IPG Cover Fluid

1. Apply IPG Cover Fluid to minimize evaporation and urea crystallization. Pipette the fluid dropwise into one end of the strip holder until one-half of the IPG strip is covered. Then pipette the fluid dropwise into the other end of the

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strip holder, adding fluid until the entire IPG strip is covered.

Placement the cover on the strip holder

1. Pressure blocks on the underside of the cover ensure that the IPG strip maintains good contact with the electrodes as the gel swells.

Rehydration of IPG strip

1. Rehydration can proceed on the bench top or on the IPGphor unit platform. Ensure that the holder is on a level surface. A minimum of 10 hours is required for rehydration; overnight is recommended. The rehydration period can be programmed as the first step of an IPGphor protocol. This is especially convenient if temperature control during rehydration is a concern.

Loading IPG strips onto the IPGphor platform

1. Ensure that the strip holders are properly positioned on the IPGphor platform. (Use the guidemarks along the sides of the platform to position each strip holder and check that the pointed end of the strip holder is over the anode [pointing to the back of the unit] and the blunt end is over the cathode.)
2. Check that both external electrode contacts on the underside of each strip holder make metal-to-metal contact with the platform.
3. Close the safety lid. At least two of the three pressure pads under the safety lid must press gently against the cover of each strip holder to ensure contact between the electrodes and the electrode areas.

Note: A maximum of 12 IPG strips can be loaded onto a single IPGphor platform

Initiation of IEF run

1. Initiate IEF by pressing “start” button on IPGphor™.
2. As isoelectric focusing proceeds, the bromophenol blue tracking dye migrates toward the anode. Note that the dye front leaves the IPG strip well before focusing is complete, so clearing of the dye is no indication that the sample is focused. If the dye does not migrate, no current is flowing. If this occurs, check the contact between the external face of the strip holder electrodes and the electrode areas on the instrument and between the rehydrated gel and the internal face of the electrodes.

Note: Record the Total Volt hours (TVhr) that the protein sample have been subjected to during IEF run for future reference.

B) Post IEF run

1. After separation by IEF in the 1st dimension, proteins are subjected to reduction and alkylation in an Immobiline™ Drystrip reswelling tray before a

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<p>Step by Step Instructions</p>	<p>second dimension separation in the SDS-PAGE phase.</p> <ol style="list-style-type: none"> Place IPG strips in 2 ml of IPG equilibration reduction buffer (Table 9.2) for 10 minutes to completely unfold the proteins and cleave disulphide bonds. Next, place the IPG strips in 2 ml of IPG equilibration alkylation buffer (Table 9.2) for 10 minutes to alkylate thiol groups on protein which prevents their reoxidation and also to minimise unwanted reactions of cysteine residues. <p>Note: Alternatively, IPG strips can be stored at -40°C to -80°C in screw-cap tubes . The 7 cm strips fit in disposable, 15 ml conical tubes; 11 cm, 13 cm, and 18 cm strips fit in 25 mm x 200 mm screw-cap culture tubes.</p> <p><u>C) Second dimension: SDS-PAGE</u></p> <ol style="list-style-type: none"> Use two pair of clean tweezers to pick up the IPG strips and carefully place it on the top of the SDS-PAGE gel facing the front of the gel cassette, by carefully sliding it down the gel cassette via the plastic laminated side of the IPG strip. <p>Note: Ensure that there is minimal contact between the gel side of IPG strip with the walls of the gel cassette as in order to prevent any picking up any contamination or debris that may affect the result of the SDS-PAGE.</p> <ol style="list-style-type: none"> Apply an appropriate amount of the protein ladder standard to a small piece of filter paper and seal it with 10 μl aliquots of 0.5% IEF sealing agarose (Refer to table 9.3) until it is completely covered. A positive control for the SDS-PAGE run can be set-up by applying an appropriate amount of the standard protein (eg. BSA) onto another small piece of filter paper and sealed with 10 μl aliquots of 0.5% IEF sealing agarose (Refer to table 9.3) until completely covered. Use a pair of clean tweezers and pick up the filter papers with protein ladder standard and standard protein (positive control) and place it on the top of the SDS-PAGE gel, next to the IPG strip. Seal the top of the gel cassettes with 0.5 % IEF agarose. Ensure that there are no formation of bubbles between the IPG strips/ filter paper and the polyacrylamide gel. If bubbles are present, tap the IPG strip/filter papers using clean forceps. <p>Note: It was important that the 0.5% IEF agarose is not too hot when used, as it would cause protein denaturation in the sample.</p> <ol style="list-style-type: none"> Place the gels into the electrophoresis tanks containing the lower tank and 2x running buffer in the upper tank. Place the lid of the electrophoresis tank on and connect the anode and cathode to the electrophoresis power supply.
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<p>Step by Step Instructions</p>	<ol style="list-style-type: none"> 8. Enter desired voltage programme and initiate electrophoresis. 9. Allow electrophoresis run to continue until the tracking dye reached the bottom of the gel . <p>Note: Ensure the electrophoresis tank is not filled beyond the maximum fill line if not short circuit to the electrophoresis system might occur and pose as an electrical hazard to user too.</p> <p>Note: Larger gel formats should be cooled with cool water of between 15°C to 20°C via the use of the cooling ducts in-built with the electrophoresis tank and with supply of cool water from MultiTemp™ III thermostatic circulator. This is due the fact that higher voltages and current used to larger gel formats may produce too much heat, which may causes the polyacrylamide gel to lose its integrity and results in poor separation.</p> <p>Optional: The dissipation of heat within the electrophoresis system can be enhanced by stirring the electrophoresis buffer in the lower tank. This can be achieved by placing the electrophoretic tank can be placed on magnetic stirrer machine and with use of magnetic stirrer within the electrophoresis tank.</p> <p><u>C) Post Electrophoresis Run</u></p> <ol style="list-style-type: none"> 1. Stop the electrophoresis run by pressing on the “End Run” button on the electrophoresis power supply. 2. Record the total amount of time required for the SDS-PAGE run for future reference. 3. Turn off the power off the electrophoresis power supply via on/off switch at the rear. 4. Disconnect anode and cathode cables of the electrophoresis tank from the electrophoresis power supply. 5. remove the gel cassettes from the electrophoresis tank 6. Ply opens the gel cassettes with a clean scraper. 7. Place gel in a tray containing of 50% Methanol /10% Acetic Acid solution for fixation of proteins within the gel prior to visualisation with any staining techniques.
<p>Clean up and Waste Disposal</p>	<ul style="list-style-type: none"> • Dispose off unwanted polyacrylamide gels and biological samples into biological waste bin. • Dispose off unwanted chemicals/buffers into halogen containing waste or non-halogen containing chemical waste bottles.

6. Key Hazards: Refer to Risk Assessment

Hazard	Harm	Controls
Chemical and biological waste spillage	Chemicals generally cause irritation to respiratory system, eyes and skin upon inhalation and contact. Biological samples might cause onset of disease or undesired immune response upon ingestion, inhalation or contact with open wounds.	<ul style="list-style-type: none"> • Don standard PPE which include laboratory coat, latex gloves and safety glasses when handling chemicals • Read MSDS prior to usage of chemicals. • Spill kits. • Hazardous substance training.
Electrical hazard from equipments	Electrical hazard	<ul style="list-style-type: none"> • Equipment manufactured to Australian standard. • Conduct visual check on equipment prior to use. • Equipment is tested and tagged periodically.

7. Emergency Procedures and Shut Down

- Inform supervisor and first aid officer/fire warden.
- For major emergencies dial x56666.
- Nearest first aid kit in room M310.
- Nearest safety shower in room M307.
- Nearest Hose reel and CO₂ fire extinguisher just outside M305.
- Nearest fire extinguishers: CO₂ fire extinguisher in rooms M305 and M307; Dry chemical extinguisher beside store room.
- Evacuate as per evacuation plan and gather in front of Chancellor Building.

8. Legislative References

[OHS Regulation 2001](#)

- Part 5.2-Design, manufacture and registration of plant, Division 1-Design of plant, Section 95 & 96; **and** Division 2-Manufacture of plant, Section 105;
- Part 5.3-Supply of plant, Division 2-sale or transfer of plant, Section 122;
- Part 5.4-Working with plant

[AS 2243.6:1990](#) – Safety in Laboratories Part 6 – Mechanical aspects

[WorkCover Code of Practice 4548](#)– Control of work-related exposure to Hepatitis and HIV (blood-borne) viruses (as per Section 43 of the OHS Act 2000 - National Code of Practice for the Control of Work-related Exposure to HIV

and Hepatitis Viruses [NOHSC: 2010 (2003)])

[WorkCover Code of Practice 963](#) – Risk Assessment

UNSW OHSMS [Training](#) requirements

9. Definitions

Troubleshooting 1st dimension IEF: IPGphor

Problem	Possible Cause	Remedy
Current too low or zero	Electrical continuity is impeded.	<p>Check the external electrode contacts: The electrodes at the bottom of the strip holder (one at each end) must make metal-to-metal contact with the appropriate electrode contact area.</p> <p>Check the internal electrode contacts: The gel (which becomes visible because of the dye in the rehydration solution) must contact both electrodes in the strip holder.</p> <p>Check that the IPG strip is fully rehydrated along its entire length. Electrical contact at the electrodes is reduced by incomplete rehydration.</p>
Voltage too low or does not reach the maximum set value	The IPGphor protocol settings are incorrect for the experiment.	<p>Check that the current limit is properly set.</p> <p>Check that the actual number of strips on the IPGphor platform equals the number of strips entered in the protocol. if sample solubility is a problem.</p>
	Conductivity/ionic strength is too high.	<p>Prepare the sample to yield a salt concentration less than 10 mM.</p> <p>The recommended IPG Buffer concentration is 0.5%. A maximum of 2% is advisable only</p>
Sparking or burning in the strips	Current limit setting is too high.	Do not exceed the maximum recommended setting of 50 μ A per IPG strip.
	The IPG strip is not fully rehydrated.	<p>Ensure that the IPG strips are rehydrated with a sufficient volume of rehydration solution.</p> <p>Remove any large bubbles trapped under the IPG strip after placing on rehydration solution.</p> <p>Check that the entire IPG strip surface is wetted.</p>
	The IPG strip dried during IEF.	Always apply IPG Cover Fluid to prevent dehydration of a rehydrated IPG strip.

Retrieved September 16, 2005, from Amersham Biosciences Web site:
<http://www1.amershambiosciences.com>

Troubleshooting 2nd dimension SDS-PAGE

Symptom	Possible cause	Remedy
No current at start of run	Insufficient volume of buffer in upper or lower reservoir.	Ensure that both reservoirs contain enough SDS electrophoresis buffer to contact both upper and lower electrode wires. Check for leaks.
The second dimension separation proceeds too slowly	SDS electrophoresis buffer is prepared incorrectly, or resolving gel buffer is prepared incorrectly.	Make fresh solutions.
	Acrylamide solution is too old.	Prepare fresh monomer stock solution.
Dye front curves up (smiles) at the edges	Gel is not properly cooled.	During electrophoresis, actively cool gel using a thermostatic circulator. Use the maximum possible volume of buffer in the lower reservoir.
	Current is too high.	Limit current to values suggested in Table 22.
Dye front curves down (frowns)	Gel is poorly polymerized near the spacers.	Degas the gel solution or increase the amount of ammonium persulphate and TEMED by 50%.
	Improper instrument assembly (SE 600).	Ensure that the gasket is not pinched.
	Leakage of upper reservoir.	Ensure that an adequate level of buffer is in the upper reservoir.
Dye front is irregular	Poor, uneven polymerization of gel.	Degas the gel solution or increase the amount of ammonium persulphate and TEMED by 50%.
	The top surface of the second-dimension gel is not flat.	Immediately after pouring the gel, overlay the surface with water-saturated butanol.

Retrieved September 16, 2005, from Amersham Biosciences Web site:
<http://www1.amershambiosciences.com>

Buffer Recipe

All solutions were prepared using Milli-Q grade water from a Milli-Q system, and stored in clean glassware (rinsed with Milli-Q water) with appropriate labelling. The following summaries the methods for preparing the solutions and reagents required in most of the experiments.

Table 9.1: IEF rehydration buffer

Constituent	Concentration	Quantity
Urea	8M	4.8 g
CHAPS	2% (w/v)	0.2 g
DTT	10 mM	0.154 g
Ampholyte	2% (v/v)	200 μ l
Bromophenol Blue	0.1% (w/v)	50 μ l

Table 9.2: Equilibration buffers

	Constituent	Concentration	Quantity
*Stock Solution (10ml)	0.5M Tris-HCl, pH 6.8	50 mM	1 ml
	Urea	6 M	3.6 g
	87% Glycerol	30 % (v/v)	3 ml
	SDS	2 % (w/v)	0.2 g
Reduction solution (5ml)	*Stock solution	-	5ml
	DTT	2 % (w/v)	0.1 g
Alkylation solution (5ml)	*Stock solution	-	5 ml
	Iodoacetamide	2.5 % (w/v)	0.125 g
	1% bromophenol blue	Trace	25 μ l

*Stock solution used to make up reduction and alkylation solution.

Table 9.3: Polyacrylamide gel solutions

	4% T	10% T	12.5 % T
	Amount	Amount	Amount
Acrylamide/bisacrylamide 40% stock	2.5 ml	25 ml	31.3 ml
Tris-HCl (1.5M, pH 8.8)	6.3 ml	-	-
Tris-HCl (0.5M, pH 6.8)	-	25 ml	25 ml
SDS (10% W/V)	250 μ l	1 ml	1 ml
APS (10% W/V)**	25 μ l	250 μ l	250 μ l
TEMED**	125 μ l	50 μ l	50 μ l
Make up to a total volume with Milli-Q water	25 ml	100 ml	100 ml

**APS and TEMED were added prior to the pouring of the gels.

Table 9.4: 0.5% IEF Agarose sealing solution

	Final Concentration	Amount
Agarose	0.5% (W/V)	0.5 g
1 X Tris/Glycine/SDS electrophoresis buffer, pH 8.3	1 X	100 ml
Bromophenol Blue	Trace	Few grains

*The solution was heated and swirled to dissolve the agarose.