

Appendix II

Complementary experimental procedures

Paraffin embedding

Samples (tissues and larvae) were placed in appropriate histological cassettes and immersed in 70% DEPC ethanol in an automatic tissue processor (Leica TP 1020). The following program for automated dehydration, clearing and embedding was set up following the instrument instructions and consisted of:

Solution	Treatment	Time (minutes)
70% Ethanol	Dehydration	10
95% Ethanol	Dehydration	30
95% Ethanol	Dehydration	30
100% Ethanol	Dehydration	60
100% Ethanol	Dehydration	60
100% Ethanol: Xylene (1:1)	Clear	60
Xylene	Clear	60
Xylene	Clear	90
Xylene:Paraffin (1:1)	Infiltration	120
Paraffin	Infiltration	120

Once the program finished paraffin blocks were prepared with the processed material using moulds of an appropriate size.

Preparation of slides with APES (aminopropyltriethoxysilane) for mounting paraffin wax sections

A batch of slides was treated each time using a glass slide rack that takes 25-50 slides.

Slides were washed by immersing in a 1% acid/alcohol solution (1% concentrated hydrochloric acid, 70% ethanol, 29% distilled water, v/v) for 30 minutes agitating occasionally. Slides were removed, the excess acid solution allowed to run off, and the slides were rinsed in running tap water for 1-2 minutes and then briefly immersed in distilled water. Slides were dried in an oven at 37°C.

Slides were coated with aminopropyltriethoxysilane (APES, Sigma-Aldrich) by immersing in acetone for 10 minutes and then in a 2% (v/v) solution of APES in acetone for 5 minutes. The excess APES solution was allowed to run off and

slides were rinsed in distilled water and dried at 37°C and boxed and stored at room temperature until required.

Tissue sections dehydration and clearing to obtain definitive preparations

On completion of staining of sectioned material, definitive preparations were prepared by dehydration, clearing (see table below), mounting in DPX (Fluka) and covering with a glass coverslip.

Solution	Treatment	Time (minutes)
Ethanol 70%	Dehydration	5
Ethanol 95%	Dehydration	5
Ethanol 100%	Dehydration	5
Xylene	Clearing	10
Xylene	Clearing	10

Preparation of agarose gels

The preparation of agarose gels for electrophoresis was similar for all procedures. The volume of agarose solutions prepared was adjusted to be appropriate to the size of the gel mould utilized.

For a 1% agarose gel of 50ml (mould 10.5×7.5×1.5cm):

Agarose (0.5g) was weighed and added to 50ml of 1×TBE buffer (appendix I). The mixture was microwaved or stirred on a hot plate until the agarose had completely dissolved and the solution was clear. The solution was allowed to cool to approximately 55°C, and 20µL of an ethidium bromide solution (0.5mg.ml⁻¹) added, the agarose gel was mixed and poured into the gel mould and allowed to solidify. The amount of agarose utilized was varied according to the final percent of agarose required. For example, a 2% solution of agarose in 50ml would correspond to 1g agarose in 50ml TBE buffer.