APPENDIX B

Reagents and solutions for 2-D DIGE

1. Sample preparation and labeling

1.1 Standard cell washing buffer

The standard cell washing buffer consisted of the following ingredients:

Reagent	Quantity	Final concentration
Tris (100 mM, pH 8.0)	5.0 ml	10 mM
Magnesium acetate (1 M)	0.25 ml	5 mM

The volume was made up to 50 ml with deionized distilled water. The buffer was filtered through 0.45 μm membrane filter and stored at 4ºC. The buffer was stable for 1 month.

1.2 Standard cell lysis buffer

The standard cell lysis buffer consisted of the following ingredients:

Reagent	Quantity	Final concentration
Tris (1M)	3.0 ml	30 mM
Thiourea (MW 76.12)	15.22 g	2 M
Urea (MW 60.06)	42.0 g	7 M
CHAPS (MW 614.89)	4 g	4% (w/v)
HCl (0.1 M)		

All reagents were mixed to dissolved before the pH was adjusted to pH 8.5 with the dilute HCl the final volume then was made up to 100 ml with distilled water. The buffer was filtered through 0.45 μm membrane filter and kept in small aliquots at –20ºC, for up to 3 months.

1.3 10 mM L-lysine

The solution was prepared by dissolving 0.018 g of L-lysine in 5 ml of deionized distilled water. The final volume was made up to 10 ml. The solution was filtered through 0.45 μm membrane filter and kept in small aliquots at –20 ºC, for up to 6 months.

1.4 1 M magnesium acetate

The solution was prepared by dissolving 21.45 g of magnesium acetate in 50 ml of deionized distilled water. The final volume was made up to 100 ml. The solution was filtered through 0.45 μm membrane filter and stored at 4 ºC, for up to 4 months.

2. First dimension IEF

2.1 Rehydration buffer

A stock solution containing the core components was prepared and aliquoted for storage at –20°C. Immediately prior to use, DTT and IPG buffer was added to this stock solution to give either 2× sample buffer or rehydration buffer.

2.1.1 Stock 2× Sample/rehydration buffer

The stock 2× sample/rehydration buffer consisted of the following ingredients:

Reagent	**Quantity**	**Final concentration**
Urea (MW 60.06)	10.5 g	7 M
Thiourea (MW 76.12)	3.8 g	2 M
CHAPS (MW 614.89)	1 g	4% (w/v)

The volume was made up to 25 ml with deionized distilled water. The buffer was filtered through 0.45μm membrane filter and kept in small aliquots (*e.g.* 2.5 ml) at –20 ºC, for up to 6 months.

2.1.2 Rehydration buffer

The rehydration buffer consisted of the following ingredients:

Reagent	**Quantity**	**Final concentration**
stock 2 × sample/rehydration buffer	2.5 ml	–
IPG buffer™, broad range pH 3-10	25 µl	1% (v/v)
DTT (MW 154.2)	5 mg	0.2% (w/v) (2 mg/ml, 13 mM)

The solution was freshly prepared before use.

2.2 Stock SDS equilibration buffer solution

The stock SDS equilibration buffer solution consisted of the following ingredients:

Reagent	**Quantity**	**Final concentration**
Tris (1.0 M, pH 8.0)	20 ml	100 mM
Urea (MW 60.06)	72.07 g	6 M
Glycerol (87% [v/v], MW 92.09)	69 ml	30% (v/v)
SDS (MW 288.38)	4 g	2% (w/v)

The volume was made up to 200 ml with deionized distilled water. This stock solution can be stored at 25 ºC. It is stable for 6 months.

2.3 Equilibration solution 1

The solution was prepared by dissolving 0.5 g of DTT in 100 ml of stock SDS equilibration buffer solution. Solution should be used freshly.

2.4 Equilibration solution 2

The solution was prepared by dissolving 0.5 g of IAA in stock SDS equilibration buffer solution. Solution should be used freshly.

3. Gel preparation and 2–D gels electrophoresis

3.1 1.5 M Tris, pH 8.8

Tris base (545 g) was dissolved in 1500 ml of deionized distilled water then the pH was adjust to 8.8 with 6 N HCl. The final volume was brought up to 3000 ml with deionized distilled water and the pH was checked to pH 8.8. The solution was filtered through a 0.2 μm membrane and store at 4ºC. It is stable for 1 month.

3.2 Sodium dodecyl sulfate (10% SDS; w/v)

This solution was prepared by dissolving 10 g of SDS (Bio-Rad, Hercules, California, USA) in 100 ml of deionized distilled water.

3.3 Ammonium persulfate (10%; w/v)

This solution was prepared just before use by dissolving 1 g of ammonium persulfate (Bio-Rad) in 10 ml deionized distilled water.

3.4 10% (v/v) TEMED

The solution was prepared by adding 0.5 ml of TEMED in 4.5 ml deionized distilled water. Solution should be prepared fresh on day of use then discard.

3.5 Water saturated butanol

The solution was prepared by mixing 50 ml of butanol and 50 ml of deionized water. Once completely separated, the top layer was used to overlay gels. The solution is store at 25ºC and is stable for 6 months.

3.6 12.5% 2–D PAGE gel for Ettan DALT

Separating polyacrylamide gel (12.5%) was prepared by mixing the following ingredients together:

Reagent	**Quantity for 900 ml of a 12.5% gel**
Acrylamide/Bis 30% (w/v)	375 ml
Tris (1.5 M, pH 8.8)	225 ml

Reagent	Quantity for 900 ml of a 12.5% gel
10% (w/v) SDS	9.0 ml
10% (w/v) APS	9.0 ml
10% (v/v) TEMED	1.24 ml

The acrylamide/Bis solution and the Tris buffer were mixed and the final volume was adjusted to 900 ml with deionized distilled water. The reagents was gently mixed and degassed under a vacuum for at least 15 min. Prior to addition of APS and TEMED, the complete solution was filtered through a 0.2 μm filter into a clean bottle. The solution was allowed to warm to 25ºC prior to addition of APS and TEMED and the gel were poured immediately.

3.7 SDS electrophoresis running buffer for Ettan DALT

The buffer contained the following reagents:

Reagent	Quantity	Final concentration
Tris (MW 121.14)	60.5 g	25 mM
Glycine (MW 75.07)	288 g	192 mM
SDS (MW 288.38)	20 g	0.1% (w/v)

The final volume was made up to 20 L with deionized distilled water. The buffer can be stored at 25ºC. It is stable for 3 months.

3.8 0.5% (w/v) agarose overlay solution

Reagent	Quantity	Final concentration
SDS electrophoresis running buffer	100 ml	–
Low melting point agarose prep	0.5 g	0.5% (w/v)
Bromophenol blue	Few grains	Trace

The components were mixed in a 250 ml conical flask and heated on a low setting in the microwave for 1 minute. Ensure all the agarose has melted. The solution was allowed to cool slightly before use. It can be stored at 25ºC not for more than one month

4. Colloidal Coomassie Brilliant Blue staining

4.1 Fixing solution

The solution was freshly prepared by mixing 10 ml of 85% *o*-phosphoric acid and 20 ml of methanol. The final volume was made to 100 ml with deionized distilled water.

4.2 Stock staining solution A

The solution was prepared by dissolving 4 g of ammonium sulfate in 20 ml of deionized distilled water then 0.95 ml of 85% *o*-phosphoric acid was added. The final volume was made to 40 ml with deionized distilled water.

4.3 Stock staining solution B

The solution was prepared by dissolving 0.5 g of Coomassie Brillient Blue G-250 in 1 ml of deionized distilled water.

4.4 Staining solution, freshly prepared:

The solution was prepared by mixing 1 ml of stock staining solution B with 40 ml of stock staining solution A. Then 10 ml of methanol was added and mixed.

4.5 Neutralization solution

The solution was prepared by dissolving 6 g of Tris-base in 250 ml of deionized distilled water. The pH was adjusted to 6.5 with *o*-phosphoric acid and the final volume was brought up to 500 ml with deionized distilled water.

4.6 Washing solution

The solution was prepared by adding 125 ml of methanol in 375 ml of deionized distilled water.

4.7 Stabilizing solution

The solution was prepared by dissolving 100 g of ammonium sulfate in 250 ml of deionized distilled water. The final volume was brought up to 500 ml with deionized distilled water.