APPENDIX A – *Media and Solution Preparation*

Appendix A

Regarding the present experimental research, a summary of recommended and standard aseptic techniques should be followed throughout every procedure, to ensure axenic conditions.

Therefore all material in use should be sterilized by autoclaving (120ºC for 20 min), and all the procedures performed on a sterile bench (disinfected with 70% alcohol). Most of the procedures should be performed in a well-ventilated area.

Gloves, goggles and lab coats should be worn when working with chemicals. Nitrile gloves are recommended when working with solvents and acids.

SOLUTIONS

AMPICILLIN, KANAMYCIN, RIFAMPICIN AND TETRACYCLINE ANTIBIOTIC SOLUTIONS

Preparation of stock solutions for solid medium (e.g. Marine Agar) is recommended. The stock solutions should be 100-300x the concentration of antibiotic required in the enriched medium.

Reagents (for a final concentration of 10^3 mg.L^{-1})

CH_3OH	10 ml
C_2H_6O	10 ml
$C_{16}H_{18}N_3NaO_4S$ (Ampicillin sodium; Sigma Chemical Co.)	0.1 g
$C_{18}H_{36}N_4O_{11}.H_2SO_4$ (Kanamicin; Sigma Chemical Co.)	0.1 g
$C_{43}H_{58}N_4O_{12}$ (Rifampicin; Sigma Chemical Co.)	0.1 g
$C_{22}H_{24}N_2O_8$ (Tetracycline; Sigma Chemical Co.)	0.1 g
NaOH	pinch

Procedures

Ampicillin & Kanamycin antibiotic solutions

1. Weigh out 0.1 g of each antibiotic powder in weighing boats;

2. Separately, dissolve the ampicillin and kanamycin powders in 10 ml distilled water. Mix very well, so that all the antibiotic powder goes into solution (to the ampicillin solution, a pinch of NaOH[a] can be added so the pH is adjusted and dissolution optimized);

3. Filter sterilise the solutions into sterile falcon tubes, using a syringe with an attached 0.2 µm filter sterilization unit;

4. Store the kanamycin solution at 4ºC and the rifampicin at -20ºC.

Rifampicin & Tetracycline antibiotic solutions

1. Dissolve the rifampicin powder per 10 ml of methanol and the tetracycline in another 10 ml of ethanol;

2. Transfer the solutions to separate sterile falcon tubes;

3. Wrap the falcon tubes in aluminium foil and immediately store them in the dark at -20ºC.

[a] If using an acidic form of Ampicillin instead of the sodium salt form as indicated in these procedures, then no NaOH should be added.

Note

Most antibiotic solutions will remain stable for up to 3 months, if stored at 0°C. However, Rifampicin should be freshly prepared for each use.

Stock solutions in water must be filter sterilized through a 0.2 µm pore filter. Antibiotics dissolved in ethanol or methanol, do not need to be filter sterilized, since bacteria do not grow in such toxic environments.

NINE-SALT SOLUTION – OLSSON *et al.* (1992)

Reagents

NaCl	17.60 g
Na_2SO_4	1.47 g
$NaHCO_3$	0.08 g
KCl	0.25 g
KBr	0.04 g
$MgCl_2 . 6H_20$	1.87 g
$CaCl_2 . 2H_20$	0.41 g
$SrCl_2 . 6H_20$	0.008 g
H_3BO_3	0.008 g

Procedure

1. Weigh out the reagents and place them in a 1 L volumetric flask;
2. Add 1000 ml of double-distilled water and mix it very well;
3. Pour into storage bottles and sterilize the solution by autoclaving at 120ºC for 15 minutes;
4. Allow them to cool, and tighten the lids.

NEUTRAL BUFFERED FORMALDEHYDE SOLUTION – HOPWOOD (1977)

Ten percent neutral buffered formaldehyde is the standard fixative used in most laboratories. It preserves a wide range of tissues and requires a relatively short fixation time. However it can also be used for long-term storage, as it produces no deleterious effects on tissue morphology with nuclear and cytoplasmic detail being adequately preserved (Cleary *et al.*, 2005).

Reagents

Formaldehyde 40%	100 ml
$NaH_2PO_4 . H_2O$	4.00 g
Na_2HPO_4	6.50 g

Procedure

1. Combine the sodium phosphate salts in 500 ml of distilled water. Add the formaldehyde while stirring;
2. Adjust the pH to 7.0 with sodium phosphate concentrated stocks if necessary;
3. Make up to 1 L with the remaining distilled water;
4. Filter the solution through a 0.45 µm pore filter and store it in a tightly capped, labelled bottle.

Notes

Formaldehyde is a known carcinogen and should be handled in such a way as to minimize exposure (Cleary *et al.*, 2005). It may irritate eyes and respiratory passages, and can cause skin and lung allergy. It has also been reported to cause impaired memory and dexterity, and can be fatal if ingested (Sheehan *et al.*, 1980).

Because of formaldehyde's hazards, specific regulations should be implemented, regarding its safe handling and storage. Safety precautions include proper ventilation and exhaust, as well as limited or restricted exposure periods. At all times goggles, gloves and lab coat should be worn.

For 10% neutral buffered formaldehyde solution to be effective, the specimen has to be completely submerged in five to ten times its volume of fixative.

This solution is usually stable for 30 days. As a precaution, the pH of old solutions should be checked (if acidic the solution shouldn't be used).

PHOSPHATE-BUFFERED SALINE SOLUTION – SAMBROOK *et al.* (2001)

Reagents (formula per 1 L)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
HCl	drops

Procedure

1. Weigh out the reagents for the selected total volume of PBS and place them in a volumetric flask;

2. Add distilled water to the required volume and mix it very well. Adjust to a 7.4 pH with HCl;

3. Dispense into storage bottles and sterilize the solution by autoclaving it at 120ºC for 15 minutes;

4. Allow to cool, and then tighten the lids.

HEPES FIX BASE

Reagents

NaCl	10.22 g
HEPES[b] (Sigma Chemical Co.)	17.85 g
$CaCl_2.2H_2O$	0.74 g

Procedure

1. Dissolve all the reagents in 400 ml distilled water, while stirring;

2. Adjust to a 7.4 pH.

3. Add distilled water to bring the volume to 500 ml;

MODIFIED KARNOVSKY'S FIXATIVE

Karnovsky originally formulated this fixative as a sodium cacodylate-buffered, calcium chloride-enhanced mixture of 5% glutaraldehyde and 4% formaldehyde (Karnovsky, 1965).

This original recipe produced a hypertonic medium (2010 mOsM) that was so hypertonic, that over the years most investigators have chosen to change it for solutions at half-strength. Prepared without the calcium chloride, with a phosphate buffer rather than sodium cacodylate buffer, and various concentrations of formaldehyde and glutaraldehyde (Salema & Santos, 1992).

Massie *et al.* (1972) indicate that HEPPES buffer is a non-toxic substitute for bicarbonate buffers.

Reagents

Paraformaldehyde	0.5 g
2 N NaOH	drops
HEPES fix base (mentioned above)	5 ml
8% Glutaraldehyde	7.9 ml

Procedure

1. Under the laminar flow hood, bring 10 ml of distilled water to approximately 60ºC and add 0.5 g paraformaldehyde powder, while stirring;

2. Add 2-3 drops of 2 N NaOH and stir the solution until it

[b] **HEPES** (N-2-ydroxyethylpiperazine-N'-2-ethane sulfonic acid), Code H3375 - Sigma Chemical Co.

clears;

3. After adding 5 ml of HEPES fix base solution, let the solution cool at room temperature;

4. Add the glutaraldehyde solution at pH 7.2-7.4 to bring the volume to 25 ml;

5. Store the solution at 4ºC.

Note

Formadehyde, although a poor x-linker, penetrates tissue at about 5x the rate of glutaraldehyde. It is thought that this fast penetration helps to stabilize the tissue structures that are later on stabilized further by the glutaraldehyde.

This fixative should not be kept for more than two weeks before use, because the formaldehyde component will begin repolymerizing into paraformaldehyde.

GROWTH AND TESTING MEDIA

MARINE BROTH

The Marine Broth 2216 (Difco Laboratories™; BD – Bioscience) is prepared according to the formula of ZoBell. The media contains all of the nutrients necessary for the growth of heterotrophic marine bacteria. It contains minerals that nearly duplicate the major mineral composition of seawater, in addition to peptone and yeast extract that provide a good source of nutrients (nitrogen, vitamins and minerals) - Retrieved [20th March, 2006 at 12:57h], from Becton, Dickinson and Company (http://www.bd.com/ds/technicalCenter/inserts/Marine_Agar_&_Broth_226.pdf).

Procedure

1. Suspend 37.4 g of the Marine Broth (MB) dehydrated powder in 1 L of distilled water. Mix thoroughly;

2. Heat under frequent agitation, and boil for 1 minute to completely dissolve the powder;

3. Transfer the media into 1 L bottles and autoclave them at 120ºC for 15 min;

4. Allow the bottles to cool and tighten the lids.

10% MARINE AGAR

The high salt content from Marine Broth 2216 (Difco Laboratories™; BD – Bioscience) helps to simulate the sea water. For that reason when preparing 10% Marine Agar, NaCl was added.

Agar is the solidifying agent.

Reagents

Marine Broth (2216 Difco Laboratories™)	3.74 g
Bacteriological Grade Agar (ICN Biomedicals)	15.00 g
NaCl	28.80 g

Procedure

1. Suspend the MB dehydrated powder in 1 L distilled water. Heat to the boiling point;

2. Add NaCl and the bacteriological grade agar, while stirring with a magnet. Let it boil for 1 minute to optimize dissolution;

3. Transfer the media into 2 L bottles. Screw on the caps loosely, and autoclave the bottles for 15 minutes at 120ºC and 15 lb/sq. in.;

4. After autoclaving, vigorously swirl the solutions in the bottles, allowing the molten agar to mix;

5. Allow the media to cool down to 50ºC (when one can hold the bottom of the flask for 10-20 s);

6. Under a laminar flow hood pour the media into sterile plastic Petri dishes (approximately 25 ml for 90 mm Petri dishes). If bubbles appear on the surface of the agar, they may be removed by briefly flaming the surface with a Bunsen burner;

7. After pouring, leave the dishes dry for 15-20 min (while still inside the laminar flow);

8. Store the Petri dishes facing down (inverted position)[c] in sealed "tape-shut" plastic sleeves, and incubate them overnight at 20ºC;

9. On the following days check for growth of bacterial contaminants. After selection, keep them stored in an incubator at room temperature.

Notes

To prepare Marine Agar medium supplemented with antibiotics (MA+R+A), Ampicillin and Rifampicin from proper sterile antibiotic stock solutions, should be added, until 10 $mg.L^{-1}$ of each are obtained in the final plates volume. When transferring the antibiotics, aseptic procedures should be followed.

The sterilized agar should be cooled to 50ºC, before the addition of heat-sensitive antibiotics (e.g. Ampicillin).

YEAST EXTRACT-PEPTONE-DEXTROSE

Complex media containing a homogenous blend of Peptone, Yeast Extract and D-Glucose in optimal concentration, is used for maintaining and propagating most *Saccharomyces cerivisiae* strains and other yeast.

The addition of protein and yeast extract hydrolysates allows a faster growth during the exponential or log-phase growth – Retrieved [30th April, 2007 at 08:33h], from Becton, Dickinson and Company (http://www.bd.com/ds/technicalCenter/inserts/YPD_Agar.pdf).

The Bacteriological Grade Agar works as the solidifying agent in solid YEPD mediums.

[c] So that any moisture that may occur, will be collected on the top of the plate without flooding the bottom where bacteria will grow.

Reagents

Yeast Extract (Thermo Fisher Scientific Inc.)	10 g
Peptone (MP Biomedicals Co.)	10 g
D-Glucose (Sigma Chemical Co.)	20 g
Bacteriological Grade Agar (MP Biomedicals Co.)	20 g

Procedure

1. Weigh out the appropriate reagents and place them into a flask at least 2 times larger than the media volume;

2. Suspend all reagents (with exception of the D-Glucose) in 950 ml of distilled water, and gently bring to the boiling point, allowing it to dissolve completely;

3. Sterilise the agar and broth media by autoclaving at 120ºC for 15 minutes;

4. Allow the media to cool down to 50ºC, and aseptically add D-Glucose to 2% (50 ml previously autoclaved). Mix thoroughly and adjust the final volume (1 L) if necessary.

Notes

Liquid YEPD medium is ready for use. With solid medium, gently pour the molten agar into sterile Petri dishes, as described in previous procedures.

Yeast extract and peptone provide carbon, nitrogen, minerals, vitamins, trace ingredients and other essential growth nutrients. The yeast extract further supplies vitamin B-complexes, which stimulate the growth of yeasts and bacteria. Dextrose (D-Glucose) is the carbohydrate source.

If adding supplements such as amino acid mixtures, care should be taken to avoid agar hydrolysis. Supplements should be added after autoclaving.

YEAST NITROGEN BASED MEDIUM

Reagents

Yeast Nitrogen Base Y1250 (Sigma Chemical Co.)	6.7 g
D-Glucose (Sigma Chemical Co.)	10 g
Uracil (Sigma Chemical Co.)	40 mg
L-Histidine (Sigma Chemical Co.)	40 mg
L-Leucine (Sigma Chemical Co.)	40 mg
L-Lysine (Sigma Chemical Co.)	40 mg

Procedure

1. Suspend the reagents in 1 litter of distilled water;

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder;

3. Dispense into suitable containers and autoclave at 120ºC for 15 minutes;

4. Allow the bottles to cool and tighten the lids.