8. Chapter Eight: Appendices

8.1 Appendix 1. Palintest Reagents

Filter all samples through a GFC filter before performing the tests. All reagents are purchased from Fisher Scientific, UK.

Phosphate:-

1) Fill a glass vial with 10ml of water sample.

2) Add one Phosphate No. 1 LR tablet and place in sonic bath until dissolved.

3) Add one Phosphate No. 2 LR tablet and place in sonic bath until dissolved.

4) Stand for 10 minutes.

5) Take test reading: PROGRAM PHOT 28; 640nm.

Sulphate:-

1) Fill a glass vial with 10ml of water sample.

2) Add one Sulphate Turb tablet and place in sonic bath until dissolved.

N.B. Cloudy solution indicates the presence of sulphate.

3) Stand for 5 minutes.

4) Take test reading: PROGRAM PHOT 32; 520nm.

Nitrite:-

1) Fill a glass vial with 10ml of water sample.

2) Add one Nitricol tablet and place in sonic bath until dissolved.

3) Stand for 10 minutes.

4) Take test reading: PROGRAM PHOT 24; 520nm.

Nitrate:-

1) Fill a glass vial with 20ml of water sample.

2) Add one level spoonful of Nitratest powder and one Nitratest tablet.

3) Replace screw cap, shake vial well for exactly 1 minute, then allow contents to settle for 1 minute.

4) Invert tube gently 3-4 times and then allow to stand for at least 2 minutes to ensure complete settlement.

5) Decant 10ml of the clear solution into a clean vial.

6) Add one Nitricol tablet and place in sonic bath until dissolved.

7) Stand for 10 minutes.

8) Take test reading: PROGRAM PHOT 23; 570nm.

Ammonia:- 1) Fill a glass vial with 10ml of water sample.

2) Add one level spoonful of Conditioning Reagent and place in sonic bath until dissolved.

N.B. If turbidity still forms, repeat using 2 level teaspoons.

3) Add one Ammonia No. 1 tablet and one Ammonia No. 2 tablet and place in sonic bath until dissolved.

4) Stand for 10 minutes.

5) Take test reading: PROGRAM PHOT 4; 640nm.

8.2 Appendix 2. Fixatives

i) Neutrally Buffered Formalin (5%)

5% Formaldehyde Solution (Fisher Scientific, UK)

ii) Stockard's Solution

5% Formaldehyde Solution (Fisher Scientific, UK)

4% Glacial Acetic Acid (Fisher Scientific, UK)

6% Glycerol (Fisher Scientific, UK)

iii) Glutaraldehyde Solution (pH7.40)

Stock Buffer Solution:

For a stock solution comprises 0.4M sodium cacodylate and 0.4mM calcium chloride.

Into a 500ml volumetric flask:

- 42.80g sodium cacodylate (TAAB Laboratories Equipment Ltd).
- 0.294g calcium chloride (BDH Laboratory Supplies).

Bring the volume up to 500ml with distilled H_2O.

3% Glutaraldehyde Fixative:
For a fixative solution of 3% Glutaraldehyde; buffered in 0.2M sodium cacodylate and 0.2mM calcium chloride.

Into a 200ml volumetric flask:

- 24ml 25% EM grade Glutaraldehyde (Agar Scientific, UK).
- 100ml stock buffer solution.

Check the pH is 7.40 (adjust with conc. HCl if necessary) and bring volume up to 200ml with distilled H_2O.

RINSING BUFFER:
For a buffering solution of 0.2M sodium cacodylate and 0.2mM calcium chloride.

Into a 200ml volumetric flask:

- 100ml stock solution.

Bring volume up to 200ml with distilled H_2O.

8.3 Appendix 3. Ninhydrin Positive Substance Analysis (Reagents)

Unknowns:

(1). Add 20 µl TCA extract into a thick glass test-tube.

(2). Add 80 µ Distilled water to each tube

(3). Add 300 µl Ninhydrin Reagent (Sigma, UK) to each tube.

(4). Vortex Mix all the tubes.

(5). Heat at 100 C for 20 minutes

(6). Add 3 ml 50% Ethanol to each tube

(7). Vortex Mix all the tubes

(8). Read absorbance at 570 nm on Spectrophotometer.

<u>Standards</u>

Dissolve 13.12 mg of Norleucine (Sigma, UK) in 50 ml 1 % TCA. (This is the 2 mM stock)

Set up a range of standards:

2 mM: 1 ml of the stock = 200 nM NPS/tube

1.5 mM: 0.75 ml stock + 0.25 ml 1% TCA = 150 nM NPS/tube

1.0 mM: 0.50 ml stock + 0.50 ml 1 % TCA = 100 nM NPS/tube

0.5 Mm: 0.25 ml stock + 0.75 ml 1 % TCA = 50 nM NPS/tube

0 mM: 1 ml 1 % TCA

Pippette 100 µl of each standard into test-tubes (in duplicate) and treat as above.

The 2 mM standard is equivalent to 200 nM per 100 µl equivalent. Multiply the results from the standard curve by 50 to get nM per egg (cos 20 µl is from the 1ml of 5 % TCA).

8.4 Appendix 4. Protein Analysis (Reagents)

Bovine Serum Albumin (BSA) standards were then prepared from a stock solution of 1g BSA (Sigma, UK) dissolved in 10ml of distilled water.

Standards were as follows:

10: 1ml of stock = 5 mg of Protein/tube

8: 800µl stock + 200 µl distilled water = 4 mg of Protein/tube

6: 600µl stock + 400 µl distilled water = 3 mg of Protein/ tube

4: 400µl stock + 600 µl distilled water = 2 mg of Protein/ tube

2: 200µl stock + 800 µl distilled water = 1mg of Protein/ tube

0: 1ml of distilled water

Biuret Reagent

3g of Potassium-Sodium Tartrate (Fisher Scientific, UK) and 0.75 g of Copper Sulphate ($CuSO4.5H_2O$) (Fisher Scientific, UK) are dissolved in 250ml of distilled water. To this 150ml of 10% Sodium Hydroxide (pellets purchased from Fisher Scientific, UK) is added and stirred well. The solution is then further diluted to 500ml with additional distilled water.

8.5 Appendix 5. Comet Assay (Reagents)

Unless otherwise stated, all compounds were supplied by Sigma (UK).

Trypan Blue Test *(test for viable cells)*

1) Prepare 0.4% Trypan Blue in Phosphate Buffered Saline (PBS)
2) Add 10ul of 0.4% Trypan Blue to 20ul of cell homogenatein PBS
3) Pippette 10ul of the stained homogenate into a haemocytometer
4) Count the number of dead and viable cells in 2 x 10 squares (dead cells stain deep blue, viable cells remain translucent) and take the mean average

Lysis Solution (per 1000ml)

Compound	Final Concentration	Weight (g)
NaCl	2.5M	146.4
Na2EDTA	100mM	37.2
Tris Base	10mM	1.2
N-Lauroyl-Sarcosine	1%	10

- Add ingredients, then add 800ml of dH2O
- Stir with magnetic stirrer and check pH
- Add NaOH to give pH 10 (approx. 8.0g)
- Make up to 1000ml
- Leave at room temp

Add immediately prior to use;

- Triton X 1% (eg. 0.5ml per 50ml)
- DMSO (eg. 5ml per 45ml)

Alkaline Electrophoresis Solution pH>13 (300mM NaOH, 1mM EDTA)

(per 1000ml)

Compound	Quantity
NaOH	12g
500mM EDTA, pH 8	2ml
dH2O	800ml

- Make up to 1L with dH2O after NaOH has dissolved
- Adjust the volume prepared based on the dimensions of the electrophoresis apparatus

Alkaline Solution, pH3 (per 50ml) – *need 100ml*

Compound	Quantity
NaOH Pellets	0.6g

200mM EDTA	250ul
dH2O	49.75ml

Stock SYBR Green

SYBR Green was purchased from Stratech (UK)

Compound	Quantity
SYBR Green Stain	1ul (2ul strengthens colour)
TE Buffer pH 7.4	10ul

TE Buffer;

Compound	Concentration	Quantity (per 250ml)
Tris-cl pH 7.5	10mM	0.394g
EDTA	1mM	0.09g

Standard Operating Procedure for the Comet Assay

1) Make up Alkaline Solution and Electrophoresis Solution (see ***Reagents***). Place the Alkaline, Electrophoresis and Lysis Solution in the dark and leave to chill at 16C
2) Put LMP Agarose (in PBS) into microwave for 2.30 min on medium, power level 5, take out after 1 min and stir. Place on heating block or in the incubator at 37C
3) Put 90ml Lysis Solution in glass measuring cylinder and add 10ml of DMSO. Then add 1000ul of Triton X in the beaker. Place beaker in fridge for 5-10mins
4) Remove 2ul of blood from each fry and dilute in 498ul of PBS. Take 10ul of the blood/PBS solution and pipette into 160ul of LMP Agarose (do one at a time) then take 50ul (for 20 sample slides) and place onto each of the circles (3 x replicates for each sample). Keep the slide in the dark while you finish placing the agarose onto the slides

5) Repeat for the other samples and leave in fridge for 10mins to set
6) Gently pour the Lysis Solution into the Petri dish with the slides. Then leave for a minium of 30mins in the fridge (preferably 45-60mins)
7) Take out slides and gently tap of excess Lysis Solution then wash out Petri dish with Ultrapure water and dry
8) Put slides back into Petri dish and add the Alkaline Solution leave in fridge for 30mins
9) Take slides out and gently tap off excess alkaline solution
10) Place the slides in the electrophoresis tanks 30V (approx. 0.7 per cm), 300ma and run for 30 mins
11) Take out slides and wash them three times in Ultrapure water, carefully not to let the agarose slip off, normally tilt the slide slighty
12) Leave then in 70% ethanol for 5 mins and then leave on the side in the dark to dry
13) Add 50ul of SYBR Green to each circle to be analysed (make sure this is done in the dark and are kept in the dark until they are analysed) then place a coverslip gentle over the slide
14) Slides are now ready to be analysed on the microscope