APPENDIX 3 (MOLECULAR TECHNIQUES)

3.2.2a) DNA extraction method as per QIAamp DNA mini kit (Qiagen, Germany)

Method

- Two hundred microliters (200 µl) of the EDTA blood was added to 200 µl of Buffer AL and 20 µl Proteinase K in Eppendorf safelock micro-centrifuge tubes (Merck).
- The mixtures were pulse-vortexed for approximately 10 s and placed in a Hagar heating block at 56 ℃ for 10 min before brief centrifugation to remove droplets from tube edges and lid.
- Two hundred microliters (200 µl) of absolute ethanol (98 %) was added to the mixtures, briefly vortexed and centrifuged.
- The mixtures were then transferred to the spin columns included with the QiAamp DNA mini kit and centrifuged at 8 000 rpm for 60 s. The flow through collecting in the collection tube was disposed of in a beaker containing 10 % (v/v) formalin and the spin column was placed into a clean collection tube.
- Five hundred microliters (500 µl) of Buffer AW1 was pipetted into the spin column, and the tubes were then centrifuged at 8 000 rpm for 60 s before discarding the flow through as above and placing the spin column into new collection tubes.
- Five hundred microliters (500 µl) of Buffer AW2 was pipetted into the spin column, before centrifugation at 13 000 rpm for 3 min. The flow-through was discarded and the spin columns were placed back into the collections tubes and centrifuged at 13 000 rpm for 60 s to eliminate Buffer AW2 carryover.
- The collection tubes were discarded and the filters were placed into Eppendorf tube with the lids cut off. Elution occurred in two identical steps, where 50 µl Buffer AE (elution buffer) was added to the spin column, allowed to stand at room temperature for 2 min and centrifuged at 8000 rpm for 1 min. This step was repeated once more before the final volume of 100 µl DNA was obtained.
- Extracted DNA was stored at -20 ºC until use.

3.2.2b) 50x TAE (Tris/acetate/EDTA) buffer

Components

242 g Tris base [40 mM]
57.1 ml Glacial acetic acid [20 mM]
100 ml 0.5 M EDTA (pH~ 8) [1 mM]

186.1 g $Na_2EDTAx2H_2O$
to 700 ml sterile water
adjust pH to 8.0 using 10 M NaOH
to 1000 ml sterile water

to 700 ml Sterile water
adjust pH to 7.9 using glacial acetic acid
to 1000 ml Sterile water

Method

- Dissolved the 186.1 g $Na_2EDTAx2H_2O$ in 700 ml of sterile distilled water to make up 0.5 M EDTA. The pH was adjusted to ~8.0 by adding 10 M NaOH until the solution reached target pH. Sterile water was added to a final volume of 1000 ml was reached.
- Dissolved 242 g of Tris base in 700 ml sterile water.
- Added 57.1 ml glacial acetic acid to the Tris solution and followed with 100 ml of 0.5 M EDTA (pH~8.0) solution.
- Checked and adjusted the pH to 7.9 using glacial acetic acid.
- Added in sterile water to make a final volume of 1000 ml of 50x TAE buffer.

3.2.2c) 2 % (w/v) TAE agarose gel (10 x 20 cm casting tray)

Components

2 g Molecular grade, low EEO (electro end osmosis point) Agarose powder (White Sci)
100 ml TAE buffer (1x)
4 µl Ethidium bromide (Biobasic Inc.) [10 mg/ml solution]

Method

- ♣ Diluted 50x TAE buffer described in 3.2.2b to a 1x TAE buffer working solution (20 ml 50x TAE buffer diluted with 980 ml sterile distilled water)
- ♣ Weighed out 2 g of molecular grade agarose powder and mixed with 100 ml of 1x TAE buffer in a 250 ml glass schott bottle and placed lid on the bottle loosely.
- ♣ Heated in microwave oven on high heat for 30 s, removed from heat and swirled vigorously and reheated for a further 30 s. (**note**: the solution must come to a boil in order to ensure all the agarose has dissolved in the TAE buffer)
- ♣ Once all the agarose had dissolved, the liquid was poured into a measuring cylinder to asses whether the volume had diminished due to evaporation. If it had dropped below the 100 ml mark sterile water was added in until the 100 ml mark was reached.
- ♣ The liquid was poured back into the schott bottle and was swirled while cooling the liquid until the bottle was warm to the touch and added in 4 µl of EtBr to the liquid while swirling.
- ♣ The casting tray (10 x 20 cm) was set up with dams in place, the warm agarose mixture was poured, and the comb/s were placed to create the wells.
- ♣ The gel was allowed to set.

3.2.5a) DNA extraction method as per QIAquick Gel Extraction Kit (Qiagen, Germany)

- Once the Agarose gel containing the gel slice has been weighed out, add 3 volumes of Buffer QG to 1 volume of Agarose gel. (1:1 (v/v) ratio).
- Incubate at 50 ℃ for 10 min in order to completely dissolve the gel, mix by vortexing every 2 – 3 min.
- Once gel is completely dissolved, ensure the mixture is yellow (if not, add 10 µl of 3 M sodium acetate to ensure the pH is 5.0).
- Add 1 gel volume of Isopropanol to sample and mix.
- Place mixture into included collection tube (800 µl at a time) and centrifuge at 13 000 rpm for 1 min.
- Add 0.5 ml of Buffer QG to spin column ad centrifuge at 13 000 rpm for 1 min.
- To wash, add 0.75 ml of Buffer PE and spin at 13 000 rpm for 1 min.
- Discard flow-through and spin for an additional 1 min at 13 000 rpm in order to remove all ethanol.
- Place spin column into a clean 1.5 ml collection tube and elute DNA in 50 µl (for concentrated DNA ~ 30 µl) of either Buffer EB or sterile distilled water. Let column stand for 1 min and spin down as before for 1 min.
- Check DNA quality by running on a 1% (w/v) TAE agarose gel.

3.2.5b) LB agar and LB broth

LB agar Components

5.0 g Tryptone (Beckton Dickenson)
2.5 g Yeast extract (Beckton Dickenson)
5.0 g NaCl (Sigma Aldrich)
7.5 g 1.5% Agar (Beckton Dickenson)

Method

- ♣ Solubilized in 500 ml sterile distilled water
- ♣ Autoclaved and let cool to 50 ℃
- ♣ Added 0.5 ml Ampicillin solution (100 mg/ml)
- ♣ Poured into sterile plates, allowed to solidify, and stored in fridge

LB broth Components

5.0 g Tryptone (Beckton Dickenson)
2.5 g Yeast extract (Beckton Dickenson)
5.0 g NaCl (Sigma Aldrich)

Method

- ♣ Solubilized in 500 ml sterile distilled water
- ♣ Autoclaved and cooled
- ♣ Added 0.5 ml Ampicillin solution (100 mg/ml)
- ♣ Poured into sterile tubes (~20 ml per tube) and stored in fridge