7. APPENDICES

Appendix 1 - Lysis Buffer I

- 30 mM Tris

- 7 M Urea

- 2 M Thiourea

- 4% w/v CHAPS

- To pH 8.5

Appendix 2 - Perfect Focus Protocol

1. Transfer 1-100µl of protein sample into a 1.5 ml microfuge tube, add 300 µl UPPA-I and vortex for 30 seconds. Incubate samples in an ice bucket (4-5^{o}C) for 15 minutes. Add 300 µl UPPA-II
2. Vortex for 30 seconds and centrifuge at 15,000xg for 5 minutes to form a tight pellet
3. Carefully without disturbing the pellet, remove entire supernatant
4. Centrifuge tube again for 30 seconds and remove remaining supernatant from the sample. Add 25 µl of pure water on top of the pellet and vortex for 30 seconds.
5. Add 1ml pre-chilled (-20^{o}C) OrgoSol buffer and 5 µl of SEED.
6. Vortex for 30 seconds to suspend the pellet, the OrgoSol buffer needs to be 10 fold in excess of the pure water added to the pellet.
7. Centrifuge the microfuge tube at 15,000xg for 5 minutes to form a tight pellet. Remove the supernatant.
8. Air dry the white pellet in the microfuge tube. Add a suitable volume of IEF loading buffer (C1 lysis buffer) and vortex tube for 30 seconds. Centrifuge the microfuge tube and collect a clear protein solution and load onto IEF gel strip

Appendix 3 - Lysis Buffer II

- 8 M Urea
- 2% CHAPS
- 33 mM DTT
- 0.5% ampholytes pH range 3-10
- ddH_2O
- Bromophenol Blue

Appendix 4 - Programming IEF Run 1st Dimension

- Turn power on
- Present method

  Press RAPID ramping
- Rehydration

  YES (select using button by arrow)
- Gel length

  7, 11 or 17 cm (select 17)
- Focus temperature

  $20^{o}c$

Press next

- Select ACTIVE rehydration @ 50 volts
- Preset temperature: $20^{o}c$

Press next

- Enter time as 10 hours
- Say NO to pause after rehydration

Press next

- S1: 250 volts 15 minutes – preset
- S2: select HRS:MIN
- S2: 10 000 volts in preset
- Enter 2 hours

Press next

- Select VOLT HOURS
- S3: 10 000 (preset)
- Enter 50, 000 VOLT HOURS
- S4: volt HOLD – YES

Press next

- Display says RAPID METHOD limited per 50 μA
- Enter number of gels (2)

Press start

Appendix 5 – Equilibration Buffer I

- 6M urea – 31.5 ml of 9.5M Urea
- 2% SDS – 5 ml of 20% SDS
- 0.375 mM Tris pH 8.8 – 3.125 ml of 1.5M Tris pH 8.8
- 20% Glycerol – 10ml of 100% Glycerol
- Add 375 µl dd H_2O to make up to final volume 50 ml
- Immediately prior to use add 1 g DDT and pinch of bromophemol blue

Appendix 6 – Equilibrium Buffer II

- 6 M Urea – 31.5 ml of 9.5M Urea
- 2% SDS – 5 ml of 20% SDS
- 0.375 mM Tris pH 8.8 – 3.125 ml of 1.5M Tris pH 8.8
- 20% Glycerol – 10ml of 100% Glycerol
- Add 375 µl ddH_2O to make up to final volume 50 ml
- Immediately prior to use add 1.25 g Idoacetamide and pinch of bromophemol blue

Appendix 7 – Resolving Gel

- For 12% large gel, 17cm

- 29.3 ml acrylamide (30%)
- 17.5 ml running gel buffer
- 23.2 ml dd H_2O
- 262 µl ammonium persulphate
- 70 µl temed

- Total Volume: 70.3 ml

Appendix 8 – Stacking Gel

- 1500 µl stock acrylamide (30%)
- 2500 µl stacking gel buffer
- 6000 µl dd H_2O
- 50 µl ammonium persulphate (10%)
- 15 µl temed

- Total Volume: 10.05 ml

Appendix 9 – Low Melting Point Agarose Gel

- 0.5% low melting point agarose in 125mM Tris pH 6.8 (stack used)
- Dissolve 1.51375 tris into 80ml ddH_2O on a hot plate stirrer
- Adjust pH to 6.8 using a pH meter and hydrochloric acid
- Add a further 30ml of ddH_2O added to solution to bring up to volume of 100 ml
- Add 0.5g low melting point agarose, heat in microwave until clear
- Store on bench

Appendix 10 Coomassie Blue

- 2.5g Coomassie blue powder
- 500 ml methanol
- 100 ml acetic acid

- Dissolve Coomassie blue in methanol, then add acetic acid and bring total volume to 1000 ml with ddH_2O

Appendix 11 De-stain Solution

- 400 ml methanol (40%)
- 100 ml acetic acid (10%)
- 500 ml ddH_2O (50%)

Appendix 12 Silver Staining Solutions

- 50% methanol and 5% acetic acid

  500 ml methanol

  450 ml dd H_2O

  50 ml acetic acid

- 50% methanol

  500 ml methanol

  500 ml ddH_2O

- 0.02% sodium thiosulphate

  0.2 g sodium thiosulphate dissolved up to a volume of 1000ml with ddH_2O

- 0.1% silver nitrate

  1 g silver nitrate dissolved up to a volume of 1000ml with H_2O

- 0.04 formalin in 2% sodium carbonate (add formalin just before use)

  0.4ml formalin

  20 g sodium carbonate dissolved up to a volume of 1000ml with ddH_2O

- 1% acetic acid

  10 ml acetic acid

  990 ml double distilled water

Appendix 13 - Tryptic Digestion with Silver Stained Gels (Protocol 1)

1. Cut out the spot of interest
2. Give the gel pieces a quick 2-minute wash in fresh water
3. Prepare a 30 mM Potassium ferricyanide solution in water
4. Prepare a 100 mM sodium thiosulfate solution in water
5. Mix 1:1 of previously prepared solutions
6. Add 40 µl to the gel and leave to de-stain at room temperature, typically for two minutes. Remove solution
7. Add 50 µl of 200 mM ammonium acetate and leave for 10 minute at room temperature and remove.
8. Add 50 µl of acetonitrile, leave for 10 minutes at room temperature and remove.
9. Repeate twice.
10. Dry gel pieces in a vacuum centrifuge.
11. Once the gel pieces have been dried, add 5-10 µl of a 10 ng/µl solution of trypsin to the gel pieces. The trypsin should be made up in water.
12. Incubate overnight at 37 ^{o}C.
13. Add 60 µl of acetonitrile and leave at room temperature for 20 minutes.
14. Collect the supernatant and dry down in a speed vacuum centrifuge.

Appendix 14 - Tryptic Digestion with Silver Stained Gels (Protocol 2)

1. 30 mM potassium ferricyaninde : 100mM sodium thiosulphate (1:1) Add 50 µl to each plug and incubate at 37 ^{o}C for 15 min. Discard supernatant. Repeat until fully destained.
2. Dehydrate plugs in 100% ACN until opaque (10µl/plug). Incubate at 37^{o}C for approx 30 mins.
3. Rehydrate in 50 mM ambic and trypsin (9 µl ambic and 1 µl trypsin stock, 100 ng/µl)
4. Incubate at 37 ^{o}C overnight (check after 30 minutes and if solution has been adsorbed add further 5-10 µl ambic
5. Stop with 2 µl formic acid (1 in 10 dilution in water)
6. Add 30 µl 60% ACN/1% TFA and place in a sonicator bath for 5 minutes. Spin briefly and collect supernatant. Add a further 30 µl 60% ACN/1% TFA to the gel pieces and sonicate again. Pool the supernatents and dry in a Speed Vac

Appendix 15 – PMF Molecular Mass Peak List

Manually generated molecular mass peak (Monoisotopic peptide masses) list for peptides, for MASCOT PMF analysis.

Name of Spot	Molecular Weight	Charge
594ms01	359.139	1
	371.166	1
	376.169	1
	381.123	1
	447.197	1
	473.264	1
	734.312	1
	739.262	1
	755.242	1
	869.234	1
594ms02	359.142	1
	381.129	1
	415.218	1
	453.172	1
	497.241	1
	511.091	1
	541.27	1
	755.247	1
	811.32	1
	869	1
201ms01	371.157	1
	381.131	1
	397.103	1
	437.2	1
	503.305	1
	515.327	1
	547.333	1
	591.359	1
	672.4	1
	686.405	1
	705.426	1
	743.437	1
	842.502	1
1880ms01	321.123	1
	337.098	1
	437.184	1
	453.159	1
	515.363	1

	637.364	1
	653.333	1
	833.198	1
1880ms02	321.123	1
	415.172	1
	437.184	1
	453.16	1
	459.17	1
	515.365	1
	637.365	1
	653.335	1
	689.225	1
	833.231	1
1880ms03	321.124	1
	415.15	1
	437.181	1
	453.16	1
	459.167	1
	470.133	1
	515.365	1
	637.366	1
	653.337	1
	833.224	1
1880ms04	437.177	1
	453.154	1
	459.162	1
	470.127	1
	515.364	1
	531.308	1
	637.362	1
	653.332	1
	671.176	1
	833.218	1
1906ms01	337.071	1
	359.113	1
	376.137	1
	381.091	1
	397.063	1
	415.167	1
	437.145	1
	453.117	1
	787.074	1
1906ms02	321.099	1
	337.072	1
	359.114	1
	376.137	1
	381.092	1
	397.065	1

	415.166	1
	437.146	1
	453.119	1
	787.07	1
1906ms03	337.073	1
	397.065	1
	437.146	1
	453.118	1
	787.066	1
1906ms04	359.115	1
	337.074	1
	381.095	1
	397.066	1
	315.169	1
	437.149	1
	453.121	1
	787.064	1