



# **Community Reference Laboratory for Crustacean Diseases**

## **Standard Operating Procedure SOP 2013 Revision Number 1**

### **Method for testing crustaceans for White Spot Disease, Yellowhead Disease and Taura Syndrome by histological analysis**

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## 1 Introduction

The Community Reference Laboratory (CRL) routinely samples crustacean tissues; their health status examined and evaluated using wax histology methods. Each laboratory will have different chemicals and equipment so details of how to use each piece of equipment have been omitted; this protocol provides an overview of the technique and chemicals used at the CRL.

## 2 Scope

This procedure provides the method for the receipt of crustacean samples and subsequent histological processing of the samples. It explains in overview form the processes of processing, embedding, cutting of wax blocks and staining of histological slides. It includes reading of slides and interpretation of results. Histological techniques enable presumptive diagnosis for viral pathogens to be made, and also enables other pathogens to be identified. Suspected viral infections require molecular and electron microscopy techniques to confirm diagnosis but this is outside the scope of this protocol.

## 3 Training (Identify any specific training linked to the SOP)

This procedure may only be carried out by staff that have received training in the use of equipment required for histological preparation.

## 4 Safety Precautions

Many hazardous chemicals are used during the histological processing of samples, before performing this procedure staff should have read and understood all relevant safety documentation.

The use of local exhaust ventilation (LEV) or fume extraction hood, where available, is advised for making of solutions and fixation of samples. Where a LEV is not available ensure chemicals are used in a well-ventilated area.

Staff should wear personal protective equipment, such as lab coat, eye protection and gloves, at all times.

Material Safety Data Sheets (MSDS) can be found on the Internet or via the suppliers.

### **Absolute Ethanol**

Eye: Causes severe eye irritation

Skin: Causes moderate skin irritation

Ingestion: May cause gastrointestinal irritation with nausea, vomiting and diarrhoea

Inhalation: Vapours may cause dizziness or suffocation



Highly Flammable  
Flash point 16.6°C

**Clarene**

Eye: May cause irritation and redness

Skin: Causes skin irritation, may cause sensitisation by skin contact

Ingestion: Nausea and stomach pain may occur

Inhalation: May cause feeling of tightness in the chest with shortness of breath



Irritant



Dangerous for the environment

**Eosin-y Alcoholic**

Eye: Causes severe eye irritation

Skin: Brief contact may cause slight irritation

Ingestion: Contains methyl alcohol; may cause headache, dizziness, diarrhea and general weakness

Inhalation: High concentrations are irritating to respiratory tract



Highly Flammable  
Flash point 18°C



Toxic

**Formaldehyde**

Eye: Causes irritation. May result in corneal injury

Skin: Causes skin irritation. Harmful if absorbed through the skin

Ingestion: Cause gastrointestinal irritation with nausea, vomiting and diarrhoea. Harmful if swallowed

Inhalation: Harmful if inhaled. Causes respiratory tract irritation.



Toxic

**Glacial Acetic Acid**

Eye: Causes severe irritation

Skin: Causes skin burns. May be harmful if absorbed through the skin

Ingestion: May cause severe and permanent damage to the digestive tract

Inhalation: Effects may be delayed. Causes chemical burns to the respiratory tract.



Flammable



Corrosive



Irritant

**Haemotoxilin Gill III**

Eye: May cause irritation

Skin: Staining of skin may occur, wash area well with soap and water

Ingestion: Harmful if swallowed, may cause irritation of throat.

Inhalation: May cause irritation of throat with tightening of chest.



Harmful

**Histosolve**

Eye: High vapour concentration may cause irritation and discomfort

Skin: Brief contact may cause slight skin irritation; prolonged contact may cause irritation or dermatitis

Ingestion: May result in vomiting

Inhalation: High concentrations are irritating to respiratory tract



Flammable



Harmful

**IMS**

Eye: Causes severe eye irritation



Highly Flammable  
Flash point 18°C



Harmful

Skin: Causes moderate skin irritation. May be absorbed through the skin in harmful amounts

Ingestion: May be fatal or cause blindness if ingested. May cause gastrointestinal irritation with nausea, vomiting and diarrhoea

Inhalation: Inhalation of high concentrations may cause central nervous system effects characterised by nausea, headache, dizziness, unconsciousness and coma. Vapours may cause dizziness or suffocation.

## 5 References/Associated documents

### 5.1 References

OIE (2006). Manual of Diagnostic Tests for Aquatic Animals, 5<sup>th</sup> edition, Paris, France, 358 p. (also available at [http://www.oie.int/eng/normes/fmanual/A\\_summry.htm](http://www.oie.int/eng/normes/fmanual/A_summry.htm))

Bower, S.M., McGladdery, S.E. & Price, I.M. (1994) Synopsis of infectious diseases and parasites of commercially exploited shellfish. Annual Review of Fish Diseases 4(1): 1-199

Registry of Aquatic Pathology (RAP), Cefas Weymouth Laboratory. [www.aquaticpathology.co.uk/](http://www.aquaticpathology.co.uk/)

## 6 Equipment /Apparatus

Processing Data Sheet

Labelled cassettes and lids

Labelled fixative pots

Davidson's Fixative (Seawater or Freshwater)

Neutral Buffered Formalin (NBF)

70% IMS

90% IMS

Absolute IMS

Histosolve

Wax

Embedding centre

Microtome

Water bath

Labelled glass slides

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Hot plate  
 Staining rack and pots  
 Haematoxylin  
 1% Acid alcohol  
 Eosin  
 Water  
 Clearene  
 Mounting media  
 Coverslips  
 Microscope  
 White Spot Disease Data sheet  
 Yellowhead Disease Data sheet  
 Taura Syndrome Data sheet  
 Results Record Sheet

## 7 Reagents

### Davidson's Seawater Fixative

#### Stock Solution

Filtered sea water	3340ml
95% Ethanol	3330ml
36 - 40% Formaldehyde	2220ml
Glycerin	1110ml

#### Working Solution

Stock solution	720ml
Glacial acetic acid	80ml

### Davidson's Freshwater Fixative

To be used for fixation of freshwater crustacean samples, for example crayfish

#### Stock Solution

Distilled water	3350ml
95% Ethanol	3300ml
36 - 40% Formaldehyde	2200ml

Working Solution

Stock solution	885ml
Glacial Acetic Acid	115ml

**Neutral Buffered Formalin**

Can be used for fixation of freshwater crustacean samples, for example crayfish

Distilled Water	9L
36-40% Formaldehyde	1L
Sodium dihydrogen orthophosphate dihydrate	40g
di-Sodium hydrogen orthophosphate anhydrous	65g

**8 Procedure**

All details of processing are recorded on the histology worksheet (see appendix 1).

**8.1 Receipt and storage of samples**

8.1.1 Upon receipt, the labelling on the pots and cassettes needs to be compared with the numbers provided on the accompanying histology worksheet. The number of pots and cassettes associated with the particular sample needs to be confirmed by comparing with information provided in the histology worksheet. Additionally, the legibility of the labelling needs to be confirmed. The accompanying sheet detailing sample history needs to be assessed to ensure that Davidson's fixative was used and tissues were fixed for at least 24 hours before transfer into IMS.

8.1.2 The person that receives the sample in the laboratory needs to initial, date and record the time on the histology worksheet that samples were received. In the event of a discrepancy or missing paperwork, inform the Technical manager or Deputy. This needs to be recorded in the departures logbook.

**8.2 Sample preparation to wax blocks**

8.2.1 All samples in cassettes are stored in 70% IMS prior to processing. To process the tissues to wax blocks, the samples are processed by being dehydrated, cleared and infiltrated with paraffin in an automatic tissue

processor. For crustacean samples the routine programme is as below:

Step	Reagent	Temperature	Time (in minutes)
1	70% IMS	Ambient	30
2	90% IMS	Ambient	30
3	Absolute IMS	Ambient	30
4	Absolute IMS	Ambient	30
5	Absolute IMS	Ambient	30
6	Absolute IMS	Ambient	30
7	Histosolve	Ambient	40
8	Histosolve	Ambient	40
9	Histosolve	Ambient	40
10	Histosolve	Ambient	40
11	Wax	65°C	45
12	Wax	65°C	45
13	Wax	65°C	50

- 8.2.2 In the process section of the histology worksheet, initial, date and record the time at which the processor is started, record the date and time at which the processor is expected to finish.
- 8.2.3 Once the processor has completed, note the actual date and time the processor ended into the histology process sheet.
- 8.2.4 Date, initial and record the time at which the processor is emptied in the process section of the histology worksheet. After the processor has completed, transfer the sample cassettes to the molten wax compartment of an embedding centre.
- 8.2.5 A clean cycle is initiated on the tissue processor.
- 8.2.6 Initial and date the process sheet of the histology worksheet. Individual cassettes are removed from the heated wax and placed on the heated surface. The lid of the cassette is removed by holding the main body of the cassette on the surface of the embedding centre and prising the lid with the other hand. Molten wax is added to a metallic mould and the crustacean tissue is transferred gently using forceps to the molten wax in the mould. The sample should be manipulated so that it is at the bottom of the mould. It can be temporarily held in place using the small press provided with the embedding centres. The mould is moved to the small cold area of the embedding centre. Once the wax in the bottom of the mould begins to cool, as denoted by a change in the colour of the wax from clear to white, the cassette is placed on top of the mould. A small volume of molten wax is then added to the top of the cassette.
- 8.2.7 The mould is moved to the main cold plate to allow the wax to harden. The wax is deemed to have hardened when the wax on the top has gone white and the block is cool to the touch. Once cooled, the paraffin

block can be removed from the mould. Excess wax is removed either manually or by using a para-trimmer.

### 8.3 *Sectioning of wax blocks*

Good sectioning requires training and experience as well as properly prepared material.

- 8.3.1 Wax blocks should be trimmed at room temperature on a microtome until the crustacean tissues are exposed. If required, the blade can be replaced if cutting is not progressing well.
- 8.3.2 Glass slides are labelled. The following information needs to be included on the slide: sample reference number, block number and the month and year of submission.
- 8.3.3 Record the microtome station number on the histology process sheet. In addition, initial and date the process section of the histology worksheet when samples are cut.
- 8.3.4 The microtome is set to 3-5µm and a section cut and floated onto a glass slide using routine sectioning methods.
- 8.3.5 Slides are dried on the hotplate until the water has evaporated and the wax at the edges of the sections appear translucent.

### 8.4 *Staining of tissue samples on glass slides*

8.4.1 Slides are deparaffinised, rehydrated and then stained with Haematoxylin and Eosin either manually or in an automatic tissue stainer. Typically the haematoxylin and eosin staining protocol is as follows:

Clearene	3 minutes
Clearene	3 minutes
100% alcohol	3 minutes
100% alcohol	3 minutes
Water	3 minutes
Haematoxylin	30 seconds
Haematoxylin	2 minutes, 30 seconds
Water	6 minutes
1% Acid alcohol	3 seconds
Water	6 minutes
Eosin	3 minutes
Water	10 seconds
70% alcohol	20-25 seconds
100% alcohol	3 minutes
100% alcohol	3 minutes
100% alcohol	3 minutes
Clearene	3 minutes
Clearene	3 minutes
Clearene	3 minutes

- 8.4.2 Stained sections are coverslipped either manually or with an automatic coverslipper. Date and initial the process section of the histology worksheet once staining and coverslipping has been completed.
- 8.4.3 Once the mounting medium has hardened, remove any excess mounting medium using a single edged razor blade if needed and clean the slides. The slides are submitted to the appropriate reader and the process sheet dated and initialled.

### *8.5 Reading, double checking and reporting of results*

- 8.5.1 Refer to White Spot Disease, Yellowhead Disease and Taura Syndrome data sheets for examples of pathologies likely to be caused by these viruses. Slides are read on a microscope equipped with bright field illumination and  $\times 4$ ,  $\times 10$ ,  $\times 40$  and  $\times 100$  objective lenses.
- 8.5.2 Readers should check at least 10% of the slides to confirm the quality of the slides. If the slides are deemed appropriate, the reader should note this on the results sheet.
- 8.5.3 Notes of the pathologies found during examination are recorded on the results sheet, an example of which is attached as appendix 2. On completion of examination, the results are summarised in the results summary sheet, which is signed and dated. An example sheet is attached as appendix 3.
- 8.5.4 Two different competent readers are required for reading of samples – one to act as primary reader who will read all the slides, and a second reader who will double check the result.
- 8.5.5 Reference slides can be obtained from the Community Reference Laboratory.
- 8.5.6 Any discrepancies between the results obtained by the primary reader and those of the double checker should be reported to the Technical Manager or deputy.
- 8.5.7 On completion of the double-checking, the histology worksheet should be signed and dated.
- 8.5.8 Report results to submitter.

### *8.6 Archiving of samples*

- 8.6.1 On completion of the test, paperwork, slides and blocks can be archived. Blocks and slides should be kept for a minimum of 5 years, after which time they can be destroyed if required.

## **9 Review**

This procedure will be reviewed as a minimum on the time scales given in the review / amendment programme. A record of the review will be made on a separate Review / Amendment Sheet which will be added to the Master Copy file of this SOP. Any amendments arising from such review or from operating requirements will result in the issue of the entire amended procedure as a new Issue.

## **10 Records**

This procedure, its review sheets and its subsequent revisions constitute records in themselves and each master copy will be retained in a file as arranged by the Quality Manager. Records will be retained for a minimum of five years unless otherwise specified.

**(List specific record sheets/books)**

**Appendix 1 An example of the process sheet for histology**

				LAB REF NO
Group(s)	1	2	3	Notes
Cassettes printed				
Sample cassetted (date, time of last cassette, initials)				
Processor protocol name				
Processor start time and date (and initials)				
Expected end date and time				
Actual end date and time				
Processor emptied (date, time, initials)				
Samples embedded (date and initials)				
All Sections cut (Date and initials)				
Stained and coverslipped (Date and initials)				
Presented (Date and initials)				



**Appendix 3 An example of the results box for histology**

LAB REF NO
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Results summary for histology

Group number	Results	Signature and Date	Confirmed (Signature, Date)	Reported (Signature, Date)