

# FINAL REPORT



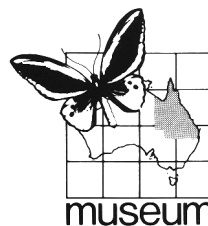
**Aquatic Animal Health Subprogram:  
Validation of DNA-based (PCR) diagnostic  
tests suitable for use in surveillance  
programs for QX disease of Sydney rock  
oysters (*Saccostrea glomerata*) in Australia**

**Dr R.D. Adlard & Dr J. Worthington Wilmer**

**Project No. 2001/630**



Australian Government  
Department of Agriculture,  
Fisheries and Forestry



Australian Government  
Fisheries Research and  
Development Corporation





**Aquatic Animal Health Subprogram: Validation of DNA-based (PCR) diagnostic tests suitable for use in surveillance programs for QX disease of Sydney rock oysters (*Saccostrea glomerata*) in Australia**

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2001/630

**Aquatic Animal Health Subprogram: Validation of DNA-based (PCR) diagnostic tests suitable for use in surveillance programs for QX disease of Sydney rock oysters (*Saccostrea glomerata*) in Australia**

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**OBJECTIVES:**

1. Production of a fully-validated, standard PCR diagnostic test for the presence of *Marteilia sydneyi* in oyster tissue capable of identifying *Marteilia sydneyi* to species level and with a high level of sensitivity.
2. Assessment of comparative cost/benefit of histological, cytological and PCR diagnostic methods for identification of *Marteilia sydneyi*.
3. Production of an Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for QX disease.

**NON TECHNICAL SUMMARY:**

**OUTCOMES ACHIEVED**

The validation of DNA-based diagnostic tests for QX disease has had an unexpected outcome. Using these sensitive diagnostic methods during the validation process, the disease agent (*Marteilia sydneyi*) has been identified in 9 estuaries from which it has never previously been reported. As a consequence, our understanding of the dynamics of QX disease has changed dramatically. QX disease management has largely been predicated on the basis that the distribution of the disease agent is limited, and where it does occur it causes significant mortalities of oysters. Hence, restriction on translocation of potentially infected stock from QX disease endemic areas was thought to be the most effective management tool. It now appears that the distribution of the disease agent is much more widespread than previously thought and that it can exist without causing epizootic disease.

*Summary:* The most significant disease of commercial rock oysters in Australia is QX disease (caused by infection with the protozoan parasite *Marteilia sydneyi*) which has led to recurrent seasonal mortalities of cultured rock oysters. Research has determined that oysters become infected over a relatively short period of time in mid to late summer, then the disease agent proliferates within the oyster and oysters die within about 6-12 weeks of infection. Outbreaks of QX disease have been recorded routinely in estuaries on the NSW central and northern coast and in SE Queensland. Management practices to limit the impact of the disease are currently predicated on restricting the translocation of potentially infected oyster stock from disease endemic areas to areas free of the disease. However, this in turn is based on our ability to detect the disease in any area through diagnostic examination of samples collected

during surveillance programs. Until now, diagnosis of the presence of the QX disease agent has been undertaken through microscopic examination of either tissue imprints of oyster digestive glands (i.e. tissue is blotted against a glass microscope slide, stained and examined) or tissue sections (i.e. routine embedding, staining and examination of thin tissue sections). These are standard pathology techniques which require training in the identification of developmental stages of the parasite.

The aim of this project (FRDC2001/630) was to enhance our capacity to diagnose QX disease by the development of a standard diagnostic protocol based on detecting the presence of the disease agent's DNA. We built on research undertaken by Kleeman & Adlard (2000) to refine the polymerase chain reaction (PCR) amplification of a short section of the parasite's DNA, a specific indicator of its presence. The DNA test was optimised to reduce cost without compromising the sensitivity of the test and then validated against 1,839 individual oysters of known or presumed disease status.

Results of these tests indicated clearly that DNA-based diagnostics were able to detect the presence of *M. sydneyi* at a higher sensitivity than microscopic analysis. In estuaries in which the disease had been diagnosed through microscopy (Georges R, Clarence R., Richmond R., Southern Moreton Bay), a total of 199 oysters were determined to be infected out of 1,000 tested. However, diagnosis using DNA-based tests on a subset of 400 of the same oysters (including all positive oysters diagnosed by microscopy) revealed a total of 223 infected oysters (absolute identity was confirmed using other tests). The difference between these diagnostic results can be explained by the number of disease agents present (i.e. the intensity of infection) in the tissues tested. There is an inverse correlation between the intensity of infection and the required sensitivity of a diagnostic test to detect it.

Furthermore, in a total of 1,340 oysters from 9 estuaries in which QX disease had not been reported previously and which were presumed to be negative from microscopic examination, 116 were determined to be positive for the disease agent through DNA-based diagnosis. On re-examination, these specimens exhibited inflammation and tissue changes consistent with that expected in oysters with early QX disease, however, no disease agent was observed. A second molecular test (DNA probe in-situ hybridisation) confirmed the presence of the disease agent albeit at an atypical developmental stage, rather than at its normal proliferative stage.

In all cases the DNA-based diagnostic protocol identified the presence of *Marteilia sydneyi* at a higher sensitivity than that provided by previous diagnostic tests based on microscopic examination. This technique also obviates the requirement for expert recognition of individual pathogens, and as such, the protocol is easily transferable between laboratories equipped for PCR diagnosis.

The ability to run DNA-based diagnostic tests in batches also confers time and cost benefits with the average test being significantly less expensive in consumable reagents than the cost of diagnosis through examination of tissue sections.

The major aim of the project, to produce a validated Standard Diagnostic Procedure for *Marteilia sydneyi* based on DNA tests, has been achieved and its use is recommended for surveillance of QX disease in commercial rock oysters.

**KEYWORDS:** Sydney rock oyster, aquaculture, QX disease.

## **ACKNOWLEDGMENTS:**

We acknowledge our major partner, NSW Fisheries, for the provision of field samples from NSW estuaries and their input into various aspects of the design of this project. Our thanks to oyster industry groups in New South Wales (NSW Oyster Farmer's Association, NSW Farmer's Federation – Oyster Section) and Queensland (Queensland Oyster Grower's Association) who provided access to their aquaculture areas and logistical support. We are grateful to the members of the NSW Oyster Research Advisory Committee for their support of this project and to the Queensland Department of Primary Industries for assisting with liaison to industry. Special thanks to Dr Stephen Wesche, Research Officer on project FRDC2001-214, for technical and operational support.

## **BACKGROUND:**

DNA-based detection techniques have offered many applications for the study of aquatic diseases. Polymerase Chain Reaction (PCR) and *in situ* hybridisation (ISH), have been the subject of significant interest for use in scientific research as well as for disease management in aquaculture. Protocols have been developed and optimised for use in the detection of haplosporidians (Fong *et al.*, 1993; Stokes & Burrenson, 1995; Stokes *et al.*, 1995), myxosporidians (Bartholomew *et al.*, 1997; Antonio *et al.*, 1998) and paramyxians (Kleeman & Adlard, 2000; Le Roux *et al.*, 1999). Such techniques are rapid, highly sensitive and reliable. Furthermore, these technologies enable the unambiguous detection of all life cycle stages of a pathogen that are otherwise unrecognisable using traditional detection methods. Nonetheless, such tests must be validated before they can be used with confidence (Hiney & Smith, 1998).

Assays developed for the diagnosis of cryptic parasite stages in host tissue that are based on nucleic acid sequences must prove: sensitive enough to detect low levels of infection and/or individual cells; unable to cross react with host tissue; able to be repeated with consistent results; and their levels of specificity among closely related species must be qualified.

This project relates directly to the FRDC project 2001/214 '*Development of a disease zoning policy for marteiliosis to support sustainable production, health certification and trade in the Sydney rock oyster*', in which the zoning policy framework developed under the Federal Government's *Aquaplan* is implemented in a practical framework. FRDC2001/214 is aimed at identifying estuaries of marteiliosis endemicity and will help develop an effective program of surveillance for the disease in oyster culture areas on the east coast of Australia.

The value of surveillance programs to identify the presence of pathogens depends on both a statistically robust sampling regime and a high confidence in diagnosis of the aetiological agent. The results of this project demonstrate unequivocally that sensitive diagnostic methods employing DNA-based presumptive diagnosis, followed by confirmatory molecular diagnosis, not only fulfil their expectations but also provide a wealth of information on the biology of the disease agent.

In the current project, samples collected under FRDC2001/214 were used to validate DNA-based (PCR) diagnostic methods for marteiliosis.

## **NEED:**

Marteiliosis (QX disease, aetiological agent the protistan parasite *Marteilia sydneyi*), typically causes serious, seasonally recurrent mortalities in farmed and wild rock oysters (*Saccostrea glomerata*) in eastern Australia. The disease is listed as notifiable by the OIE and is included on the Australian National List of Reportable Disease of Aquatic Animals.

The global animal health body (Office Internationale des Epizooties, OIE) has recently adopted the concept of zoning to facilitate trade and to prevent spread of disease within a country. In turn, Australia has recognised the value of zoning in its aquaculture industries with the adoption and endorsement of *Zoning Policy Guidelines* by the Standing Committee on Fisheries and Aquaculture.

The establishment of scientifically defensible zoning and translocation policies, particularly in relation to QX disease control, is critical to the long-term development of the rock oyster aquaculture industry. State authorities in New South Wales and Queensland currently prohibit movement of oysters from known QX-infected estuaries to those thought to be free of infection. However, given the many millions of rock oysters translocated annually between NSW estuaries of undetermined disease status, there is an urgent need to accurately identify free and infected zones. This, in turn, depends upon the availability of standardised, validated diagnostic tests.

Diagnostic techniques for molluscan pathogens are relatively limited, with most based on histological and ultrastructural examination (OIE Manual, 2002). The OIE manual (2002) recommends that surveillance is routinely performed by histology, but that various presumptive diagnostic methods e.g. cytology (tissue imprints), can be used in addition. Furthermore, when a pathogen is encountered during screening or mortality outbreaks, electron microscopy and/or molecular probes should be used for specific identification, if available (OIE Manual, 2002).

Recently, there have been major advances in development of PCR tests for marteiliosis (Berthe *et al.*, 2000; Kleeman and Adlard 2000). The validation of PCR-based tests for marteiliosis through this project will assess whether PCR may provide: a more sensitive alternative for presumptive diagnosis obtained by either histopathology or cytology; a relatively inexpensive method for mass screening of oysters for the presence of *Marteilia sydneyi*.

## **OBJECTIVES:**

1. Production of a fully-validated, standard PCR diagnostic test for the presence of *Marteilia sydneyi* in oyster tissue capable of identifying *Marteilia sydneyi* to species level and with a high level of sensitivity.
2. Assessment of comparative cost/benefit of histological, cytological and PCR diagnostic methods for identification of *Marteilia sydneyi*.
3. Production of an Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) for *Marteilia sydneyi*.

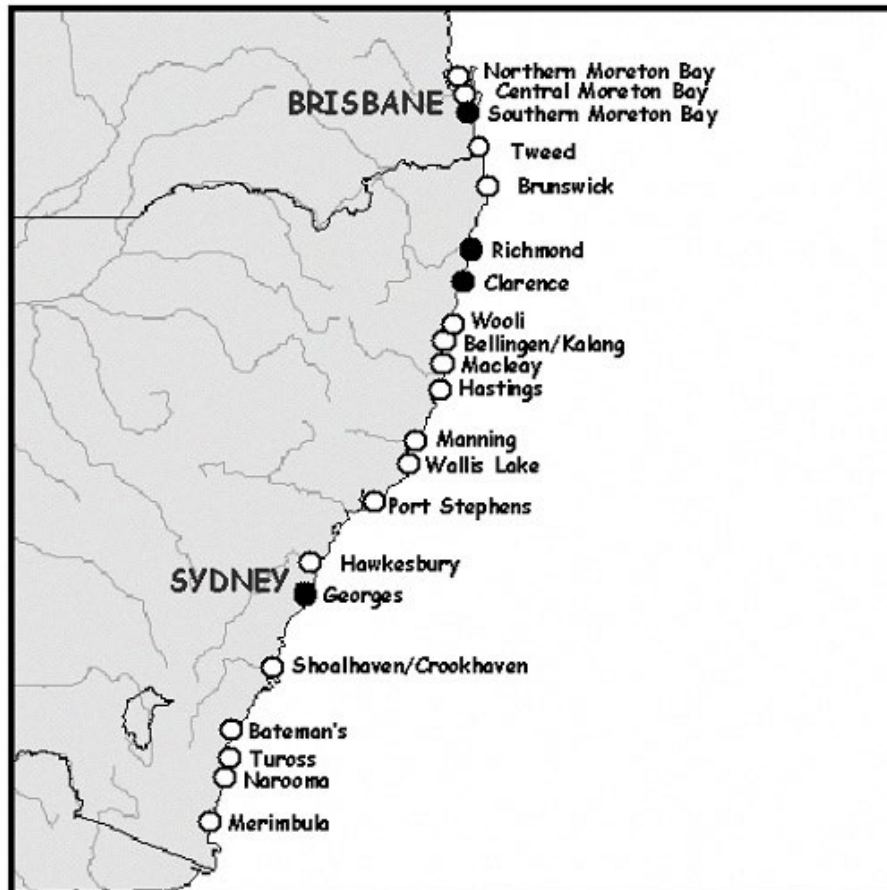
## **METHODS:**

### *Sampling and specimen selection:*

Oysters collected under project FRDC2001/214 from 18 estuaries in NSW and 3 areas in SE Queensland were screened by microscopic examination of tissue imprints and the disease status recorded under that project. All specimens were given a unique identifier against which collection data were recorded. Tissue samples from each oyster were preserved in 100% ethanol for DNA extraction and 10% buffered formalin for histological examination if required. These specimens then became the basis for validating the PCR diagnostic test for *Marteilia sydneyi*.

Figure 1 shows collection areas from which 4 groups of specimens were selected on disease status. Group 1: Known QX disease-positive oysters from Southern Moreton Bay (N=2), Clarence River (N=55), Richmond River (N=102) and Georges River (N=40) (i.e. positive oysters, total N=199); Group 2: presumed negative oysters from estuaries known to be positive for QX disease - Southern Moreton Bay, Clarence River, Georges River (N=100 from each estuary), (i.e. negatives from positive

estuaries, total N=300); Group 3: presumed negative oysters from estuaries with no record of QX disease – Merimbula (N=140), Narooma (N=100), Tuross (N=100), Bateman's Bay (N=100), Crookhaven/Shoalhaven (N=100), Hawkesbury River (N=100), Port Stephens (N=100), Wallis Lake (N=100), (i.e. negatives from negative estuaries, total N=840); Group 4: strategic estuaries sampled in response to results from Group 3 above – Hastings River (N=100), Brunswick River (N=100), Macleay River (N=100), Northern Moreton Bay (N=100), Central Moreton Bay (N=100), i.e. presumed negative oysters from strategic estuaries, N=500).



*Figure 1: Estuaries in which oyster collection was undertaken, filled circles indicate estuaries in which recurrent epizootics of QX disease occur and in which microscopic examination of imprints in 2002 samples identified the presence of Marteilia sydneyi.*

#### *Presumptive Diagnosis – PCR screening:*

##### *1) DNA Extraction:*

Total genomic DNA was extracted from a small subsample of tissue excised from the digestive gland of each oyster. Extractions were performed using commercially available standard DNA tissue extraction kits (DNeasy Tissue Kit, Qiagen) but standard Proteinase-K digestion, phenol/chloroform extraction and ethanol precipitation as described in Kleeman and Adlard (2000) and Kleeman *et al.* (2002) may be substituted.



## 2) Polymerase Chain Reaction.(PCR)

- a) Double stranded PCR reactions were performed on all DNA extracts using *Marteilia sydneyi* specific primers which target an ~200 base pair (bp) section of ITS1 (internal transcribed spacer) (see Table 1). For each batch of PCR reactions performed, a negative control i.e. a PCR reaction containing no DNA template, and a positive control i.e. a PCR reaction containing DNA from a known *Marteilia sydneyi* infected sample, were included. The former is run to ensure that the presence of a amplified product was not due to contamination of the PCR reagents, and the latter to ensure that the absence of any amplified product was not a result of a failed reaction.

**Table 1 – *Marteilia sydneyi* ITS1 PCR Primers**

<b>Primer Name</b>	<b>Sequence 5'-3'</b>	<b>Source/Ref</b>
LEG1 (forward)	CGA TCT GTG TAG TCG GAT TCC GA	Kleeman and Adlard, 2000
PRO2 (reverse)	TCA AGG GAC ATC CAA CGG TC	Kleeman and Adlard, 2000

- b) Reaction parameters were initially taken from Kleeman & Adlard (2000) where the environment was based on research outcomes. In this project we extended that research and aimed to maximise the cost/benefit of PCR tests for the presence of *M. sydneyi* under a standard diagnostic regime, where PCR tests could be applied potentially to routine surveillance for QX disease. As such, reaction parameters were optimised to minimise cost without compromising the efficacy of the test.

Using a subset (n=7) of marteiliosis positive DNA samples, threshold detection and optimisation of the original PCR protocol developed by Kleeman and Adlard (2000) was investigated. According to this protocol PCR amplifications were originally performed in a 50µl reaction volume under the following conditions, expressed as final concentrations:

1x *Taq* polymerase buffer;  
2mM dNTPs;  
2mM MgCl<sub>2</sub>;  
1µM each primer;  
3 units *Taq* polymerase

The thermal cycling parameters were as follows: denature at 95°C for 1 minute; anneal at 55°C for 1 minute; extend at 72°C for 1 minute, repeated for 30 cycles with a final extension at 72°C for 7 minutes (Kleeman and Adlard, 2000).

The lower threshold limits of each of the key reagents and reaction parameters of the protocol was realised by altering each component individually while holding all other parameters constant. These lower threshold limits were defined as the concentration of reagent at which a PCR product was no longer successfully amplified or whereby the yield was such that the results could be considered unreliable (see results).

## 3) Gel Electrophoresis

The examination for the presence or absence of an amplified product (presumptive diagnosis of *Marteilia sydneyi*) was conducted by electrophoresis of 10µl of PCR product through submarine 1.4% (w/v) agarose gels and examined and

photographed under ultra-violet light. A molecular weight standard (100bp DNA ladder, MBI Fermentas) was used to estimate the size of the products.

## RESULTS:

### *Optimisation and Threshold Detection Experiments.*

The original protocol (Kleeman & Adlard, 2000) was run in a reduced total reaction volume of 25 $\mu$ l, currently a more widely accepted reaction volume for PCR. Following successful amplification under these conditions, the duration of each cycle parameter was reduced from 1 minute to 30 seconds and a further 5 cycles added according to standard PCR protocols. The use of a hot start Taq polymerase dictated an initial denaturation cycle of 95°C for 10 minutes which was included into the cycling parameters. Again successful amplification was achieved in all samples. Subsequently, all reagent titration experiments were performed using the new final 25 $\mu$ l reaction volume and the new thermal cycling conditions. After experimental results were assessed from the titration of each reagent, the chosen optimised value of that reagent was used in the subsequent threshold experiments (see Tables 2-5).

*Table 2: Results of titration experiments of Taq polymerase (\* indicates the concentration of reagent selected for use in the optimised protocol).*

Reagent	Final concentration per 25 $\mu$ l reaction	Results (n=7 samples)
Taq polymerase Applied Biosystems Hot Start AmpliTaq Gold	3U (original protocol)	7/7
	1U	7/7
	0.75U*	7/7
	0.5U	7/7 decrease in yield
	0.25U	5/7
	0.1U	1/7

*Table 3: Results of titration experiments of oligonucleotide primers (\* indicates the concentration of reagent selected for use in the optimised protocol).*

Reagent	Final concentration per 25 $\mu$ l reaction	Results (n=7 samples)
Primers (each)	1 $\mu$ M (original protocol)	7/7
	0.5 $\mu$ M	7/7
	0.4 $\mu$ M*	7/7
	0.3 $\mu$ M	7/7 decrease in yield
	0.2 $\mu$ M	7/7 decrease in yield
	0.1 $\mu$ M	5/7
	0.05 $\mu$ M	2/7

*Table 4: Results of titration experiments of di-nucleotide tri-phosphates (A,C,G,T) (\* indicates the concentration of reagent selected for use in the optimised protocol).*

Reagent	Final concentration per 25µl reaction	Results (n=7 samples)
dNTPs	2mM (original protocol)	7/7
	1 mM	7/7
	0.75 mM (0.8mM*)	7/7
	0.5 mM	7/7 slight decrease in yield
	0.25 mM	7/7 slight decrease in yield
	0.1 mM	7/7 slight decrease in yield
	0.075 mM	6/7 large decrease in yield
	0.05 mM	5/7 decrease in yield
	0.025 mM	0/7
	0.01 mM	0/7

*Table 5: Results of titration experiments of MgCl<sub>2</sub> (\* indicates the concentration of reagent selected for use in the optimised protocol).*

Reagent	Final concentration per 25µl reaction	Results (n=7 samples)
MgCl <sub>2</sub>	2.0 mM* (original protocol)	7/7
	1.5 mM	7/7
	1.0 mM	5/7
	0.5 mM	0/7
	0 mM	0/7

In conjunction with the MgCl<sub>2</sub> titrations, PCR amplifications were run across a 20 degree temperature gradient (Gradient Thermal Cycler, Corbett Research) consisting of 12 different temperature points ranging from 45°C to 65°C (i.e. 45.0, 47.0, 48.8, 50.7, 52.5, 54.1, 55.8, 57.5, 59.2, 61.1, 63.0, 65.0°C). Amplification in the annealing temperature range 45-48.8°C was inconsistent, 50.7-59.2°C was consistent for all samples and showed normal yields, 61.1-65.0°C was consistent but yields were reduced (qualitative assessment from amplified product intensity on gels). These results indicated that no change in the 55°C annealing temperature nor the magnesium chloride concentration described in the original protocol would be required.

The cost/benefit result of these optimisation experiments was a four-fold reduction in expense of individual PCR tests from approximately \$2.80 to \$0.70 per sample (based on current 2003 costs). Subsequent validation was undertaken on a total of 1,839 oysters representing 17 different locations in NSW and SE Queensland (see Fig 1, Appendix 2 for raw data) and was conducted under the newly developed, optimised protocol detailed below.

### **Optimised PCR protocol:**

All PCR reactions, including the controls, are carried out in 25µl volume under the following reaction conditions:

#### *Reagents*

<b>PCR reagents/sample</b>	<b>Volume</b>	<b>Final conc./sample</b>
Water (molecular biology grade)	14.35µl	
10x <i>Taq</i> polymerase buffer	2.5µl	1x
10mM dNTPS	2.0µl	0.8mM
10µM Primer LEG1	1.0µl	0.4µM
10µM Primer PRO2	1.0µl	0.4µM
25mM MgCl <sub>2</sub>	2.0µl	2.0mM
<i>Taq</i> polymerase	0.15µl	0.75 Units
Genomic DNA template	2.0µl	20-50ng

#### *PCR Cycling Parameters*

The *Taq* polymerase used in the optimised reaction protocol is a hot-start polymerase (e.g. Applied Biosystems *AmpliTaq* Gold) that requires a 5-10 minute initial denaturation cycle prior to the commencement of the remaining cycle parameters.

- a) Initial denaturation 95°C for 10 min.
- b) Denaturation .....95°C for 30 sec  
   Annealing .....55°C for 30 sec  
   Extension .....72°C for 30 sec } x 35 cycles
- c) Final Extension..... 72°C for 5 min  
   .....22°C for 30 sec

#### *Field validation of PCR protocol*

PCR was applied to all individual oysters representing the four experimental Groups (Table 6). Oysters which had been identified as positive through microscopic identification of *M. sydneyi* (Group 1), returned 2 false negative results in PCR of the total 199 known positive individuals tested. The inconsistency of this result may be explained either by degeneration of parasite DNA through autolytic processes (i.e. the tested oysters were in advanced stages of post-mortem degradation) and/or the tissue excised for DNA extraction did not contain oyster digestive gland or other infected tissues. The former hypothesis can be easily overcome by rejecting from testing oysters that are dead, while the latter can be avoided by sub-sampling of digestive gland tissue prior to preservation (and concomitant bleaching of the tissues) in ethanol.

A third potential hypothesis that '*the PCR test is not as sensitive as microscopic examination*' was discarded based on the overwhelming evidence provided by presumed negative (through microscopic examination, Groups 2, 3 and 4) oysters.

*Table 6: Summary results of PCR tests for 4 experimental groups of oyster samples.*

<b>Group</b>	<b>Designation</b>	<b>Total Oysters Screened</b>	<b>Positive PCR Results</b>
1	Known QX positives	N = 199	N = 197
2	Negative oysters from QX positive estuaries	N = 300	N = 25
3	Negative oysters from QX negative estuaries	N = 840	N = 58
4	Strategic response estuaries	N = 500	N = 59
		Total N=1,839	Total N=339

A total of 300 oysters from Group 2 were tested by PCR to determine whether the detection limits of microscopic examination from estuaries in which the disease occurs were at a level which detected all infections. A further 25 oysters proved to be positive in PCR tests from this Group, suggesting that infection intensity in these individuals was too low to be identified through microscopic examination.

Finally, in presumed negative Groups 3 and 4, 117 oysters representing 13 different localities (see Table 7) proved to be positive for *M. sydneyi* in PCR of a total 1,340 oysters tested. To prevent diagnostic acceptance of false positives, a subset of these oysters were sectioned for histological examination. All showed varying degrees of inflammation throughout the connective tissue and associated with the digestive epithelia. Pathological abnormalities were consistent with, but not specifically indicative of, infection with *M. sydneyi*. DNA probe in-situ hybridisation (ISH) following the technique described in Kleeman, Adlard & Lester (2002) was applied to these samples and early stages of *M. sydneyi* were then observed in connective tissue surrounding the digestive tubules. Their identity was confirmed by Dr Sarah Kleeman (Biosecurity Australia).

*Table 7: Breakdown of oyster cultivation area against results of cytological and PCR diagnostic tests for *Marteilia sydneyi*.*

<b>Estuary</b>	<b>Group Assignment</b>	<b>Imprint Positives</b>	<b>PCR Positives</b>
Southern Moreton Bay	1 and 2	2	18
Central Moreton Bay	4	0	6
Northern Moreton Bay	4	0	7
Clarence River	1 and 2	55	62
Georges River	1 and 2	40	40
Richmond River	1	102	102
Merimbula	3	0	1
Narooma	3	0	25
Tuross Lake	3	0	19
Bateman's Bay	3	0	4
Crookhaven/Shoalhaven	3	0	0
Hawkesbury River	3	0	0
Port Stephens	3	0	2
Wallis Lake	3	0	7
Hastings River	4	0	3
Brunswick River	4	0	8
Macleay River	4	0	35

### Cost/Benefit Analysis

The cost of consumables for an individual PCR test (from subsample of tissue, includes DNA extraction) is calculated at \$5.75/oyster with this figure being exclusive of time or capital costs. A similar calculation for the cost of consumables for producing stained tissue imprints of oyster digestive gland is \$0.36/oyster. To allow direct cost/benefit comparison of cytology, PCR and histology (which is out-sourced from the Queensland Museum to veterinary pathology at the University of Queensland) the calculations in Table 8 are based on batches of 150 oysters (sample size aligned to allow detection at 2% prevalence). Note that costs for time (Table 8) are based on actual hands-on processes (e.g. PCR machine run-time not included) and hourly rates are current Queensland Museum rates calculated on basic technical ability for preparation of imprints (casual rate \$22.39/hr), higher technical ability for DNA extraction and PCR processes (casual rate \$27.82/hr) and professional expertise in identifying disease agents through microscopy (casual rate \$37.23/hr). These calculations assume that appropriately trained staff are available; they do not include infrastructure or administrative on-costs which vary between institutions and should be used for comparative purposes only.

*Table 8: Estimated comparison of costs for cytology (tissue imprints), histology and PCR based diagnostic testing of a batch of 150 oysters.*

	Cytology	Histology	PCR
Consumables	\$54.00	-	\$862.50
Cost/Time to product (includes time or outsource cost for histology)	8.125hrs @\$22.39/hr =\$181.92	150 @\$10/block =\$1,500.00	12.5hrs @\$27.82/hr =\$348
Minutes to result	12.5hrs @\$37.23/hr =\$465.37	12.5hrs @\$37.23/hr =\$465.37	-
Total Cost for comparison	\$701.29	\$1,965.37	\$1,210.25

Cytological diagnosis costs only 58% of that using PCR tests, however, the benefit of PCR tests has been demonstrated through this project (and FRDC2001/214) by the identification of *Marteilia sydneyi* in oyster samples from an extra 13 estuaries presumed to be negative (= below detectable limits) from cytological examination. As such, we would recommend the adoption of PCR as a presumptive test for QX disease.

### CONCLUSION:

Through our optimisation of the original PCR test for *Marteilia sydneyi* provided by Kleeman & Adlard (2000) and its validation against field samples, we are confident that the resultant protocol is appropriate for use as a standard diagnostic procedure for QX disease. Cost/benefit analysis supports PCR testing as a preferred diagnostic procedure in surveillance activities. It is further recommended that confirmatory diagnosis based on DNA probe in situ hybridisation (ISH) or transmission electron microscopy (see OIE Diagnostic Manual) be undertaken on a subset of PCR positive samples from any one zone/area regardless of a lack of amplification in standard negative controls. See Appendix 1 for a full Standard Diagnostic Procedure.

### BENEFITS:

The outcomes of this research will benefit directly the edible oyster industry in New South Wales and Queensland, by providing a validated and sensitive technique for detection of *Marteilia sydneyi*. The scientific benefit of this diagnostic technique has already been demonstrated even during the validation process, by offering novel insights into the biology and distribution of the disease.

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## APPENDIX 1.

Australian and New Zealand Standard  
Diagnostic Procedure:  
QX Disease (Infection with *Marteilia  
sydneyi*)

**DRAFT FOR ENDORSEMENT**



## QX Disease

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### Summary

*QX disease (marteiliosis) of Australian commercial rock oysters (Saccostrea glomerata) is caused by the protistan parasite Marteilia sydneyi (Phylum Paramyxea). It infects oysters through the epithelium of the gills and palps, is found as a transient stage in the connective tissue surrounding the gut and gonads of the oyster, and sporulates in the epithelium of the digestive tubules.*

*The disease is associated with rapid loss of condition in the oyster, resorption of gonad, and disruption of the cellular integrity of the digestive tubules resulting in oyster death due to starvation.*

*QX disease is seasonal, with initial infections occurring in a short 'pulse' of 2-4 weeks duration in the southern mid to late summer (January-February). Two proliferative phases follow infection, the first in the gills and palps, and the second in the basal lamina of the epithelial cells of the digestive tubules, finally producing sporonts containing 2 tricellular spores. The complete lifecycle of Marteilia sydneyi is unknown but the presence of an alternate host is strongly suspected. It has not been possible to transmit QX disease directly between oysters.*

**Identification of the agent:** *Marteilia sydneyi can be detected in tissue imprints, histological preparations and through PCR diagnostic tests with specific confirmation provided by DNA probe in situ hybridisation or transmission electron microscopy.*

**Status of Australia and New Zealand:** *M. sydneyi is endemic to some areas of rock oyster culture on the east coast of mainland Australia and has been found in naturally recruited S. glomerata on the east coast and at a low prevalence in natural beds in Western Australia. There have been no reports of M. sydneyi from New Zealand.*

### **Significance**

QX disease, or marteiliosis of the Sydney Rock Oyster (*Saccostrea glomerata*), is caused by the protistan parasite, *Marteilia sydneyi* (Phylum Paramyxea). The disease typically results in serious and seasonally recurrent mortalities in farmed and wild rock oysters in estuaries of eastern Australia. In severe epizootics the disease has resulted in stock mortalities exceeding 90% on some aquaculture sites (Nell & Smith, 1988). It is listed as a notifiable disease by the Office International des Epizooties (OIE) and is included on the Australian National List of Reportable Disease of Aquatic Animals.

### **Aetiology**

In the oyster, infection is through the epithelium of the gills and palps in which proliferation occurs followed by a short transient phase in the connective tissues then establishment in the basal lamina of the epithelial cells of the digestive tubules (Kleeman, Adlard & Lester, 2002). Sporulation occurs in these tissues and may persist for 6-12 weeks prior to oyster death. In some specimens spores may be identified for months after initial infection (Adlard, pers obs.).

### **Geographic distribution**

QX disease has been identified in estuaries extending from the Great Sandy Strait (25°30'S) in southern Queensland, to the Macleay River (31°S) in northern New South Wales with the last reported extension in its southern range occurring in the Georges River estuary (34°S) in 1994 (Adlard & Ernst, 1995). It has also been identified from natural populations of *S. glomerata* in Western Australia at low prevalence (Hine, pers comm).

### **Limitation statement**

Discussion in this paper will be restricted to QX Disease (marteiliosis) of *Saccostrea glomerata* in Australia. A related pathogen, *Marteilia refringens* (Aber Disease) the type species of the genus, is a lethal parasite of the European flat oyster, *Ostrea edulis*. Other species of the genus occur in a range of invertebrates, mostly identified from Europe. Further information on these species may be found in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE, 2000).

### **The Disease**

#### **Host range:**

QX Disease has been unambiguously identified only from the Sydney Rock Oyster, *Saccostrea glomerata* (synonyms – *Saccostrea commercialis*, *Crassostrea commercialis*) and is a lethal parasite of this oyster. Wolf (1979) speculated that *M. sydneyi* may also infect *Saccostrea echinata* since the range of this oyster overlapped with that of *S. glomerata*, however he reported only that 'cells resembling *M. sydneyi* were found in one *S. echinata*' and the report remains unconfirmed.

There has been much speculation and some scientific evidence (e.g. absence of transmission from oyster to oyster) that an alternate (or intermediate) host forms part of the lifecycle of *Marteilia sydneyi*. The most compelling evidence for the existence of a second host was provided by Audemard *et al.* (2002) who showed the involvement of the copepod, *Paracartia grani*, in the lifecycle

of *Marteilia refringens* in Europe. A second host for *M. sydneyi* remains to be identified.

### **Clinical signs**

Infection in QX disease outbreaks is associated with poor condition and emaciation of infected oysters, resorption of gonads, discolouration of the digestive gland (pale in terminal stage sporulation), cessation of growth, and mortality. In the final stages of parasite development within the oyster, secondary infection with bacteria is relatively common and presents as abscesses on gills and mantle with infected oysters appearing typically thin and translucent.

### **Occurrence – seasonality**

Contrary to early reports (Wolf, 1979) QX disease shows a strongly seasonal infection pattern. Initial infections occur in the southern mid to late summer (Adlard & Lester, 1996; Kleeman, Adlard & Lester, 2002) and are of short duration (2-4 weeks), which affords a local disease management opportunity with stock being brought into susceptible locations after the infection risk period. Once infected, QX disease develops in the oyster in the gills and palps, connective tissue, and finally the epithelium of the digestive tubules with spores released prior to oyster death. The time to death post-infection can vary from 2-4 months with spores being recognisable in some individuals as late as 6-7 months post-infection.

### **Pathology**

Initial infection and development of *M. sydneyi* in the basal lamina of the epithelium of the gills and palps of the oyster results in epithelial hyperplasia associated with an infiltration of haemocytes in the adjacent connective tissue. Hypertrophy and fusion of gill filaments may also be observed (Kleeman, Adlard & Lester, 2002).

In contrast, little host reaction was associated with the presence of individual parasites in the connective tissue of the palps, gonad, mantle and digestive gland. However, development of parasites in the basal lamina of the digestive tubules produced both focal and extensive haemocytic infiltration, with epithelial cells tending cuboidal and metaplastic. Diapedesis of haemocytes through the epithelium of the digestive gland is common in intermediate stages of disease progression with host reaction subsiding as development reaches more terminal stages (sporulation).

## **Diagnostic tests**

### **Specimens and fixation required**

Specimens should be collected using a statistically robust sampling regime. Currently, the collection of 150 oysters selected at random by GPS generated sites within digitised oyster growing areas of an estuary (zone) provides the level of detection of 2% or greater population prevalence stipulated by the OIE (Angus Cameron, AusVet).

Collected specimens should be prepared by dissection of the oyster into 2 longitudinal halves in a plane parallel with the oyster valve margins, bisecting the palps and gills, such that representative samples of all tissues occur in each half. This allows processing and fixation of the oyster tissue without exclusion of any of the available diagnostic techniques.

### **Wet preparations**

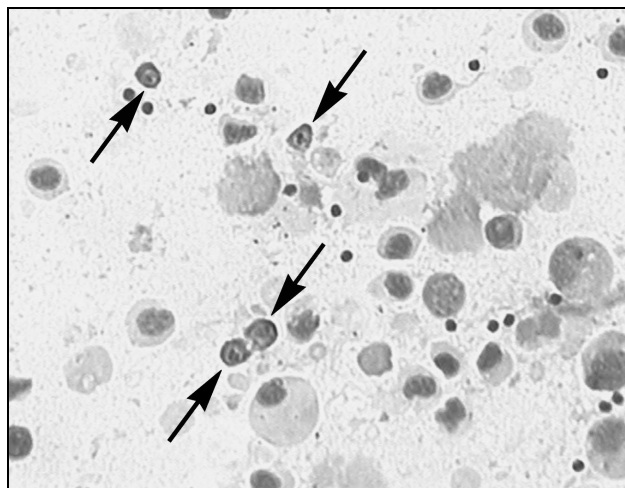
Traditionally, wet preparations of oyster digestive gland were made by squashing a small piece of tissue under a glass cover-slip on a microscope slide. This technique can provide a simple and rapid diagnosis of terminal stage infections by the identification of distinctive refringent granules and spores within sporonts. The technique is limited in its resolution and can only be applied to terminal stage infections with any confidence, as such it is not recommended for surveillance programs.

### **Cytological examination - tissue imprints**

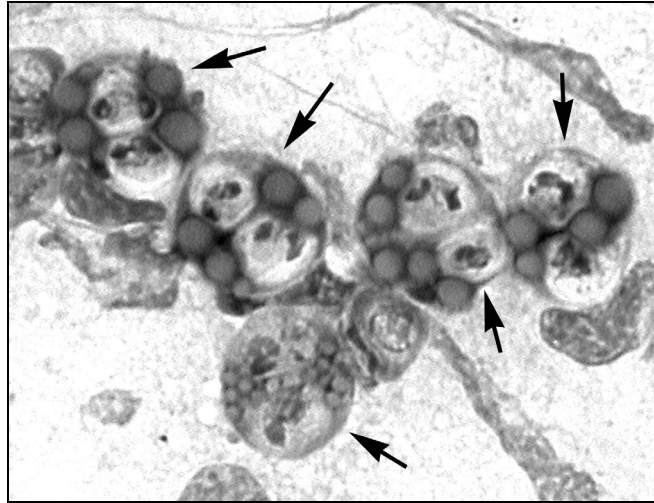
Imprints are prepared by excision of approximately a 2mm<sup>3</sup> piece of digestive gland (from one half of the bisected oyster – see above), remove excess water by blotting the sample on absorbent paper, then make a series of imprints against a microscope slide sufficient to fill the area of a 20x40mm cover slip. The slides are air-dried and then fixed in methanol (2-3 minutes). The samples are stained using a commercially available staining kit for blood cells (e.g. Hemacolor, Merck) in accordance with the manufacturer's instructions. After staining, rinse in tap water, allow to dry completely and mount with a cover-slip using an appropriate synthetic resin.

The parasite is 3-4 µm in size in early uninucleate/daughter cell stages (Figure 1) and may reach up to 30 µm during sporulation (Figure 2). The cytoplasm of the cells stains densely basophilic, the nucleus is eosinophilic. An observation time of 10 minutes per slide is considered sufficient to identify these stages.

Cytological examination of tissue imprints is recommended as a presumptive test for maritelliosis by the OIE Diagnostic Manual for Aquatic Animal Disease (OIE, 2000).



**Figure 1:** *Uninucleate cells of M. sydneyi (arrows) in stained tissue imprint (mag x400) - note peripheral cytoplasm is dense and darkly stained.*



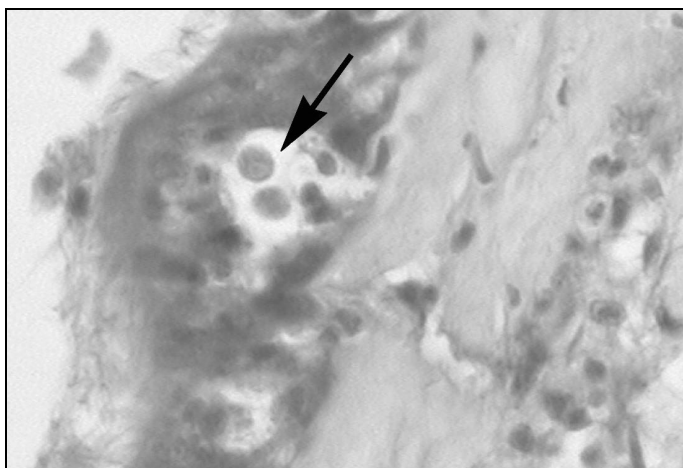
**Figure 2:** Stained tissue imprint of terminal stage infection. Two tricellular spores of *M. sydneyi* developing within each sporont (arrows). Note spherical refringent granules within sporont membrane (mag x1000).

### **Histopathology**

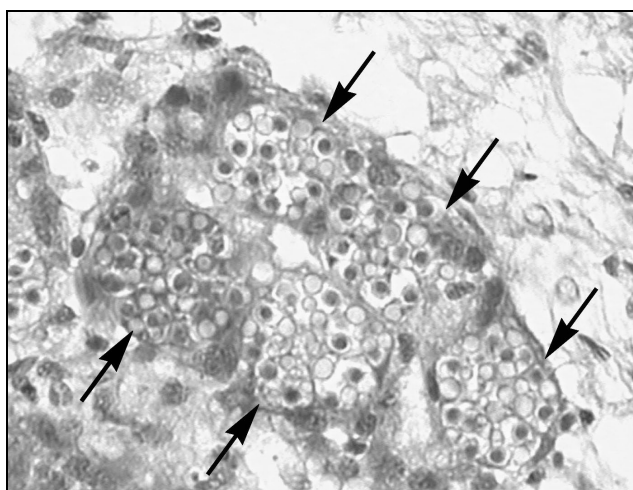
One half of the bisected oyster should be preserved in formalin (10%v/v) made up in artificial seawater for histology and/or DNA probe in situ hybridisation (ISH) if required for confirmatory diagnosis. Note that neither Davidson's nor Carson's fixatives (see OIE, 2000) are recommended if confirmatory diagnosis is to be based on ISH.

The samples are subsequently handled in accordance with classical histological methods, paraffin embedding, 5-7 $\mu$ m sectioning, staining with haematoxylin/eosin (see Chapter 1.2. of OIE Diagnostic Manual for Aquatic Animal Disease, 2000).

Early developmental stages of *Marteilia sydneyi* are present in the epithelia of the gills and palps and in the connective tissue surrounding the digestive glands (Figure 3). Later developmental stages can be found in the epithelia of the digestive tubules where sporonts containing 2 spores and distinctive refringent bodies develop within plasmodia (Figure 4). The unique feature of internal cleavage to produce cells within cells during sporulation differentiates *Marteilia* spp. from all other protista (OIE, 2000).



**Figure 3: Haematoxylin and eosin stained tissue section showing early stage infection (arrow) of *M. sydneyi* in the epithelium of the gills (mag x1000)**



**Figure 4: H&E stained tissue section showing plasmodia (arrows) in the epithelium of the digestive tubules of *S. glomerata*. Within the plasmodia are developing sporonts and spores of *M. sydneyi*. Late stage infection (mag x400).**

### **PCR standard protocol**

The following standard PCR protocol, which targets an approximately 200bp region of the ITS1 of rDNA, has proven to be the most sensitive test for the presence of *M. sydneyi*, will detect the disease at subclinical levels, and is recommended for presumptive diagnosis of QX disease in surveillance programs. It has been shown to be specific for *Marteilia sydneyi* when compared against congener *M. refringens* and member of the sister genus *Marteiliodes chungmuensis* (see Kleeman, Le Roux, Berthe & Adlard, 2002).

Excise approximately a 2mm<sup>3</sup> piece of digestive gland from the remaining bisected half oyster and place directly into DNA extraction buffer (50mM Tris pH8, 5mM EDTA pH8, 100mM NaCl) in a micro-centrifuge tube. The remainder of this sample should be preserved in 90-100% (v/v) ethanol as a DNA archive.

### **DNA extraction**

Total genomic DNA is extracted from sub-samples of digestive gland tissue from each oyster. Extractions may be performed using either commercially available standard DNA tissue extraction kits (e.g. DNeasy Tissue Kit, Qiagen) or standard Proteinase-K digestion, phenol/chloroform extraction and ethanol precipitation as described in Kleeman and Adlard (2000) and Kleeman, Adlard & Lester (2002).

#### *PCR protocol*

For each batch of PCR reactions performed, a negative control (i.e. a PCR reaction containing no DNA template), and a positive control (i.e. a PCR reaction containing DNA from a known *Marteilia sydneyi* infected sample), should be included. The former to ensure that the presence of an amplified product is not due to contamination of the PCR reagents, and the latter to ensure that the absence of any amplified product is not a result of a failed reaction.

All PCR reactions, including the controls, are carried out in 25µl volume under the following reaction conditions:

#### *Reagents*

PCR reagents/sample	Volume	Final conc./sample
Water (molecular biology grade)	14.35µl	
10x <i>Taq</i> polymerase buffer	2.5µl	1x
10mM dNTPS	2.0µl	0.8mM
10µM Primer LEG1	1.0µl	0.4µM
10µM Primer PRO2	1.0µl	0.4µM
25mM MgCl <sub>2</sub>	2.0µl	2.0mM
<i>Taq</i> polymerase	0.15µl	0.75 Units
Genomic DNA template	2.0µl	20-50ng

#### *Primer sequences (ITS1 region)*

The following primers have been shown to confer species level specificity to the PCR test (see Kleeman & Adlard, 2000):

Primer Name	Primer direction	Primer Sequence 5'-3'
LEG1	forward	CGA TCT GTG TAG TCG GAT TCC GA
PRO2	reverse	TCA AGG GAC ATC CAA CGG TC

#### *PCR Cycling Parameters*

The *Taq* polymerase used in the optimised reaction protocol is a hot-start polymerase (e.g. Applied Biosystems *AmpliTaq* Gold) that requires a 5-10 minute initial denaturation cycle prior to the commencement of the remaining cycle parameters.

- a) Initial denaturation 95°C for 10 min.

- b) Denaturation 95°C for 30 sec  
 Annealing ... 55°C for 30 sec  
 Extension .... 72°C for 30 sec } x 35 cycles
- c) Final Extension 72°C for 5 min  
 ..... 22°C for 30 sec

#### *Visualising amplified products*

The examination for the presence or absence of an amplified product of approximately 200bp in length (presumptive diagnosis of *Marteilia sydneyi*) is conducted by electrophoresis of 10µl of PCR product through submarine agarose gels (1.4% w/v) and examined and photographed under ultra-violet light. A molecular weight standard (e.g. 100bp DNA ladder, MBI Fermentas) should be used to estimate the size of any products.

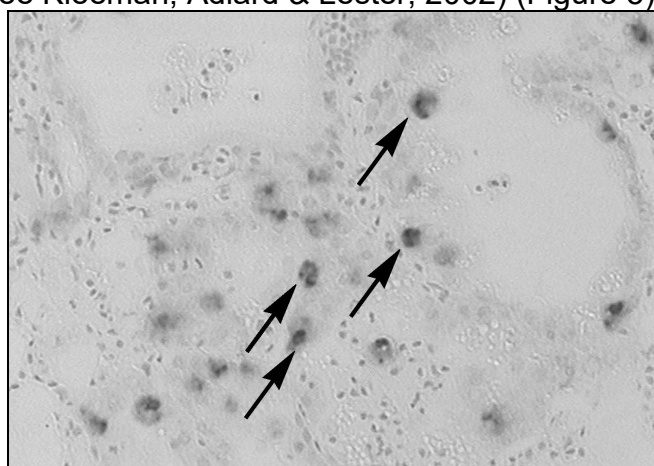
### **Confirmatory testing**

#### **In situ DNA probe hybridisation**

It is recommended that the DNA probe developed within the internal transcribed spacer region (ITS1) of the rDNA gene cluster be used for in situ hybridisation tests (see protocol detailed in Kleeman & Adlard, 2000) as confirmation of the specific identity of *Marteilia sydneyi*.

ISH should be carried out for specific confirmation on a subset of PCR, histological or cytological positive samples from any one zone/area. PCR positives should not be accepted as valid, regardless of a lack of amplification in standard negative controls, until available confirmatory tests have been undertaken. Subsequent histological examination may provide histopathological signs consistent with infection by *M. sydneyi* but may fail to identify unambiguously the presence of the parasite.

ISH will provide visualisation of individual parasites at all stages of development (see Kleeman, Adlard & Lester, 2002) (Figure 5).



**Figure 5:** DNA probe in situ hybridisation to *M. sydneyi* stages (arrows) during initial phase of infection in the epithelium of the digestive tubules of *S. glomerata*. Counterstain Bismark Brown (mag x200).

### **Transmission electron microscopy**



The OIE Diagnostic Manual for Aquatic Animal Disease (OIE, 2000) recommends transmission electron microscopy for confirmatory diagnosis of *Marteilia* spp. (see Chapter I.2. of the Manual). *Marteilia sydneyi* can be differentiated from *M. refringens* by a lack of striated inclusions in the plasmodia, formation of eight to sixteen sporangial primordia in each plasmodium (instead of eight for *M. refringens*) (OIE, 2000). In contrast to *Marteilia refringens* (Aber disease of the European flat oyster) in which sporonts contain 4 spores, *M. sydneyi* develops sporonts with consistently only 2 spores, a specific diagnostic feature also observable in well-prepared and stained tissue imprints.

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# APPENDIX 2.

## Staff List

Dr Robert D. Adlard – Principle Investigator

Dr Jessica Worthington Wilmer – Research Officer (this project)

Dr Stephen Wesche – Research Officer (FRDC2001/214)

Dr Malcolm Bryant – Senior Museum Technician

## APPENDIX 3.

### DNA / PCR Screening Raw Data















Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	
Georges River (GR)	8459	positive	1x	multiple (optimisation)	positive		
Georges River (GR)	8465	positive	1x	multiple (optimisation)	positive		
Georges River (GR)	8474	positive	1x	1x	positive		
Georges River (GR)	8482	positive	1x	1x	positive		
Georges River (GR)	8483	positive	1x	1x	positive		
Georges River (GR)	8485	positive	1x	1x	positive		
Georges River (GR)	8487	positive	1x	1x	positive		
Georges River (GR)	8488	positive	1x	1x	positive		
Georges River (GR)	8491	positive	1x	1x	positive		
Georges River (GR)	8501	positive	1x	1x	positive		
Georges River (GR)	8504	positive	1x	2x	positive	positive	
Georges River (GR)	8508	positive	1x	3x	positive	positive	positive
Georges River (GR)	8515	positive	1x	1x	positive		
Georges River (GR)	8520	positive	1x	1x	positive		
Georges River (GR)	8523	positive	1x	1x	positive		
Georges River (GR)	8524	positive	1x	1x	positive		
Georges River (GR)	8539	positive	1x	1x	positive		
Georges River (GR)	8552	positive	1x	1x	positive		
Georges River (GR)	8555	positive	1x	1x	positive		
Georges River (GR)	8557	positive	1x	1x	positive		
Georges River (GR)	8566	positive	1x	1x	positive		
Georges River (GR)	8574	positive	1x	1x	positive		
Georges River (GR)	8584	positive	1x	2x	positive	positive	
Georges River (GR)	8593	positive	1x	1x	positive		
Georges River (GR)	8599	positive	1x	1x	positive		
Georges River (GR)	8602	positive	1x	2x	positive	positive	
Georges River (GR)	8607	positive	1x	1x	positive		
Georges River (GR)	8614	positive	1x	1x	positive		
Georges River (GR)	8617	positive	1x	1x	positive		
Georges River (GR)	10371	positive	1x	1x	positive		
Georges River (GR)	10373	positive	1x	1x	positive		
Georges River (GR)	10374	positive	1x	1x	positive		
Georges River (GR)	10382	positive	1x	1x	positive		
Georges River (GR)	10395	positive	1x	1x	positive		
Georges River (GR)	10413	positive	1x	1x	positive		
Georges River (GR)	10427	positive	1x	1x	positive		
Georges River (GR)	10432	positive	1x	1x	positive		
Georges River (GR)	10434	positive	1x	1x	positive		
Georges River (GR)	10446	positive	1x	1x	positive		
Georges River (GR)	10447	positive	1x	1x	positive		
Georges River (GR)	8458	negative	1x	1x	Negative		
Georges River (GR)	8460	negative	1x	1x	Negative		
Georges River (GR)	8461	negative	1x	1x	Negative		
Georges River (GR)	8462	negative	1x	1x	Negative		
Georges River (GR)	8463	negative	1x	1x	Negative		
Georges River (GR)	8466	negative	1x	1x	Negative		
Georges River (GR)	8467	negative	1x	1x	Negative		
Georges River (GR)	8468	negative	1x	1x	Negative		
Georges River (GR)	8469	negative	1x	1x	Negative		
Georges River (GR)	8470	negative	1x	1x	Negative		
Georges River (GR)	8471	negative	1x	1x	Negative		
Georges River (GR)	8472	negative	1x	1x	Negative		
Georges River (GR)	8473	negative	1x	1x	Negative		
Georges River (GR)	8475	negative	1x	1x	Negative		
Georges River (GR)	8476	negative	1x	1x	Negative		
Georges River (GR)	8477	negative	1x	1x	Negative		
Georges River (GR)	8478	negative	1x	1x	Negative		
Georges River (GR)	8479	negative	1x	1x	Negative		
Georges River (GR)	8480	negative	1x	1x	Negative		
Georges River (GR)	8481	negative	1x	1x	Negative		
Georges River (GR)	8484	negative	1x	1x	Negative		
Georges River (GR)	8486	negative	1x	1x	Negative		
Georges River (GR)	8489	negative	1x	1x	Negative		
Georges River (GR)	8490	negative	1x	1x	Negative		
Georges River (GR)	8492	negative	1x	1x	Negative		
Georges River (GR)	8493	negative	1x	1x	Negative		
Georges River (GR)	8494	negative	1x	1x	Negative		
Georges River (GR)	8495	negative	1x	1x	Negative		
Georges River (GR)	8496	negative	1x	1x	Negative		
Georges River (GR)	8497	negative	1x	1x	Negative		



Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result
Clarence River (CR)	5263	positive	1x	multiple (optimisation)	positive	
Clarence River (CR)	5266	positive	1x	multiple (optimisation)	positive	
Clarence River (CR)	5267	positive	1x	2x	positive	positive
Clarence River (CR)	5270	positive	1x	1x	positive	
Clarence River (CR)	5271	positive	1x	2x	positive	positive
Clarence River (CR)	5277	positive	1x	1x	positive	
Clarence River (CR)	5279	positive	1x	1x	positive	
Clarence River (CR)	5283	positive	1x	2x	positive	positive
Clarence River (CR)	5290	positive	1x	1x	positive	
Clarence River (CR)	5293	positive	1x	1x	positive	
Clarence River (CR)	5295	positive	1x	1x	positive	
Clarence River (CR)	5296	positive	1x	2x	positive	positive
Clarence River (CR)	5301	positive	1x	1x	positive	
Clarence River (CR)	5307	positive	1x	1x	positive	
Clarence River (CR)	5309	positive	1x	2x	positive	positive
Clarence River (CR)	5312	positive	1x	1x	positive	
Clarence River (CR)	5314	positive	1x	1x	positive	
Clarence River (CR)	5326	positive	1x	1x	positive	
Clarence River (CR)	5328	positive	1x	1x	positive	
Clarence River (CR)	5331	positive	1x	1x	positive	
Clarence River (CR)	5340	positive	1x	2x	positive	positive
Clarence River (CR)	5342	positive	1x	1x	positive	
Clarence River (CR)	5350	positive	2x	Extn 1 - 3x; extn 2 - 1x	negative	negative
Clarence River (CR)	5352	positive	1x	1x	positive	
Clarence River (CR)	5358	positive	2x	Extn 1 - 3x; extn 2 - 1x	negative	negative
Clarence River (CR)	5360	positive	1x	1x	positive	
Clarence River (CR)	5368	positive	1x	1x	positive	
Clarence River (CR)	5369	positive	1x	1x	positive	
Clarence River (CR)	5370	positive	1x	2x	positive	positive
Clarence River (CR)	5373	positive	1x	1x	positive	
Clarence River (CR)	5375	positive	1x	1x	positive	
Clarence River (CR)	5380	positive	1x	1x	positive	
Clarence River (CR)	5387	positive	1x	2x	positive	positive
Clarence River (CR)	5396	positive	1x	1x	positive	
Clarence River (CR)	5398	positive	1x	1x	positive	
Clarence River (CR)	5405	positive	1x	1x	positive	
Clarence River (CR)	5407	positive	1x	1x	positive	
Clarence River (CR)	5413	positive	1x	1x	positive	
Clarence River (CR)	5414	positive	1x	1x	positive	
Clarence River (CR)	5428	positive	1x	1x	positive	
Clarence River (CR)	5431	positive	1x	1x	positive	
Clarence River (CR)	5442	positive	1x	1x	positive	
Clarence River (CR)	5443	positive	1x	1x	positive	
Clarence River (CR)	5445	positive	1x	1x	positive	
Clarence River (CR)	5449	positive	1x	2x	positive	positive
Clarence River (CR)	5452	positive	1x	1x	positive	
Clarence River (CR)	5457	positive	1x	1x	positive	
Clarence River (CR)	5461	positive	1x	1x	positive	
Clarence River (CR)	5469	positive	1x	1x	positive	
Clarence River (CR)	5471	positive	1x	1x	positive	
Clarence River (CR)	5482	positive	1x	3x	positive	positive
Clarence River (CR)	5484	positive	1x	2x	positive	positive
Clarence River (CR)	5493	positive	1x	1x	positive	
Clarence River (CR)	5495	positive	1x	1x	positive	
Clarence River (CR)	5500	positive	1x	1x	positive	
Clarence River (CR)	5262	negative	1x	1x	negative	
Clarence River (CR)	5264	negative	1x	1x	negative	
Clarence River (CR)	5265	negative	1x	2x	positive	positive
Clarence River (CR)	5268	negative	1x	1x	negative	
Clarence River (CR)	5269	negative	1x	1x	negative	
Clarence River (CR)	5272	negative	1x	1x	negative	
Clarence River (CR)	5273	negative	1x	1x	negative	
Clarence River (CR)	5274	negative	1x	1x	negative	
Clarence River (CR)	5275	negative	1x	1x	negative	
Clarence River (CR)	5276	negative	1x	1x	negative	
Clarence River (CR)	5278	negative	1x	1x	negative	
Clarence River (CR)	5280	negative	1x	1x	negative	
Clarence River (CR)	5281	negative	1x	1x	negative	
Clarence River (CR)	5282	negative	1x	1x	negative	
Clarence River (CR)	5284	negative	1x	1x	negative	
Clarence River (CR)	5285	negative	1x	1x	negative	
Clarence River (CR)	5286	negative	1x	1x	negative	
Clarence River (CR)	5287	negative	1x	1x	negative	
Clarence River (CR)	5288	negative	1x	1x	negative	
Clarence River (CR)	5289	negative	1x	1x	negative	
Clarence River (CR)	5291	negative	1x	1x	negative	
Clarence River (CR)	5292	negative	1x	1x	negative	
Clarence River (CR)	5294	negative	1x	1x	negative	



Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	PCR after 2nd extn
Richmond River (RR)	6012	positive	1x	multiple (optimisation)	positive		
Richmond River (RR)	6014	positive	1x	multiple (optimisation)	positive		
Richmond River (RR)	6016	positive	1x	1x	positive		
Richmond River (RR)	6017	positive	1x	1x	positive		
Richmond River (RR)	6018	positive	1x	2x	negative	positive	
Richmond River (RR)	6022	positive	2x	3x	negative	negative	positive
Richmond River (RR)	6026	positive	1x	1x	positive		
Richmond River (RR)	6030	positive	1x	1x	positive		
Richmond River (RR)	6031	positive	1x	1x	positive		
Richmond River (RR)	6033	positive	1x	1x	positive		
Richmond River (RR)	6037	positive	1x	2x	negative	positive	
Richmond River (RR)	6040	positive	1x	1x	positive		
Richmond River (RR)	6041	positive	1x	1x	positive		
Richmond River (RR)	6042	positive	1x	1x	positive		
Richmond River (RR)	6043	positive	1x	1x	positive		
Richmond River (RR)	6045	positive	1x	multiple (optimisation)	positive		
Richmond River (RR)	6046	positive	1x	1x	positive		
Richmond River (RR)	6050	positive	1x	1x	positive		
Richmond River (RR)	6052	positive	1x	2x	negative	positive	
Richmond River (RR)	6054	positive	1x	1x	positive		
Richmond River (RR)	6055	positive	1x	2x	negative	positive	
Richmond River (RR)	6057	positive	1x	2x	negative	positive	
Richmond River (RR)	6062	positive	1x	1x	positive		
Richmond River (RR)	6064	positive	1x	1x	positive		
Richmond River (RR)	6065	positive	1x	2x	negative	positive	
Richmond River (RR)	6066	positive	1x	1x	positive		
Richmond River (RR)	6069	positive	1x	1x	positive		
Richmond River (RR)	6073	positive	1x	1x	positive		
Richmond River (RR)	6074	positive	1x	2x	negative	positive	
Richmond River (RR)	6076	positive	1x	2x	negative	positive	
Richmond River (RR)	6082	positive	1x	1x	positive		
Richmond River (RR)	6086	positive	1x	1x	positive		
Richmond River (RR)	6090	positive	1x	1x	positive		
Richmond River (RR)	6091	positive	1x	1x	positive		
Richmond River (RR)	6093	positive	1x	1x	positive		
Richmond River (RR)	6094	positive	1x	1x	positive		
Richmond River (RR)	6099	positive	1x	1x	positive		
Richmond River (RR)	6104	positive	1x	1x	positive		
Richmond River (RR)	6109	positive	1x	2x	negative	positive	
Richmond River (RR)	6111	positive	1x	1x	positive		
Richmond River (RR)	6112	positive	1x	1x	positive		
Richmond River (RR)	6117	positive	1x	2x	negative	positive	
Richmond River (RR)	6119	positive	1x	2x	negative	positive	
Richmond River (RR)	6120	positive	1x	1x	positive		
Richmond River (RR)	6124	positive	1x	2x	negative	positive	
Richmond River (RR)	6132	positive	1x	1x	positive		
Richmond River (RR)	6138	positive	1x	2x	negative	positive	
Richmond River (RR)	6141	positive	1x	1x	positive		
Richmond River (RR)	6143	positive	1x	1x	positive		
Richmond River (RR)	6147	positive	1x	1x	positive		
Richmond River (RR)	6148	positive	1x	1x	positive		
Richmond River (RR)	6150	positive	1x	1x	positive		
Richmond River (RR)	6152	positive	1x	1x	positive		
Richmond River (RR)	6167	positive	1x	1x	positive		
Richmond River (RR)	6168	positive	1x	1x	positive		
Richmond River (RR)	6171	positive	1x	2x	negative	positive	
Richmond River (RR)	6188	positive	1x	1x	positive		
Richmond River (RR)	6189	positive	1x	1x	positive		
Richmond River (RR)	6190	positive	1x	1x	positive		
Richmond River (RR)	6192	positive	1x	1x	positive		
Richmond River (RR)	6193	positive	1x	1x	positive		
Richmond River (RR)	6194	positive	1x	1x	positive		
Richmond River (RR)	6197	positive	1x	1x	positive		
Richmond River (RR)	6198	positive	1x	1x	positive		
Richmond River (RR)	6199	positive	1x	1x	positive		
Richmond River (RR)	6201	positive	1x	1x	positive		
Richmond River (RR)	6202	positive	1x	1x	positive		
Richmond River (RR)	6204	positive	1x	1x	positive		

Richmond River (RR)	6205	positive	1x	1x	positive		
Richmond River (RR)	6206	positive	1x	1x	positive		
Richmond River (RR)	6207	positive	1x	1x	positive		
Richmond River (RR)	6208	positive	1x	1x	positive		
Richmond River (RR)	6209	positive	1x	1x	positive		
Richmond River (RR)	6210	positive	1x	1x	positive		
Richmond River (RR)	6212	positive	2x	3x	negative	negative	positive
Richmond River (RR)	6214	positive	1x	1x	positive		
Richmond River (RR)	6216	positive	1x	1x	positive		
Richmond River (RR)	6217	positive	1x	1x	positive		
Richmond River (RR)	6219	positive	1x	1x	positive		
Richmond River (RR)	6221	positive	1x	1x	positive		
Richmond River (RR)	6226	positive	1x	1x	positive		
Richmond River (RR)	6229	positive	1x	1x	positive		
Richmond River (RR)	6232	positive	1x	1x	positive		
Richmond River (RR)	6236	positive	1x	1x	positive		
Richmond River (RR)	6237	positive	1x	1x	positive		
Richmond River (RR)	6241	positive	1x	1x	positive		
Richmond River (RR)	6242	positive	1x	1x	positive		
Richmond River (RR)	6243	positive	1x	1x	positive		
Richmond River (RR)	6244	positive	1x	1x	positive		
Richmond River (RR)	6245	positive	1x	1x	positive		
Richmond River (RR)	6246	positive	1x	1x	positive		
Richmond River (RR)	6248	positive	1x	1x	positive		
Richmond River (RR)	6249	positive	1x	1x	positive		
Richmond River (RR)	6250	positive	1x	1x	positive		
Richmond River (RR)	6251	positive	1x	1x	positive		
Richmond River (RR)	6252	positive	1x	1x	positive		
Richmond River (RR)	6253	positive	2x	3x	negative	negative	positive
Richmond River (RR)	6254	positive	1x	1x	positive		
Richmond River (RR)	6255	positive	1x	1x	positive		
Richmond River (RR)	6258	positive	1x	1x	positive		
Richmond River (RR)	6259	positive	1x	1x	positive		







Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	PCR after 2nd extrn
Narooma (NA)	9870	negative	1x	1x	negative		
Narooma (NA)	9871	negative	2x	3x	positive	positive	negative
Narooma (NA)	9872	negative	2x	3x	positive	positive	negative
Narooma (NA)	9873	negative	1x	1x	negative		
Narooma (NA)	9874	negative	1x	1x	negative		
Narooma (NA)	9875	negative	1x	1x	negative		
Narooma (NA)	9876	negative	1x	1x	negative		
Narooma (NA)	9877	negative	1x	1x	negative		
Narooma (NA)	9878	negative	1x	1x	negative		
Narooma (NA)	9879	negative	1x	1x	negative		
Narooma (NA)	9880	negative	2x	3x	positive	positive	positive
Narooma (NA)	9881	negative	2x	3x	positive	positive	positive
Narooma (NA)	9882	negative	2x	3x	positive	positive	negative
Narooma (NA)	9883	negative	2x	3x	positive	positive	negative
Narooma (NA)	9884	negative	1x	1x	negative		
Narooma (NA)	9885	negative	1x	1x	negative		
Narooma (NA)	9886	negative	2x	3x	positive	positive	negative
Narooma (NA)	9887	negative	1x	1x	negative		
Narooma (NA)	9888	negative	1x	1x	negative		
Narooma (NA)	9889	negative	2x	3x	positive	positive	negative
Narooma (NA)	9890	negative	1x	1x	negative		
Narooma (NA)	9891	negative	1x	2x	positive	negative	
Narooma (NA)	9892	negative	1x	1x	negative		
Narooma (NA)	9893	negative	1x	1x	negative		
Narooma (NA)	9894	negative	1x	1x	negative		
Narooma (NA)	9895	negative	1x	1x	negative		
Narooma (NA)	9896	negative	1x	1x	negative		
Narooma (NA)	9897	negative	1x	1x	negative		
Narooma (NA)	9898	negative	1x	1x	negative		
Narooma (NA)	9899	negative	1x	1x	negative		
Narooma (NA)	9900	negative	1x	1x	negative		
Narooma (NA)	9901	negative	1x	1x	negative		
Narooma (NA)	9902	negative	1x	1x	negative		
Narooma (NA)	9903	negative	1x	1x	negative		
Narooma (NA)	9904	negative	1x	1x	negative		
Narooma (NA)	9905	negative	1x	1x	negative		
Narooma (NA)	9906	negative	1x	1x	negative		
Narooma (NA)	9907	negative	1x	1x	negative		
Narooma (NA)	9908	negative	1x	1x	negative		
Narooma (NA)	9909	negative	1x	1x	negative		
Narooma (NA)	9910	negative	1x	1x	negative		
Narooma (NA)	9911	negative	1x	1x	negative		
Narooma (NA)	9912	negative	1x	1x	negative		
Narooma (NA)	9913	negative	1x	2x	positive	negative	
Narooma (NA)	9914	negative	1x	1x	negative		
Narooma (NA)	9915	negative	1x	1x	negative		
Narooma (NA)	9916	negative	2x	3x	positive	positive	negative
Narooma (NA)	9917	negative	1x	1x	negative		
Narooma (NA)	9918	negative	2x	3x	positive	positive	positive
Narooma (NA)	9919	negative	1x	1x	negative		
Narooma (NA)	9920	negative	1x	1x	negative		
Narooma (NA)	9921	negative	1x	1x	negative		
Narooma (NA)	9922	negative	2x	3x	positive	positive	positive
Narooma (NA)	9923	negative	1x	1x	negative		
Narooma (NA)	9924	negative	1x	1x	negative		
Narooma (NA)	9925	negative	1x	1x	negative		
Narooma (NA)	9926	negative	1x	1x	negative		
Narooma (NA)	9927	negative	2x	3x	positive	positive	positive
Narooma (NA)	9928	negative	1x	2x	positive	negative	

Narooma (NA)	9929	negative	2x	3x	positive	positive	negative
Narooma (NA)	9930	negative	1x	1x	negative		
Narooma (NA)	9931	negative	1x	1x	negative		
Narooma (NA)	9932	negative	2x	3x	positive	positive	positive
Narooma (NA)	9933	negative	1x	1x	negative		
Narooma (NA)	9934	negative	1x	1x	negative		
Narooma (NA)	9935	negative	1x	1x	negative		
Narooma (NA)	9936	negative	1x	1x	negative		
Narooma (NA)	9937	negative	2x	3x	positive	positive	positive
Narooma (NA)	9938	negative	2x	3x	positive	positive	positive
Narooma (NA)	9939	negative	1x	1x	negative		
Narooma (NA)	9940	negative	2x	3x	positive	positive	positive
Narooma (NA)	9941	negative	1x	1x	negative		
Narooma (NA)	9942	negative	1x	1x	negative		
Narooma (NA)	9943	negative	1x	1x	negative		
Narooma (NA)	9944	negative	1x	2x	positive	negative	
Narooma (NA)	9945	negative	1x	1x	negative		
Narooma (NA)	9946	negative	1x	1x	negative		
Narooma (NA)	9947	negative	2x	3x	positive	positive	positive
Narooma (NA)	9948	negative	1x	1x	negative		
Narooma (NA)	9949	negative	1x	1x	negative		
Narooma (NA)	9950	negative	1x	1x	negative		
Narooma (NA)	9951	negative	2x	3x	positive	positive	positive
Narooma (NA)	9952	negative	2x	3x	positive	positive	positive
Narooma (NA)	9953	negative	1x	1x	negative		
Narooma (NA)	9954	negative	1x	1x	negative		
Narooma (NA)	9955	negative	2x	3x	positive	positive	positive
Narooma (NA)	9956	negative	1x	1x	negative		
Narooma (NA)	9957	negative	1x	1x	negative		
Narooma (NA)	9958	negative	1x	1x	negative		
Narooma (NA)	9959	negative	1x	1x	negative		
Narooma (NA)	9960	negative	1x	1x	negative		
Narooma (NA)	9961	negative	1x	1x	negative		
Narooma (NA)	9962	negative	1x	1x	negative		
Narooma (NA)	9963	negative	2x	3x	positive	positive	positive
Narooma (NA)	9964	negative	1x	1x	negative		
Narooma (NA)	9965	negative	2x	3x	positive	positive	negative
Narooma (NA)	9966	negative	1x	1x	negative		
Narooma (NA)	9967	negative	2x	3x	positive	positive	positive
Narooma (NA)	9968	negative	1x	1x	negative		
Narooma (NA)	9969	negative	2x	3x	positive	positive	negative

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	PCR after 2nd extn
Tuross Lake (TL)	9370	negative	1x	1x	negative		
Tuross Lake (TL)	9371	negative	1x	1x	negative		
Tuross Lake (TL)	9372	negative	1x	1x	negative		
Tuross Lake (TL)	9373	negative	1x	1x	negative		
Tuross Lake (TL)	9374	negative	1x	1x	negative		
Tuross Lake (TL)	9375	negative	1x	1x	negative		
Tuross Lake (TL)	9376	negative	1x	1x	negative		
Tuross Lake (TL)	9377	negative	1x	1x	negative		
Tuross Lake (TL)	9378	negative	1x	1x	negative		
Tuross Lake (TL)	9379	negative	1x	1x	negative		
Tuross Lake (TL)	9380	negative	1x	1x	negative		
Tuross Lake (TL)	9381	negative	1x	1x	negative		
Tuross Lake (TL)	9382	negative	1x	1x	negative		
Tuross Lake (TL)	9383	negative	1x	1x	negative		
Tuross Lake (TL)	9384	negative	1x	1x	negative		
Tuross Lake (TL)	9385	negative	1x	1x	negative		
Tuross Lake (TL)	9386	negative	1x	1x	negative		
Tuross Lake (TL)	9387	negative	1x	1x	negative		
Tuross Lake (TL)	9388	negative	1x	1x	negative		
Tuross Lake (TL)	9389	negative	1x	1x	negative		
Tuross Lake (TL)	9390	negative	1x	1x	negative		
Tuross Lake (TL)	9391	negative	1x	1x	negative		
Tuross Lake (TL)	9392	negative	1x	1x	negative		
Tuross Lake (TL)	9393	negative	1x	1x	negative		
Tuross Lake (TL)	9394	negative	1x	1x	negative		
Tuross Lake (TL)	9395	negative	1x	1x	negative		
Tuross Lake (TL)	9396	negative	1x	1x	negative		
Tuross Lake (TL)	9397	negative	1x	1x	negative		
Tuross Lake (TL)	9398	negative	1x	1x	negative		
Tuross Lake (TL)	9399	negative	1x	1x	negative		
Tuross Lake (TL)	9400	negative	1x	1x	negative		
Tuross Lake (TL)	9401	negative	1x	1x	negative		
Tuross Lake (TL)	9402	negative	1x	1x	negative		
Tuross Lake (TL)	9403	negative	1x	1x	negative		
Tuross Lake (TL)	9404	negative	1x	1x	negative		
Tuross Lake (TL)	9405	negative	1x	1x	negative		
Tuross Lake (TL)	9406	negative	1x	1x	negative		
Tuross Lake (TL)	9407	negative	1x	1x	negative		
Tuross Lake (TL)	9408	negative	1x	1x	negative		
Tuross Lake (TL)	9409	negative	1x	1x	negative		
Tuross Lake (TL)	9410	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9411	negative	1x	1x	negative		
Tuross Lake (TL)	9412	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9413	negative	1x	1x	negative		
Tuross Lake (TL)	9414	negative	1x	1x	negative		
Tuross Lake (TL)	9415	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9416	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9417	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9418	negative	1x	1x	negative		
Tuross Lake (TL)	9419	negative	1x	2x	positive	positive	positive
Tuross Lake (TL)	9420	negative	1x	1x	negative		
Tuross Lake (TL)	9421	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9422	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9423	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9424	negative	1x	1x	negative		
Tuross Lake (TL)	9425	negative	1x	1x	negative		
Tuross Lake (TL)	9426	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9427	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9428	negative	1x	1x	negative		

Tuross Lake (TL)	9429	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9430	negative	1x	1x	negative		
Tuross Lake (TL)	9431	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9432	negative	2x	3x	positive	positive	positive
Tuross Lake (TL)	9433	negative	1x	2x	positive	negative	
Tuross Lake (TL)	9434	negative	1x	1x	negative		
Tuross Lake (TL)	9435	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9436	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9437	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9438	negative	1x	1x	negative		
Tuross Lake (TL)	9439	negative	1x	1x	positive		
Tuross Lake (TL)	9440	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9441	negative	1x	2x	positive	positive	negative
Tuross Lake (TL)	9442	negative	1x	1x	negative		
Tuross Lake (TL)	9443	negative	1x	1x	negative		
Tuross Lake (TL)	9444	negative	1x	1x	negative		
Tuross Lake (TL)	9445	negative	1x	1x	negative		
Tuross Lake (TL)	9446	negative	1x	1x	negative		
Tuross Lake (TL)	9447	negative	1x	1x	negative		
Tuross Lake (TL)	9448	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9449	negative	1x	1x	negative		
Tuross Lake (TL)	9450	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9451	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9452	negative	1x	1x	negative		
Tuross Lake (TL)	9453	negative	1x	1x	negative		
Tuross Lake (TL)	9454	negative	1x	1x	negative		
Tuross Lake (TL)	9455	negative	1x	1x	negative		
Tuross Lake (TL)	9456	negative	1x	1x	negative		
Tuross Lake (TL)	9457	negative	1x	1x	negative		
Tuross Lake (TL)	9458	negative	1x	1x	negative		
Tuross Lake (TL)	9459	negative	1x	1x	negative		
Tuross Lake (TL)	9460	negative	1x	1x	negative		
Tuross Lake (TL)	9461	negative	1x	1x	negative		
Tuross Lake (TL)	9462	negative	1x	1x	negative		
Tuross Lake (TL)	9463	negative	1x	1x	negative		
Tuross Lake (TL)	9464	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9465	negative	2x	3x	positive	positive	negative
Tuross Lake (TL)	9466	negative	1x	1x	negative		
Tuross Lake (TL)	9467	negative	1x	1x	negative		
Tuross Lake (TL)	9468	negative	1x	1x	negative		
Tuross Lake (TL)	9469	negative	1x	1x	negative		

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	PCR after 2nd extn
Batemans Bay (BB)	10158	negative	1x	1x	negative		
Batemans Bay (BB)	10159	negative	1x	1x	negative		
Batemans Bay (BB)	10160	negative	1x	1x	negative		
Batemans Bay (BB)	10161	negative	1x	1x	negative		
Batemans Bay (BB)	10162	negative	1x	1x	negative		
Batemans Bay (BB)	10163	negative	1x	2x	positive	negative	
Batemans Bay (BB)	10164	negative	1x	1x	negative		
Batemans Bay (BB)	10165	negative	1x	2x	positive	negative	
Batemans Bay (BB)	10166	negative	1x	1x	negative		
Batemans Bay (BB)	10167	negative	1x	1x	negative		
Batemans Bay (BB)	10168	negative	1x	1x	negative		
Batemans Bay (BB)	10169	negative	1x	1x	negative		
Batemans Bay (BB)	10170	negative	2x	3x	positive	positive	negative
Batemans Bay (BB)	10171	negative	1x	1x	negative		
Batemans Bay (BB)	10172	negative	1x	1x	negative		
Batemans Bay (BB)	10173	negative	1x	1x	negative		
Batemans Bay (BB)	10174	negative	1x	1x	negative		
Batemans Bay (BB)	10175	negative	1x	1x	negative		
Batemans Bay (BB)	10176	negative	1x	1x	negative		
Batemans Bay (BB)	10177	negative	1x	1x	negative		
Batemans Bay (BB)	10178	negative	2x	3x	positive	positive	negative
Batemans Bay (BB)	10179	negative	1x	1x	negative		
Batemans Bay (BB)	10180	negative	1x	1x	negative		
Batemans Bay (BB)	10181	negative	1x	1x	negative		
Batemans Bay (BB)	10182	negative	1x	1x	negative		
Batemans Bay (BB)	10183	negative	1x	1x	negative		
Batemans Bay (BB)	10184	negative	1x	1x	negative		
Batemans Bay (BB)	10185	negative	1x	1x	negative		
Batemans Bay (BB)	10186	negative	1x	1x	negative		
Batemans Bay (BB)	10187	negative	2x	3x	positive	positive	negative
Batemans Bay (BB)	10188	negative	1x	1x	negative		
Batemans Bay (BB)	10189	negative	1x	1x	negative		
Batemans Bay (BB)	10190	negative	1x	1x	negative		
Batemans Bay (BB)	10191	negative	1x	1x	negative		
Batemans Bay (BB)	10192	negative	1x	1x	negative		
Batemans Bay (BB)	10193	negative	1x	1x	negative		
Batemans Bay (BB)	10194	negative	1x	1x	negative		
Batemans Bay (BB)	10195	negative	1x	1x	negative		
Batemans Bay (BB)	10196	negative	1x	1x	negative		
Batemans Bay (BB)	10197	negative	1x	1x	negative		
Batemans Bay (BB)	10198	negative	1x	1x	negative		
Batemans Bay (BB)	10199	negative	1x	1x	negative		
Batemans Bay (BB)	10200	negative	1x	1x	negative		
Batemans Bay (BB)	10201	negative	1x	1x	negative		
Batemans Bay (BB)	10202	negative	1x	1x	negative		
Batemans Bay (BB)	10203	negative	1x	1x	negative		
Batemans Bay (BB)	10204	negative	1x	1x	negative		
Batemans Bay (BB)	10205	negative	1x	1x	negative		
Batemans Bay (BB)	10206	negative	1x	1x	negative		
Batemans Bay (BB)	10207	negative	1x	1x	negative		
Batemans Bay (BB)	10208	negative	1x	1x	negative		
Batemans Bay (BB)	10209	negative	1x	1x	negative		
Batemans Bay (BB)	10210	negative	1x	1x	negative		
Batemans Bay (BB)	10211	negative	1x	1x	negative		
Batemans Bay (BB)	10212	negative	1x	1x	negative		
Batemans Bay (BB)	10213	negative	1x	1x	negative		
Batemans Bay (BB)	10214	negative	1x	1x	negative		
Batemans Bay (BB)	10215	negative	1x	1x	negative		
Batemans Bay (BB)	10216	negative	1x	1x	negative		
Batemans Bay (BB)	10217	negative	1x	1x	negative		

Batemans Bay (BB)	10218	negative	1x	1x	negative		
Batemans Bay (BB)	10219	negative	1x	1x	negative		
Batemans Bay (BB)	10220	negative	1x	1x	negative		
Batemans Bay (BB)	10221	negative	1x	1x	negative		
Batemans Bay (BB)	10222	negative	1x	1x	negative		
Batemans Bay (BB)	10223	negative	1x	1x	negative		
Batemans Bay (BB)	10224	negative	1x	1x	negative		
Batemans Bay (BB)	10225	negative	1x	1x	negative		
Batemans Bay (BB)	10226	negative	1x	1x	negative		
Batemans Bay (BB)	10227	negative	1x	1x	negative		
Batemans Bay (BB)	10228	negative	1x	1x	negative		
Batemans Bay (BB)	10229	negative	1x	1x	negative		
Batemans Bay (BB)	10230	negative	1x	1x	negative		
Batemans Bay (BB)	10231	negative	1x	1x	negative		
Batemans Bay (BB)	10232	negative	1x	1x	negative		
Batemans Bay (BB)	10233	negative	1x	1x	negative		
Batemans Bay (BB)	10234	negative	1x	1x	negative		
Batemans Bay (BB)	10235	negative	1x	1x	negative		
Batemans Bay (BB)	10236	negative	1x	1x	negative		
Batemans Bay (BB)	10237	negative	1x	1x	negative		
Batemans Bay (BB)	10238	negative	1x	1x	negative		
Batemans Bay (BB)	10239	negative	1x	1x	negative		
Batemans Bay (BB)	10240	negative	1x	1x	negative		
Batemans Bay (BB)	10241	negative	1x	1x	negative		
Batemans Bay (BB)	10242	negative	1x	1x	negative		
Batemans Bay (BB)	10243	negative	1x	1x	negative		
Batemans Bay (BB)	10244	negative	1x	1x	negative		
Batemans Bay (BB)	10245	negative	1x	1x	negative		
Batemans Bay (BB)	10246	negative	1x	1x	negative		
Batemans Bay (BB)	10247	negative	1x	1x	negative		
Batemans Bay (BB)	10248	negative	1x	1x	negative		
Batemans Bay (BB)	10249	negative	2x	3x	positive	positive	negative
Batemans Bay (BB)	10250	negative	1x	1x	negative		
Batemans Bay (BB)	10251	negative	2x	3x	positive	negative	negative
Batemans Bay (BB)	10252	negative	1x	1x	negative		
Batemans Bay (BB)	10253	negative	1x	1x	negative		
Batemans Bay (BB)	10254	negative	1x	1x	negative		
Batemans Bay (BB)	10255	negative	1x	1x	negative		
Batemans Bay (BB)	10256	negative	1x	1x	negative		
Batemans Bay (BB)	10257	negative	1x	1x	negative		













Port Stephens (PS)	8271	negative	1x	1x	negative		
Port Stephens (PS)	8272	negative	1x	1x	negative		
Port Stephens (PS)	8273	negative	1x	1x	negative		
Port Stephens (PS)	8274	negative	1x	1x	negative		
Port Stephens (PS)	8275	negative	1x	1x	negative		
Port Stephens (PS)	8276	negative	1x	1x	negative		
Port Stephens (PS)	8277	negative	1x	1x	negative		
Port Stephens (PS)	8278	negative	1x	1x	negative		
Port Stephens (PS)	8279	negative	1x	1x	negative		
Port Stephens (PS)	8280	negative	1x	1x	negative		
Port Stephens (PS)	8281	negative	1x	1x	negative		
Port Stephens (PS)	8282	negative	1x	1x	negative		
Port Stephens (PS)	8283	negative	1x	1x	negative		
Port Stephens (PS)	8284	negative	1x	1x	negative		
Port Stephens (PS)	8285	negative	1x	1x	negative		
Port Stephens (PS)	8286	negative	1x	1x	negative		
Port Stephens (PS)	8287	negative	1x	1x	negative		
Port Stephens (PS)	8288	negative	1x	1x	negative		
Port Stephens (PS)	8289	negative	1x	1x	negative		
Port Stephens (PS)	8290	negative	1x	1x	negative		
Port Stephens (PS)	8291	negative	1x	1x	negative		
Port Stephens (PS)	8292	negative	1x	1x	negative		
Port Stephens (PS)	8293	negative	1x	1x	negative		
Port Stephens (PS)	8294	negative	1x	1x	negative		
Port Stephens (PS)	8295	negative	1x	1x	negative		
Port Stephens (PS)	8296	negative	1x	1x	negative		
Port Stephens (PS)	8297	negative	2x	3x	positive	positive	negative
Port Stephens (PS)	8298	negative	1x	1x	negative		
Port Stephens (PS)	8299	negative	1x	1x	negative		
Port Stephens (PS)	8300	negative	1x	1x	negative		
Port Stephens (PS)	8301	negative	1x	1x	negative		
Port Stephens (PS)	8302	negative	1x	1x	negative		
Port Stephens (PS)	8303	negative	1x	1x	negative		
Port Stephens (PS)	8304	negative	1x	1x	negative		
Port Stephens (PS)	8305	negative	1x	2x	positive	negative	
Port Stephens (PS)	8306	negative	1x	1x	negative		
Port Stephens (PS)	8307	negative	1x	1x	negative		

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result	PCR after 2nd extrn
Wallis Lakes (WL)	7620	negative	1x	1x	negative		
Wallis Lakes (WL)	7621	negative	2x	3x	positive	positive	positive
Wallis Lakes (WL)	7622	negative	1x	1x	negative		
Wallis Lakes (WL)	7623	negative	1x	1x	negative		
Wallis Lakes (WL)	7624	negative	2x	3x	positive	positive	positive
Wallis Lakes (WL)	7625	negative	2x	3x	positive	positive	
Wallis Lakes (WL)	7626	negative	1x	1x	negative		
Wallis Lakes (WL)	7627	negative	1x	1x	negative		
Wallis Lakes (WL)	7628	negative	1x	1x	negative		
Wallis Lakes (WL)	7629	negative	1x	1x	negative		
Wallis Lakes (WL)	7630	negative	1x	1x	negative		
Wallis Lakes (WL)	7631	negative	1x	1x	negative		
Wallis Lakes (WL)	7632	negative	1x	1x	negative		
Wallis Lakes (WL)	7633	negative	1x	1x	negative		
Wallis Lakes (WL)	7634	negative	1x	1x	negative		
Wallis Lakes (WL)	7635	negative	1x	1x	negative		
Wallis Lakes (WL)	7636	negative	1x	1x	negative		
Wallis Lakes (WL)	7637	negative	1x	1x	negative		
Wallis Lakes (WL)	7638	negative	1x	1x	negative		
Wallis Lakes (WL)	7639	negative	1x	1x	negative		
Wallis Lakes (WL)	7640	negative	1x	1x	negative		
Wallis Lakes (WL)	7641	negative	1x	1x	negative		
Wallis Lakes (WL)	7642	negative	1x	1x	negative		
Wallis Lakes (WL)	7643	negative	1x	1x	negative		
Wallis Lakes (WL)	7644	negative	1x	1x	negative		
Wallis Lakes (WL)	7645	negative	1x	1x	negative		
Wallis Lakes (WL)	7646	negative	1x	1x	negative		
Wallis Lakes (WL)	7647	negative	1x	1x	negative		
Wallis Lakes (WL)	7648	negative	1x	1x	negative		
Wallis Lakes (WL)	7649	negative	1x	1x	negative		
Wallis Lakes (WL)	7650	negative	1x	1x	negative		
Wallis Lakes (WL)	7651	negative	1x	1x	negative		
Wallis Lakes (WL)	7652	negative	1x	1x	negative		
Wallis Lakes (WL)	7653	negative	1x	1x	negative		
Wallis Lakes (WL)	7654	negative	1x	1x	negative		
Wallis Lakes (WL)	7655	negative	1x	1x	negative		
Wallis Lakes (WL)	7656	negative	1x	1x	negative		
Wallis Lakes (WL)	7657	negative	1x	1x	negative		
Wallis Lakes (WL)	7658	negative	1x	1x	negative		
Wallis Lakes (WL)	7659	negative	1x	1x	negative		
Wallis Lakes (WL)	7660	negative	2x	3x	positive	positive	negative
Wallis Lakes (WL)	7661	negative	2x	3x	positive	positive	positive
Wallis Lakes (WL)	7662	negative	1x	1x	negative		
Wallis Lakes (WL)	7663	negative	1x	1x	negative		
Wallis Lakes (WL)	7664	negative	1x	1x	negative		
Wallis Lakes (WL)	7665	negative	1x	1x	negative		
Wallis Lakes (WL)	7666	negative	2x	3x	positive	positive	negative
Wallis Lakes (WL)	7667	negative	1x	1x	negative		
Wallis Lakes (WL)	7668	negative	1x	1x	negative		
Wallis Lakes (WL)	7669	negative	1x	1x	negative		
Wallis Lakes (WL)	7670	negative	1x	1x	negative		
Wallis Lakes (WL)	7671	negative	1x	1x	negative		
Wallis Lakes (WL)	7672	negative	1x	1x	negative		
Wallis Lakes (WL)	7673	negative	1x	1x	negative		
Wallis Lakes (WL)	7674	negative	1x	1x	negative		
Wallis Lakes (WL)	7675	negative	1x	1x	negative		
Wallis Lakes (WL)	7676	negative	1x	1x	negative		
Wallis Lakes (WL)	7677	negative	1x	1x	negative		
Wallis Lakes (WL)	7678	negative	1x	1x	negative		
Wallis Lakes (WL)	7679	negative	1x	1x	negative		
Wallis Lakes (WL)	7680	negative	1x	1x	negative		
Wallis Lakes (WL)	7681	negative	1x	1x	negative		

Wallis Lakes (WL)	7682	negative	1x	1x	negative		
Wallis Lakes (WL)	7683	negative	1x	1x	negative		
Wallis Lakes (WL)	7684	negative	1x	1x	negative		
Wallis Lakes (WL)	7685	negative	1x	1x	negative		
Wallis Lakes (WL)	7686	negative	1x	1x	negative		
Wallis Lakes (WL)	7687	negative	1x	1x	negative		
Wallis Lakes (WL)	7688	negative	1x	1x	negative		
Wallis Lakes (WL)	7689	negative	1x	1x	negative		
Wallis Lakes (WL)	7690	negative	1x	1x	negative		
Wallis Lakes (WL)	7691	negative	1x	1x	negative		
Wallis Lakes (WL)	7692	negative	1x	1x	negative		
Wallis Lakes (WL)	7693	negative	1x	1x	negative		
Wallis Lakes (WL)	7694	negative	1x	1x	negative		
Wallis Lakes (WL)	7695	negative	1x	1x	negative		
Wallis Lakes (WL)	7696	negative	1x	1x	negative		
Wallis Lakes (WL)	7697	negative	1x	1x	negative		
Wallis Lakes (WL)	7698	negative	1x	1x	negative		
Wallis Lakes (WL)	7699	negative	1x	1x	negative		
Wallis Lakes (WL)	7700	negative	1x	1x	negative		
Wallis Lakes (WL)	7701	negative	1x	1x	negative		
Wallis Lakes (WL)	7702	negative	1x	1x	negative		
Wallis Lakes (WL)	7703	negative	1x	1x	negative		
Wallis Lakes (WL)	7704	negative	1x	1x	negative		
Wallis Lakes (WL)	7705	negative	1x	1x	negative		
Wallis Lakes (WL)	7706	negative	1x	1x	negative		
Wallis Lakes (WL)	7707	negative	2x	3x	positive	positive	positive
Wallis Lakes (WL)	7708	negative	1x	1x	negative		
Wallis Lakes (WL)	7709	negative	1x	1x	negative		
Wallis Lakes (WL)	7710	negative	1x	1x	negative		
Wallis Lakes (WL)	7711	negative	1x	1x	negative		
Wallis Lakes (WL)	7712	negative	1x	1x	negative		
Wallis Lakes (WL)	7713	negative	1x	1x	negative		
Wallis Lakes (WL)	7714	negative	1x	1x	negative		
Wallis Lakes (WL)	7715	negative	1x	1x	negative		
Wallis Lakes (WL)	7716	negative	1x	1x	negative		
Wallis Lakes (WL)	7717	negative	1x	1x	negative		
Wallis Lakes (WL)	7718	negative	1x	1x	negative		
Wallis Lakes (WL)	7719	negative	1x	1x	negative		

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result
Hastings River (HS)	7370	negative	1x	1x	negative
Hastings River (HS)	7371	negative	1x	1x	negative
Hastings River (HS)	7372	negative	1x	1x	negative
Hastings River (HS)	7373	negative	1x	1x	negative
Hastings River (HS)	7374	negative	1x	1x	negative
Hastings River (HS)	7375	negative	1x	1x	positive
Hastings River (HS)	7376	negative	1x	1x	negative
Hastings River (HS)	7377	negative	1x	1x	negative
Hastings River (HS)	7378	negative	1x	1x	negative
Hastings River (HS)	7379	negative	1x	1x	negative
Hastings River (HS)	7380	negative	1x	1x	negative
Hastings River (HS)	7381	negative	1x	1x	negative
Hastings River (HS)	7382	negative	1x	1x	negative
Hastings River (HS)	7383	negative	1x	1x	positive
Hastings River (HS)	7384	negative	1x	1x	negative
Hastings River (HS)	7385	negative	1x	1x	negative
Hastings River (HS)	7386	negative	1x	1x	negative
Hastings River (HS)	7387	negative	1x	1x	negative
Hastings River (HS)	7388	negative	1x	1x	negative
Hastings River (HS)	7389	negative	1x	1x	negative
Hastings River (HS)	7390	negative	1x	1x	negative
Hastings River (HS)	7391	negative	1x	1x	negative
Hastings River (HS)	7392	negative	1x	1x	negative
Hastings River (HS)	7393	negative	1x	1x	negative
Hastings River (HS)	7394	negative	1x	1x	negative
Hastings River (HS)	7395	negative	1x	1x	negative
Hastings River (HS)	7396	negative	1x	1x	negative
Hastings River (HS)	7397	negative	1x	1x	negative
Hastings River (HS)	7398	negative	1x	1x	negative
Hastings River (HS)	7399	negative	1x	1x	negative
Hastings River (HS)	7400	negative	1x	1x	negative
Hastings River (HS)	7401	negative	1x	1x	negative
Hastings River (HS)	7402	negative	1x	1x	negative
Hastings River (HS)	7403	negative	1x	1x	negative
Hastings River (HS)	7404	negative	1x	1x	negative
Hastings River (HS)	7405	negative	1x	1x	negative
Hastings River (HS)	7406	negative	1x	1x	negative
Hastings River (HS)	7407	negative	1x	1x	negative
Hastings River (HS)	7408	negative	1x	1x	negative
Hastings River (HS)	7409	negative	1x	1x	negative
Hastings River (HS)	7410	negative	1x	1x	negative
Hastings River (HS)	7411	negative	1x	1x	negative
Hastings River (HS)	7412	negative	1x	1x	negative
Hastings River (HS)	7413	negative	1x	1x	negative
Hastings River (HS)	7414	negative	1x	1x	negative
Hastings River (HS)	7415	negative	1x	1x	negative
Hastings River (HS)	7416	negative	1x	1x	positive
Hastings River (HS)	7417	negative	1x	1x	negative
Hastings River (HS)	7418	negative	1x	1x	negative
Hastings River (HS)	7419	negative	1x	1x	negative
Hastings River (HS)	7420	negative	1x	1x	negative
Hastings River (HS)	7421	negative	1x	1x	negative
Hastings River (HS)	7422	negative	1x	1x	negative
Hastings River (HS)	7423	negative	1x	1x	negative
Hastings River (HS)	7424	negative	1x	1x	negative
Hastings River (HS)	7425	negative	1x	1x	negative
Hastings River (HS)	7426	negative	1x	1x	negative



Hastings River (HS)	7427	negative	1x	1x	negative
Hastings River (HS)	7428	negative	1x	1x	negative
Hastings River (HS)	7429	negative	1x	1x	negative
Hastings River (HS)	7430	negative	1x	1x	negative
Hastings River (HS)	7431	negative	1x	1x	negative
Hastings River (HS)	7432	negative	1x	1x	negative
Hastings River (HS)	7433	negative	1x	1x	negative
Hastings River (HS)	7434	negative	1x	1x	negative
Hastings River (HS)	7435	negative	1x	1x	negative
Hastings River (HS)	7436	negative	1x	1x	negative
Hastings River (HS)	7437	negative	1x	1x	negative
Hastings River (HS)	7438	negative	1x	1x	positive
Hastings River (HS)	7439	negative	1x	1x	negative
Hastings River (HS)	7440	negative	1x	1x	negative
Hastings River (HS)	7441	negative	1x	1x	negative
Hastings River (HS)	7442	negative	1x	1x	negative
Hastings River (HS)	7443	negative	1x	1x	positive
Hastings River (HS)	7444	negative	1x	1x	negative
Hastings River (HS)	7445	negative	1x	1x	negative
Hastings River (HS)	7446	negative	1x	1x	negative
Hastings River (HS)	7447	negative	1x	1x	positive
Hastings River (HS)	7448	negative	1x	1x	negative
Hastings River (HS)	7449	negative	1x	1x	negative
Hastings River (HS)	7450	negative	1x	1x	positive
Hastings River (HS)	7451	negative	1x	1x	positive
Hastings River (HS)	7452	negative	1x	1x	negative
Hastings River (HS)	7453	negative	1x	1x	negative
Hastings River (HS)	7454	negative	1x	1x	negative
Hastings River (HS)	7455	negative	1x	1x	negative
Hastings River (HS)	7456	negative	1x	1x	negative
Hastings River (HS)	7457	negative	1x	1x	positive
Hastings River (HS)	7458	negative	1x	1x	positive
Hastings River (HS)	7459	negative	1x	1x	negative
Hastings River (HS)	7460	negative	1x	1x	negative
Hastings River (HS)	7461	negative	1x	1x	negative
Hastings River (HS)	7462	negative	1x	1x	negative
Hastings River (HS)	7463	negative	1x	1x	negative
Hastings River (HS)	7464	negative	1x	1x	negative
Hastings River (HS)	7465	negative	1x	1x	negative
Hastings River (HS)	7466	negative	1x	1x	negative
Hastings River (HS)	7467	negative	1x	1x	negative
Hastings River (HS)	7468	negative	1x	1x	negative
Hastings River (HS)	7469	negative	1x	1x	negative

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result
Hastings River (HS)	7370	negative	1x	1x	negative
Hastings River (HS)	7371	negative	1x	1x	negative
Hastings River (HS)	7372	negative	1x	1x	negative
Hastings River (HS)	7373	negative	1x	1x	negative
Hastings River (HS)	7374	negative	1x	1x	negative
Hastings River (HS)	7375	negative	1x	1x	positive
Hastings River (HS)	7376	negative	1x	1x	negative
Hastings River (HS)	7377	negative	1x	1x	negative
Hastings River (HS)	7378	negative	1x	1x	negative
Hastings River (HS)	7379	negative	1x	1x	negative
Hastings River (HS)	7380	negative	1x	1x	negative
Hastings River (HS)	7381	negative	1x	1x	negative
Hastings River (HS)	7382	negative	1x	1x	negative
Hastings River (HS)	7383	negative	1x	1x	positive
Hastings River (HS)	7384	negative	1x	1x	negative
Hastings River (HS)	7385	negative	1x	1x	negative
Hastings River (HS)	7386	negative	1x	1x	negative
Hastings River (HS)	7387	negative	1x	1x	negative
Hastings River (HS)	7388	negative	1x	1x	negative
Hastings River (HS)	7389	negative	1x	1x	negative
Hastings River (HS)	7390	negative	1x	1x	negative
Hastings River (HS)	7391	negative	1x	1x	negative
Hastings River (HS)	7392	negative	1x	1x	negative
Hastings River (HS)	7393	negative	1x	1x	negative
Hastings River (HS)	7394	negative	1x	1x	negative
Hastings River (HS)	7395	negative	1x	1x	negative
Hastings River (HS)	7396	negative	1x	1x	negative
Hastings River (HS)	7397	negative	1x	1x	negative
Hastings River (HS)	7398	negative	1x	1x	negative
Hastings River (HS)	7399	negative	1x	1x	negative
Hastings River (HS)	7400	negative	1x	1x	negative
Hastings River (HS)	7401	negative	1x	1x	negative
Hastings River (HS)	7402	negative	1x	1x	negative
Hastings River (HS)	7403	negative	1x	1x	negative
Hastings River (HS)	7404	negative	1x	1x	negative
Hastings River (HS)	7405	negative	1x	1x	negative
Hastings River (HS)	7406	negative	1x	1x	negative
Hastings River (HS)	7407	negative	1x	1x	negative
Hastings River (HS)	7408	negative	1x	1x	negative
Hastings River (HS)	7409	negative	1x	1x	negative
Hastings River (HS)	7410	negative	1x	1x	negative
Hastings River (HS)	7411	negative	1x	1x	negative
Hastings River (HS)	7412	negative	1x	1x	negative
Hastings River (HS)	7413	negative	1x	1x	negative
Hastings River (HS)	7414	negative	1x	1x	negative
Hastings River (HS)	7415	negative	1x	1x	negative
Hastings River (HS)	7416	negative	1x	1x	positive
Hastings River (HS)	7417	negative	1x	1x	negative
Hastings River (HS)	7418	negative	1x	1x	negative
Hastings River (HS)	7419	negative	1x	1x	negative
Hastings River (HS)	7420	negative	1x	1x	negative
Hastings River (HS)	7421	negative	1x	1x	negative
Hastings River (HS)	7422	negative	1x	1x	negative
Hastings River (HS)	7423	negative	1x	1x	negative
Hastings River (HS)	7424	negative	1x	1x	negative
Hastings River (HS)	7425	negative	1x	1x	negative
Hastings River (HS)	7426	negative	1x	1x	negative

Hastings River (HS)	7427	negative	1x	1x	negative
Hastings River (HS)	7428	negative	1x	1x	negative
Hastings River (HS)	7429	negative	1x	1x	negative
Hastings River (HS)	7430	negative	1x	1x	negative
Hastings River (HS)	7431	negative	1x	1x	negative
Hastings River (HS)	7432	negative	1x	1x	negative
Hastings River (HS)	7433	negative	1x	1x	negative
Hastings River (HS)	7434	negative	1x	1x	negative
Hastings River (HS)	7435	negative	1x	1x	negative
Hastings River (HS)	7436	negative	1x	1x	negative
Hastings River (HS)	7437	negative	1x	1x	negative
Hastings River (HS)	7438	negative	1x	1x	positive
Hastings River (HS)	7439	negative	1x	1x	negative
Hastings River (HS)	7440	negative	1x	1x	negative
Hastings River (HS)	7441	negative	1x	1x	negative
Hastings River (HS)	7442	negative	1x	1x	negative
Hastings River (HS)	7443	negative	1x	1x	positive
Hastings River (HS)	7444	negative	1x	1x	negative
Hastings River (HS)	7445	negative	1x	1x	negative
Hastings River (HS)	7446	negative	1x	1x	negative
Hastings River (HS)	7447	negative	1x	1x	positive
Hastings River (HS)	7448	negative	1x	1x	negative
Hastings River (HS)	7449	negative	1x	1x	negative
Hastings River (HS)	7450	negative	1x	1x	positive
Hastings River (HS)	7451	negative	1x	1x	positive
Hastings River (HS)	7452	negative	1x	1x	negative
Hastings River (HS)	7453	negative	1x	1x	negative
Hastings River (HS)	7454	negative	1x	1x	negative
Hastings River (HS)	7455	negative	1x	1x	negative
Hastings River (HS)	7456	negative	1x	1x	negative
Hastings River (HS)	7457	negative	1x	1x	positive
Hastings River (HS)	7458	negative	1x	1x	positive
Hastings River (HS)	7459	negative	1x	1x	negative
Hastings River (HS)	7460	negative	1x	1x	negative
Hastings River (HS)	7461	negative	1x	1x	negative
Hastings River (HS)	7462	negative	1x	1x	negative
Hastings River (HS)	7463	negative	1x	1x	negative
Hastings River (HS)	7464	negative	1x	1x	negative
Hastings River (HS)	7465	negative	1x	1x	negative
Hastings River (HS)	7466	negative	1x	1x	negative
Hastings River (HS)	7467	negative	1x	1x	negative
Hastings River (HS)	7468	negative	1x	1x	negative
Hastings River (HS)	7469	negative	1x	1x	negative

Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result
Brunswick River (BR)	6370	negative	1X	1x	negative	
Brunswick River (BR)	6371	negative	1X	1x	negative	
Brunswick River (BR)	6372	negative	1X	1x	negative	
Brunswick River (BR)	6373	negative	1X	1x	negative	
Brunswick River (BR)	6374	negative	1X	1x	negative	
Brunswick River (BR)	6375	negative	1X	1x	negative	
Brunswick River (BR)	6376	negative	1X	1x	negative	
Brunswick River (BR)	6377	negative	1X	2x	positive	positive
Brunswick River (BR)	6378	negative	1X	1x	negative	
Brunswick River (BR)	6379	negative	1X	1x	negative	
Brunswick River (BR)	6380	negative	1X	1x	negative	
Brunswick River (BR)	6381	negative	1X	1x	negative	
Brunswick River (BR)	6382	negative	1X	1x	negative	
Brunswick River (BR)	6383	negative	1X	2x	positive	positive
Brunswick River (BR)	6384	negative	1X	2x	positive	positive
Brunswick River (BR)	6385	negative	1X	2x	positive	positive
Brunswick River (BR)	6386	negative	1X	1x	negative	
Brunswick River (BR)	6387	negative	1X	1x	negative	
Brunswick River (BR)	6388	negative	1X	1x	negative	
Brunswick River (BR)	6389	negative	1X	1x	negative	
Brunswick River (BR)	6390	negative	1X	1x	negative	
Brunswick River (BR)	6391	negative	1X	1x	negative	
Brunswick River (BR)	6392	negative	1X	2x	positive	positive
Brunswick River (BR)	6393	negative	1X	1x	negative	
Brunswick River (BR)	6394	negative	1X	1x	negative	
Brunswick River (BR)	6395	negative	1X	1x	negative	
Brunswick River (BR)	6396	negative	1X	1x	negative	
Brunswick River (BR)	6397	negative	1X	1x	negative	
Brunswick River (BR)	6398	negative	1X	1x	negative	
Brunswick River (BR)	6399	negative	1X	1x	negative	
Brunswick River (BR)	6400	negative	1X	1x	negative	
Brunswick River (BR)	6401	negative	1X	1x	negative	
Brunswick River (BR)	6402	negative	1X	2x	positive	positive
Brunswick River (BR)	6403	negative	1X	1x	negative	
Brunswick River (BR)	6404	negative	1X	1x	negative	
Brunswick River (BR)	6405	negative	1X	1x	negative	
Brunswick River (BR)	6406	negative	1X	1x	negative	
Brunswick River (BR)	6407	negative	1X	1x	negative	
Brunswick River (BR)	6408	negative	1X	1x	negative	
Brunswick River (BR)	6409	negative	1X	1x	negative	
Brunswick River (BR)	6410	negative	1X	1x	negative	
Brunswick River (BR)	6411	negative	1X	1x	negative	
Brunswick River (BR)	6412	negative	1X	1x	negative	
Brunswick River (BR)	6413	negative	1X	2x	positive	negative
Brunswick River (BR)	6414	negative	1X	2x	positive	negative
Brunswick River (BR)	6415	negative	1X	2x	positive	negative
Brunswick River (BR)	6416	negative	1X	1x	negative	
Brunswick River (BR)	6417	negative	1X	1x	negative	
Brunswick River (BR)	6418	negative	1X	1x	negative	
Brunswick River (BR)	6419	negative	1X	1x	negative	
Brunswick River (BR)	6420	negative	1X	1x	negative	
Brunswick River (BR)	6421	negative	1X	1x	negative	
Brunswick River (BR)	6422	negative	1X	1x	negative	
Brunswick River (BR)	6423	negative	1X	1x	negative	
Brunswick River (BR)	6424	negative	1X	1x	negative	
Brunswick River (BR)	6425	negative	1X	2x	positive	negative
Brunswick River (BR)	6426	negative	1X	1x	negative	



Population	Sample Code	Imprint Classification	DNA Extracted	PCR	PCR Result	2nd PCR Result
Macleay River (MR)	7120	negative	1x	2x	positive	positive
Macleay River (MR)	7121	negative	1x	1x	negative	
Macleay River (MR)	7122	negative	1x	1x	negative	
Macleay River (MR)	7123	negative	1x	2x	positive	positive
Macleay River (MR)	7124	negative	1x	2x	positive	positive
Macleay River (MR)	7125	negative	1x	2x	positive	negative
Macleay River (MR)	7126	negative	1x	2x	positive	negative
Macleay River (MR)	7127	negative	1x	2x	positive	negative
Macleay River (MR)	7128	negative	1x	1x	negative	
Macleay River (MR)	7129	negative	1x	1x	negative	
Macleay River (MR)	7130	negative	1x	1x	negative	
Macleay River (MR)	7131	negative	1x	1x	negative	
Macleay River (MR)	7132	negative	1x	2x	positive	positive
Macleay River (MR)	7133	negative	1x	1x	negative	
Macleay River (MR)	7134	negative	1x	1x	negative	
Macleay River (MR)	7135	negative	1x	2x	positive	positive
Macleay River (MR)	7136	negative	1x	2x	positive	positive
Macleay River (MR)	7137	negative	1x	1x	negative	
Macleay River (MR)	7138	negative	1x	1x	negative	
Macleay River (MR)	7139	negative	1x	1x	negative	
Macleay River (MR)	7140	negative	1x	1x	negative	
Macleay River (MR)	7141	negative	1x	1x	negative	
Macleay River (MR)	7142	negative	1x	1x	negative	
Macleay River (MR)	7143	negative	1x	1x	negative	
Macleay River (MR)	7144	negative	1x	2x	positive	positive
Macleay River (MR)	7145	negative	1x	2x	positive	positive
Macleay River (MR)	7146	negative	1x	1x	negative	
Macleay River (MR)	7147	negative	1x	1x	negative	
Macleay River (MR)	7148	negative	1x	2x	positive	positive
Macleay River (MR)	7149	negative	1x	1x	negative	
Macleay River (MR)	7150	negative	1x	1x	negative	
Macleay River (MR)	7151	negative	1x	2x	positive	positive
Macleay River (MR)	7152	negative	1x	2x	positive	positive
Macleay River (MR)	7153	negative	1x	1x	negative	
Macleay River (MR)	7154	negative	1x	1x	negative	
Macleay River (MR)	7155	negative	1x	1x	negative	
Macleay River (MR)	7156	negative	1x	1x	negative	
Macleay River (MR)	7157	negative	1x	1x	negative	
Macleay River (MR)	7158	negative	1x	2x	positive	positive
Macleay River (MR)	7159	negative	1x	1x	negative	
Macleay River (MR)	7160	negative	1x	2x	positive	positive
Macleay River (MR)	7161	negative	1x	2x	positive	negative
Macleay River (MR)	7162	negative	1x	2x	positive	positive
Macleay River (MR)	7163	negative	1x	2x	positive	negative
Macleay River (MR)	7164	negative	1x	2x	positive	positive
Macleay River (MR)	7165	negative	1x	1x	negative	
Macleay River (MR)	7166	negative	1x	2x	positive	positive
Macleay River (MR)	7167	negative	1x	2x	positive	positive
Macleay River (MR)	7168	negative	1x	1x	negative	
Macleay River (MR)	7169	negative	1x	2x	positive	positive
Macleay River (MR)	7170	negative	1x	2x	positive	negative
Macleay River (MR)	7171	negative	1x	2x	positive	positive
Macleay River (MR)	7172	negative	1x	2x	positive	positive
Macleay River (MR)	7173	negative	1x	2x	positive	negative
Macleay River (MR)	7174	negative	1x	2x	positive	negative
Macleay River (MR)	7175	negative	1x	2x	positive	positive
Macleay River (MR)	7176	negative	1x	2x	positive	positive

Macleay River (MR)	7177	negative	1x	2x	positive	positive
Macleay River (MR)	7178	negative	1x	2x	positive	positive
Macleay River (MR)	7179	negative	1x	1x	negative	
Macleay River (MR)	7180	negative	1x	1x	negative	
Macleay River (MR)	7181	negative	1x	1x	negative	
Macleay River (MR)	7182	negative	1x	1x	negative	
Macleay River (MR)	7183	negative	1x	2x	positive	positive
Macleay River (MR)	7184	negative	1x	2x	positive	positive
Macleay River (MR)	7185	negative	1x	2x	positive	positive
Macleay River (MR)	7186	negative	1x	1x	negative	
Macleay River (MR)	7187	negative	1x	1x	negative	
Macleay River (MR)	7188	negative	1x	2x	positive	positive
Macleay River (MR)	7189	negative	1x	1x	negative	
Macleay River (MR)	7190	negative	1x	1x	negative	
Macleay River (MR)	7191	negative	1x	1x	negative	
Macleay River (MR)	7192	negative	1x	1x	negative	
Macleay River (MR)	7193	negative	1x	2x	positive	positive
Macleay River (MR)	7194	negative	1x	1x	negative	
Macleay River (MR)	7195	negative	1x	1x	negative	
Macleay River (MR)	7196	negative	1x	1x	negative	
Macleay River (MR)	7197	negative	1x	2x	positive	positive
Macleay River (MR)	7198	negative	1x	1x	negative	
Macleay River (MR)	7199	negative	1x	1x	negative	
Macleay River (MR)	7200	negative	1x	1x	negative	
Macleay River (MR)	7201	negative	1x	1x	negative	
Macleay River (MR)	7202	negative	1x	1x	negative	
Macleay River (MR)	7203	negative	1x	1x	negative	
Macleay River (MR)	7204	negative	1x	1x	negative	
Macleay River (MR)	7205	negative	1x	1x	negative	
Macleay River (MR)	7206	negative	1x	1x	negative	
Macleay River (MR)	7207	negative	1x	1x	negative	
Macleay River (MR)	7208	negative	1x	1x	negative	
Macleay River (MR)	7209	negative	1x	2x	positive	positive
Macleay River (MR)	7210	negative	1x	2x	positive	negative
Macleay River (MR)	7211	negative	1x	2x	positive	positive
Macleay River (MR)	7212	negative	1x	2x	positive	positive
Macleay River (MR)	7213	negative	1x	2x	positive	negative
Macleay River (MR)	7214	negative	1x	2x	positive	positive
Macleay River (MR)	7215	negative	1x	1x	negative	
Macleay River (MR)	7216	negative	1x	2x	positive	positive
Macleay River (MR)	7217	negative	1x	1x	negative	
Macleay River (MR)	7218	negative	1x	1x	negative	
Macleay River (MR)	7219	negative	1x	1x	negative	