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Aquatic Animal Health and Biosecurity Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Contents

Figures

Tables

Abbreviations

18S	18S Ribosomal RNA
AbHV	Abalone herpesvirus (=*Haliotid herpesvirus 1*)
ACDP	CSIRO Australian Centre for Disease Preparedness
AbHV	Abalone herpesvirus
AFDL	ACDP Fish Diseases Laboratory
AHPND	Acute hepatopancreatic necrosis disease
ANQAP	Australian National Quality Assurance Program
ANZSDP	Australian and New Zealand Standard Diagnostic Procedure
ASe	Analytical sensitivity
ATL/PK	Qiagen tissue lysis buffer
CMNV	Covert Mortality Nodavirus
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	Cycle threshold or Threshold cycle
DAWE	Department of Agriculture, Water and the Environment
DIV1	Decapod Iridovirus 1 (SHIV, Shrimp Haemocyte Iridovirus)
DNA	deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EF1	Penaeid shrimp elongation factor 1
EHNV	Epizootic Haematopoietic Necrosis Virus
EHP	*Enterocytozoon hepatopenaei*
ELF1	Salmonid elongation factor 1
EU	European Union
IHNV	Infectious haematopoietic necrosis virus
IPC	Internal positive control
ISAV	Infectious salmon anaemia virus
KHV	Koi Herpesvirus
LEADDR	Laboratories for Emergency Animal Disease Diagnosis and Response
MCP	Major capsid protein
mRNA	messenger RNA
µL	microlitre
NEC	Negative extraction control
NHP	Necrotising hepatopancreatitis
NNV	Nervous Necrosis Virus
NTC	No template control
nPCR	nested PCR

NQC	Network quality control
NTC	No template control
OIE	World Organisation for Animal Health
OsHV-1	Ostreid herpesvirus 1
PCR	Polymerase chain reaction
PEC	Positive extraction control
PMMS	*Penaeus monodon* mortality syndrome
POMV	Pilchard Orthomyxovirus
QC	Quality Control
qPCR	real-time PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	Reverse transcriptase PCR
RT-qPCR	Reverse transcriptase real-time PCR
SAV	Salmonid Alphavirus
SD	Standard Deviation
SDDV	Scale-Drop Disease Virus
SHIV	Shrimp iridescent virus 1 (=Decapod iridovirus 1)
spp.	Species (multiple)
SVCV	Spring Viraemia of Carp Virus
TABV	Tasmanian Aquabirnavirus
TE	Solution containing 10mM Tris, 1mM EDTA
TiLV	Tilapia Lake Virus
TSRV	Tasmanian Salmon Reovirus
TSV	Taura Syndrome Virus
VHSV	Viral Haemorrhagic Septicaemia Virus
WSSV	White spot syndrome virus
$\bar{x}$	mean
YHV1	Yellow head virus genotype 1

Executive Summary

The ACDP Fish Diseases Laboratory (AFDL), located at the CSIRO Australian Centre for Disease Preparedness (ACDP), Geelong, Victoria has generated a range of stable, non-infectious positive and internal controls for molecular diagnostic tests that can be obtained on request for use by diagnostic laboratories. The pathogen-specific positive controls have been developed for pathogens on Australia's National List of Reportable Diseases of Aquatic Animals and new and emerging pathogens of aquatic animals in Australia and the region. These controls can be used by laboratories not only to assist in implementation of diagnostic tests but also as control material for use when undertaking diagnostic testing of samples submitted during surveillance activities, disease investigations and proficiency testing activities. There are several advantages to using these controls including: they are semi-quantitative (and so are useful for monitoring test performance over time), they are distinguishable from genomic nucleic acid sequences of pathogens of interest, they are non-infectious and can be used safely by front line diagnostic laboratories, and they are stable over extended periods of time.

The positive controls are artificially produced nucleic acid sequences (of priority pathogens) that have been generated using modern molecular techniques, so that they can be distinguished from pathogen genomic nucleic acid.

Background

In order for molecular diagnostic test results to be valid there is a requirement that the positive control that is included during the running of the test produces the expected result. In the past, for molecular tests such as PCR assays, positive control material has been generated from clinically infected material and so contains genomic nucleic acid of the target pathogen. Use of this material to generate positive controls creates a positive control that is indistinguishable from the target in the test/diagnostic sample. This makes it difficult, if not impossible, to distinguish between a true-positive and a false-positive result – where the false-positive result is due to cross-contamination of samples with positive control material.

False-positive results can have wide-reaching consequences such as consuming resources needed for the required subsequent disease investigation and may also have catastrophic implications for Australia's disease-free status and trade. While not reducing the risk of positive control contamination, the use of synthetic DNA and RNA positive controls provides a rapid and simple way to conclusively and unambiguously differentiate the positive control from the target detected in the test sample.

Objectives

1. Produce quantified synthetic RNA positive control material for conventional and real-time RT-PCR assays, available on request.

2. Produce quantified synthetic DNA positive control material for conventional and real-time PCR assays, available on request.

3. Optimised universal internal control based on plant viral RNA and DNA and/or species-specific genes for use in molecular assays completed and implemented.

4. Technology transferred and adopted by participating laboratories.

Methodology

For each real-time PCR assay targeting DNA viruses, the sequence between the forward and reverse PCR primers was selected from a well-characterised strain of the target pathogen and an artificial probe sequence was inserted into the target sequence, either 5' or 3' to the target probe sequence, depending on the position of the specific probe.

For real-time PCR assays targeting RNA viruses the same approach was used, however 9 bases 5' of the forward primer and 9 bases 3' from the reverse primer of the target sequence were included. This was to ensure efficient transcription of the target DNA into RNA and it was not considered optimal to have this commencing or terminating immediately prior to or after a primer-binding sequence.

For conventional PCR and RT-PCR assays, the same approach as that used for the real-time PCR assays was used except a unique 72 bp sequence was inserted into the centre of the target sequence to replace the existing pathogen-specific sequence. When nucleotides from the 72 bp sequence are translated into amino acid sequence the positive control sequence can be easily distinguished from the real genomic sequence as the artificial insert, used in all conventional PCR plasmid positive controls, spells "PLASMIDMADEATAAHLPLASMID".

Controls were prepared from plasmids purchased from a commercial supplier. Based on transcript length and concentration, stock plasmids were made to 10^9 or 10^8 copies/µL. After determination of analytical sensitivity (ASe), and assessment of suitability, a standard procedure was used for the preparation of stock and working dilutions. Quality assured reagents are available to diagnostic laboratories, nationally and internationally on request.

Results/key findings

Limits of detection or analytical sensitivity for the real-time assays varied from 2 to 200 copies regardless of whether the plasmid was diluted in water or host DNA, with a mode of 20 and 2 copies, respectively. The ASe for the plasmids targeted by the conventional PCR assays was a lot more variable than that for real-time PCR assays. This is due to the greater variation in primer design parameters, cycle number and amplicon length for these assays. As expected, addition of a nested PCR significantly increased assay sensitivity.

Thirty-two positive control plasmids (22 for real-time assays and 10 for conventional assays) have been prepared and are in routine use in over 20 laboratories nationally and internationally. A further 10 plasmid positive controls (8 for real-time assays and 2 for conventional assays) are undergoing final quality checks. Therefore, a total of 42 plasmid positive controls for 25 different pathogens have been generated as a result of this project.

In addition, T4 and QBeta bacteriophages (phages) have been evaluated as heterologous internal positive controls for DNA and RNA targets, respectively, for use in establishing that generic aspects of PCR testing (e.g. nucleic acid extraction and absence of PCR inhibitors) are performing as expected. Implementation of the use of the T4 and QBeta phages as internal positive controls has improved the quality of molecular testing, through more sensitive assessment of the effect of PCR inhibitors and confidence in results generated when testing atypical samples (i.e. plankton, dirt, feed).

Implications for relevant stakeholders

The use of these controls in diagnostic testing will assist diagnostic laboratories to monitor the performance of current methods and assist with technology transfer of new methods. This will, in turn, provide laboratories, industry, regulators (managers and policy makers), the general public and trade partners with enhanced confidence in Australia's diagnostic capability for important exotic and endemic aquatic pathogens.

Keywords

Molecular diagnostic testing, non-infectious positive controls, heterologous internal controls, diagnostic capability, PCR

Introduction

The last 15 years has seen a rapid expansion of molecular assays using polymerase chain reaction (PCR) amplification for the specific detection of nucleic acid, derived from both pathogenic agents (e.g. viruses, bacteria) and host organisms (e.g. prawn, salmon), for both surveillance of aquatic animal pathogens and diagnosis of aquatic animal disease. This is partly due to an increase in the number of characterised pathogens of aquatic animals, the increased availability of assays through publication of PCR methods in the literature, the speed, utility and ability to process large numbers of samples by real-time PCR and easier access to the required equipment and reagents.

For diagnostic laboratory PCR assays, there is an expectation that the following essential quality control elements are included in each PCR test run, to ensure validity of the test results:

- Positive control nucleic acid from the pathogen being targeted by the PCR assay, to demonstrate the assay is performing as expected with respect to detection of a specific nucleic acid sequence. Failure to obtain a positive test result for a positive control will result in a non-valid test due to the possibility of generating false-negative results.

- A negative control, or no template control (NTC) to identify cross-contamination during reaction set-up. Failure to obtain a negative test result for a NTC will result in a non-valid test due to the possibility of false-positive results.

- An internal positive control (IPC) to demonstrate the tissue-processing and nucleic acid extraction process has been completed according to standard operating procedures, resulting in extraction of nucleic acids and that these are free of PCR inhibitors (e.g. excessive host template, inhibitory compounds). Failure to obtain a positive test result for an internal positive control would usually result in a non-valid test due to the possibility of false-negative results. The exception is when a pathogen-specific PCR assay generates a positive result and the test can then be considered valid.

- A negative extraction control (NEC) which is a blank (no nucleic acid present) sample run through the extraction process to demonstrate extraction consumables and reagents are not contaminated. Failure to obtain a negative test result for a NEC will result in a non-valid test due to the possibility of false-positive results.

Furthermore, there has been an increasing demand for inactivated (non-infectious) positive control material from Australian state and commercial laboratories for diagnostic purposes, particularly as more new and emerging pathogens are described. There are also increasing requests for positive control material from national reference laboratories and collaborators' laboratories internationally. Positive control material can be obtained through participation in proficiency testing programs (e.g. LEADDR and ANQAP), particularly for pathogenic agents that are exotic to Australia. Unfortunately, non-finfish agents (i.e. those from crustacean or molluscan hosts) cannot be cultured *in vitro*, so stocks of positive control material are limited. If infectious material is available from crustacean or molluscan hosts this needs to be amplified *in vivo*, which is expensive, time-consuming and requires the use of bio-secure aquarium facilities. While the proficiency test panel material supplied is a valuable source of positive control material, this has been generated from clinically infected material so contains pathogenic agent genomic nucleic acid, often at high concentrations. Use of this material to generate positive controls creates a positive control that is indistinguishable from the target that would be detected during a real disease event and makes it difficult, if not impossible, to distinguish between a true-positive result and a false-positive result – due to cross-contamination of samples with positive control material.

False-positive results, caused by contamination of test samples with positive control nucleic acid, can have wide-reaching consequences such as consuming resources (i.e. consumables, reagents and time) required for the subsequent disease investigation, and may also have catastrophic implications for Australia's disease-free status and trade. In the past 7 years, AFDL has been involved in investigations on at least three occasions

where false-positive results have occurred. While very difficult to prove, contamination of test samples with positive controls generated from clinically infected material was the most likely cause of the samples testing positive on all three occasions. These investigations have been required to maintain the standing of Australia's disease-free status.

While not reducing the risk of positive control contamination, the use of synthetic DNA and RNA positive controls provides a rapid and simple way to conclusively and unambiguously differentiate the positive control from the target detected in the test sample. This is achieved by the use of synthetic controls which are detectable by the target pathogen primers and probe (for qPCR and RT-qPCR assays) and which also contain an additional artificial sequence, specific for the synthetic positive control probe. A positive result in a test sample for both the pathogen-specific probe and artificial probe would indicate cross-contamination of the test samples with the synthetic positive control. Moreover, standardised synthetic positive controls can also be produced in bulk, undergo rigorous quality assurance and be more accurately quantified than pathogen genomic nucleic acid sourced from infected animals. One disadvantage of synthetic positive controls is failure of detection if primers and/or probe sequences are modified or the nucleic acid region targeted has changed, for example, due to mutation. However, the requirement to change controls is reduced by implementation of properly developed and validated assays. By removing the reliance on positive controls based on pathogen genomic material, issues associated with the generation of false-positive results from this genomic material are removed.

The use of synthetic RNA and plasmid DNA positive controls enables accurate quantification of targets and control over the level of positive template (i.e. controls can be added at levels approaching the limits of detection of the assay), allowing for consistency between test runs as well as greatly assisting troubleshooting should contamination occur. They also eliminate the need to source infected animals for positive control material. As AFDL implements diagnostic assays based on OIE and EU standards and ANZSDPs, the risk of test changes requiring redevelopment of positive controls is reduced, as these assays have generally undergone appropriate sensitivity and specificity testing. One drawback is that due to their specificity, they cannot be used for other assays targeting the same pathogen. However, the relative ease of design, purchase, evaluation, quality assurance checks and long-term stability of aliquots outweighs this negative aspect. The positive controls are also readily available from AFDL.

Currently, AFDL has over 170 molecular assays in routine use for the detection of pathogen and host nucleic acids and this list is growing as more pathogens emerge and assays become available (e.g. tilapia lake virus, scale drop disease virus, covert mortality nodavirus, salmon alphavirus, shrimp EF1). Through implementation of these tests several limitations of widely-used endogenous internal positive control assays have been identified including: the failure of the most common 18S real-time PCR assay to detect crustacean DNA, the propensity for 18S contamination in negative control wells due to its ubiquitous nature and the failure of the OIE Decapod PCR to detect all decapod species. For example, the OIE Decapod PCR (OIE, 2019) does not detect nucleic acid derived from western king prawns (*Melicertus latisulcatus*) or freshwater crayfish (*Cherax* spp.), which required the evaluation and implementation of additional assays. A number of species-specific endogenous internal positive control assays have been developed; however, these assays are limited to the most commonly cultured species. For example, a salmon ELF1 mRNA endogenous control RT-qPCR is used by AFDL when samples derived from salmon or trout are tested. Given the number of different species from which samples are obtained, from finfish, crustacean and mollusc hosts, it is prohibitive to evaluate and implement species-specific assays for all species from which samples may be derived, unless required for specific purposes (e.g. contracted export certification testing). In cases where tests have not been validated on specific species, additional evaluation is required.

A number of generic approaches have been adopted to establish a more reliable, robust and cost-effective internal positive control strategy which relies on exogenous internal positive controls. This approach involves using an unrelated (heterologous) template to spike (i.e. introduction of a quantified amount) every sample. Exogenous heterologous internal positive controls are the most suitable for aquatic animal health assays (due to the wide range of different species from which samples can be derived) as they are dependent on the use of separate primers and probe and one control could be used for all RNA assays and another for all DNA assays. Use of standardised heterologous internal positive controls assays within a laboratory network also

facilitates troubleshooting of test failures, training of new staff, and comparative evaluation of new reagents and platforms. The basis for an exogenous heterologous control was identified in the form of bacteriophages (phages) which would enable development of one internal control assay for RNA and one for DNA, regardless of the species the samples are derived from. Optimisation of each assay would still be required if multiplexed, however, one internal control and one set of primers/probe for any assay reduces costs, eliminates 18S contamination issues and is applicable for all classes of host (i.e. finfish, mollusc, crustacean).

Objectives

1. Produce quantified synthetic RNA positive control material for conventional and real-time RT-PCR assays, available on request.

2. Produce quantified synthetic DNA positive control material for conventional and real-time PCR assays, available on request.

3. Optimised universal internal control based on plant viral RNA and DNA and/or species-specific genes for use in molecular assays completed and implemented.

4. Technology transferred and adopted by participating laboratories.

Methods

Objectives 1 and 2: Produce quantified synthetic RNA and DNA positive control material for conventional and real-time RT-PCR assays, available on request

1.1 *In silico* analysis and design of controls

1.1.1 Real-time PCR and RT-PCR positive controls

For each real-time PCR assay targeting DNA viruses (i.e. qPCR tests), the sequence between the forward and reverse PCR primers was selected from a well-characterised strain of the target pathogen. The artificial universal positive control probe sequence described by Snow et al (2009), 5'- **VIC**-ACC GTC TAG CAT CCA GT-**TAMRA**-3, was then inserted into the target sequence at either 5' or 3' of the target probe sequence depending on the position of the specific probe. The construct used for the OIE Martenot OsHV-1 qPCR is provided as an example of the positive control approach for DNA targets (Figure 1).

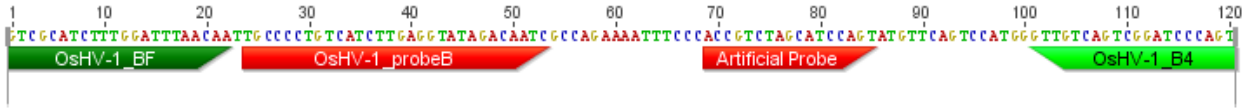


Figure 1. Plasmid construct designed for the OIE Martenot OsHV-1 qPCR

For real-time PCR assays targeting RNA viruses (i.e. RT-qPCR tests) the same approach was used, however nine bases 5' of the forward primer and nine bases 3' from the reverse primer of the target sequence were included. This was to ensure efficient transcription of the target DNA into RNA and it was not considered optimal to have this commencing or terminating immediately prior to or after a primer-binding sequence. The construct used for the CSIRO Tasmanian Atlantic salmon reovirus (TSRV) RT-qPCR is provided as an example of the positive control approach for DNA targets (Figure 2).

Figure 2. Plasmid construct designed for the CSIRO TSRV RT-qPCR

Sequences were submitted to the Integrated DNA Technologies[1] website for commercial preparation of the plasmids containing the inserts. DNA targets were prepared in the pIDTSMART plasmids, and RNA targets in the pIDTBlue plasmid which contains T3 and T7 promoters for *in vitro* RNA transcription, if required and obtained from Integrated DNA Technologies. Plasmid construct details and references for real-time PCR assays are in Appendix 1.

1.1.2 Conventional PCR and RT-PCR positive controls

For positive controls for conventional PCR and RT-PCR assays, the same approach as that used for the real-time PCR assays was used except, except, an alternate 72 bp unique sequence was inserted into the centre of the target sequence to replace the existing sequence. When nucleotides from the 72 bp sequence are translated into amino acids the positive control sequence can be easily distinguished from the real genomic sequence as the alternate artificial 72bp sequence, used in all conventional PCR plasmid positive controls, spells "PLASMIDMADEATAAHLPLASMID" when the nucleic acid sequence is are translated to an amino acid sequence. The translated amino acid construct used for the OIE YHV/GAV RT-nPCR is provided as an example

[1] https://sg.idtdna.com/

of the positive control approach for conventional PCR targets (Figure 3). Plasmid construct details and references for conventional PCR assays are in Appendix 2.

YHV1 Plasmid Control: YYAKLPYYNTSQIYFEKSTPLASMIDMADEATAAHLPLASMIDYHTLENRYQSEKAKYLG

YHV1 Genome: YYAKLPYYNTSQIYFEKSTTVIAYNGPQNKLSNMYTDNIKPFPYHTLENRYQSEKAKYLG

Figure 3. Amino acid translation of the YHV1 plasmid positive control containing the 72 bp inserted sequenced compared to the corresponding amino acid translation of the real genomic YHV1 sequence.

1.2 Preparation of assay-specific positive controls

Controls were prepared from plasmids purchased from the commercial supplier (Integrated DNA Technologies, https://sg.idtdna.com/site). Based on transcript length and concentration, determined by Qubit Fluorometer (Thermo Fisher), stock plasmids were made to 10^9 or 10^8 copies/µL. After determination of analytical sensitivity (ASe), and assessment of suitability, a standard procedure was used for the preparation of stock and working dilutions. Preparation of the plasmid dilutions and volumes stored for the OIE OsHV-1 Martenot qPCR is provided, as a typical example, in Table 1. Final working stock positive controls were diluted in TE diluent (10 mM Tris, 1 mM EDTA) containing 50 ng/µL transfer RNA as a stabiliser and to maintain homogeneity by reducing non-specific binding of the plasmid to the plastic tube. Plasmids were not linearised as results of repetitive testing, including quality control checks (homogeneity, stability and repeatability checks), indicated that this was not required. Linearisation of plasmids can improve other processes, including transcription of RNA and transfection, but opinion is divided as to whether it is really required for plasmid positive controls. The type of plasmid may have an impact and as no issues were identified with the use of non-linearised plasmids, no plasmids were linearised.

Regarding the preparation and routine use of synthetic RNA (as positive controls for RNA viruses rather than DNA plasmids) a proof-of-concept study was undertaken. Synthetic RNA was transcribed from the ISAV EUNA8 and USyd NNV plasmids using the MAXIscript Kit (Applied Biosystems). To avoid potential waste, no other synthetic RNA was transcribed until homogeneity and stability testing had been completed for this transcribed RNA. All plasmid and synthetic RNA stocks and working dilutions were stored at $\leq$ -40°C. Further work on synthetic RNA was discontinued since studies on a generic exogenous RNA control (QBeta) showed promise and concerns about stability of synthetic RNA have not been addressed. Evidence of incomplete removal of plasmid DNA from the transcribed RNA following DNAse treatment also raised concerns with the consistency of producing an RNA population free of plasmid DNA for subsequent analysis

1.3 Determination of analytical sensitivity (ASe)

For real-time PCR assays, to determine the ASe (or limit of detection) for each plasmid, 10-fold serial dilutions were prepared in both water and a stock of DNA derived from the host species. The host DNA was generated from known negative, pooled material, at a concentration representative of that produced from a typical diagnostic sample. Each 10-fold dilution was tested using five replicates, with positive results required from at least three replicates for that dilution to be considered positive. For conventional assays, 10-fold dilutions were prepared as above. Each dilution was tested in singlicate. The aim of determining the ASe for each of the plasmids was to assess the sensitivity of the molecular test used as part of the evaluation of the molecular assay. For this reason, plasmids were diluted in host DNA prior to testing and were not diluted in negative tissue homogenates, extracted and then tested.

Plasmids were tested according to standard procedures in place in AFDL:

- Real-time PCR assays targeting DNA (qPCR): each 25 µl reaction mix is made up of 2 µl extracted nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular-grade water. The qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value. Specific protocol details are provided with the plasmids.

- Real-time PCR assays targeting RNA (RT-qPCR): each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× RT-PCR buffer, 1 µl 25× RT-PCR enzyme mix (AgPath-ID™ One-Step RT-PCR, Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular grade water. The RT-qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 30 min reverse transcription at 48°C followed by an initial 10 min denaturation at 95°C followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value. Specific protocol details are provided with the plasmids.

- Conventional PCR targeting DNA (PCR): each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl HotStarTaq Master Mix (Qiagen) and 360 nM of each primer. Cycling conditions vary according to the specific assay and specific protocol details are provided with the plasmids.

- Conventional PCR targeting RNA (RT-PCR): each 25 µl reaction mix is made up of 2 µl extracted nucleic acid, 12.5 µl 2× reaction mix and 1 µl Superscript III/Platinum Taq mix (Invitrogen) and a final concentration of 180 nM of each primer. Cycling conditions vary according to the specific assay and specific protocol details are provided with the plasmids.

Table 1. Preparation of plasmid dilutions and volumes stored.

pOsHV-1_Martenot_qPCR

Transcript length:	**2127**	nucleotides
Concentration:	**10.8**	ng/µL
Concentration:	**1.08E-08**	g/µL

Calculation: (X g/µL DNA / [plasmid length in basepairs x 660]) x 6.022×10^{23} = Y molecules/µL

Calculation: (1.18×10^{-6} g/µL / [2792x 660]) x 6.022×10^{23}

Part 1:	**7.69329E-15**
Part 2:	**4,632,901,654** molecules/µL

Dilutions to be done to prepare Master Stock in water:

	C1	V1	C2	V2	Dilution factor
	4,632,901,654	10	1,000,000,000	46.3	4.632901654
Therefore	10 µL (Neat) +	36.3	diluent =	46.3	µL (10^9 copies/µL)

Analytical sensitivity (ASe):

Stock of 10^9 copies/µL is used to prepare replicate 10-fold serial dilutions to 10^{-1} in water and host DNA to determine the analytical sensitivity (ASe), efficiency etc. of the PCR

Dilutions for Positive Control aliquots

Diluent: TE (10mM Tris, 1mM EDTA, pH8) with tRNA (50ng/µL)

			Storage location	Volume stored
10^8 copies/µL	100 µL (10^9 copies/µL) + 900 µL diluent	(1000 µL)	Store in Liquid Nitrogen	1 x 900µL
10^7 copies/µL	100 µL (10^8 copies/µL + 900 µL diluent	(1000 µL)	Store in Liquid Nitrogen	1 x 900µL
10^6 copies/µL	100 µL (10^7 copies/µL) + 900 µL diluent	(1000 µL)	Store in Liquid Nitrogen	1 x 900µL
10^5 copies/µL	500 µL (10^6 copies/µL) + 4500 µL diluent	(5000 µL)	Store at -40°C	4 x 1mL
10^4 copies/µL	1000 µL (10^5 copies/µL) + 9000 µL diluent	(10000 µL)	Store at -40°C	48 x 200µL
10^3 copies/µL	500 µL (10^4 copies/µL) + 4500 µL diluent	(5000 µL)	Store at -40°C	4 x 1mL
10^2 copies/µL	1000 µL (10^3 copies/µL) + 9000 µL diluent	(10000 µL)	Store at -40°C	48 x 200µL
10^1 copies/µL	100 µL (10^2 copies/µL) + 900 µL diluent	(1000 µL)	Not stored	
10^0 copies/µL	100 µL (10^1 copies/µL) + 900 µL diluent	(1000 µL)	Not stored	
10^{-1} copies/µL	100 µL (10^0 copies/µL) + 900 µL diluent	(1000 µL)	Not stored	

1.4 Preparation of working stocks for distribution and quality assurance testing

Based on very consistent results for the real-time assays, and previous experience providing plasmids as Network Quality Controls (NQCs) for the Laboratories for Emergency Animal Disease Diagnosis and Response (LEADDR) network, positive controls at concentrations of approximately 10^4 and 10^2 copies/µL were prepared for each assay. For convenience these are referred to as NQC-1 and NQC-2 (for each assay). Two controls were prepared to reduce the need for assay retests in the event of only one control being outside acceptable limits. Both controls producing results outside acceptable limits would be indicative of a serious test failure, requiring retesting of all test samples, whereas one positive control failing would most likely be due to operator error and retesting of all test samples would not necessarily be required. Due to only one reading being used for the initial determination of the concentration of the reconstituted plasmid, the plasmids are not considered to be accurately quantified for use in standard curves to determine target template copy number.

Forty-eight aliquots have been prepared for each concentration of each plasmid and stored at ≤ -40°C. For each assay, aliquots were tested for each of the 10^4 and 10^2 copies/µL concentrations with the specific pathogen probe and artificial probe as singleplex and multiplex assays. Both assay formats were tested to assess the effect of multiplexing on assay sensitivity.

During repeatability testing of the real-time PCR plasmids, issues were identified with homogeneity for some plasmids. It was observed that replicates would occasionally produce C_T values well below the expected value. Extensive troubleshooting, including detailed discussions with the supplier, indicated that this was most likely caused by aggregation of the plasmid, with detection of the aggregates being a random event. This was investigated and the issue resolved by heating the reconstituted plasmid stock to 95°C for 5 minutes prior to any quantitation, dilution and testing.

Objective 3. Optimised universal internal control based on plant viral RNA and DNA and/or species-specific genes for use in molecular assays completed and implemented

2.1 Literature search for internal control assays

Through implementation of assays detecting pathogens from finfish, crustacean and molluscan hosts, several limitations to accepted endogenous internal positive control assays have been identified, in particular the failure of the most common 18S rDNA real-time assay to detect crustacean DNA, the propensity for 18S contamination in negative control wells, and the failure of the OIE Decapod PCR to detect all decapod species. Given the number of different host species from which samples are obtained it is cost- and time-prohibitive to develop, evaluate and implement assays for each and every species from which samples may be derived. Additionally, there is an unrealistic cost associated with maintenance of primers and probe for assays that are used infrequently, and which would require regular check-testing as part of a laboratory quality system. Literature searches using Science Direct and Web of Science were undertaken to determine the availability of potential internal control assays.

2.2 Preliminary evaluation of endogenous and heterologous internal positive control assays

Literature searches using Science Direct and Web of Science yielded very few assays specifically designed as internal control, housekeeping gene assays for molecular diagnostics tests for aquatic animals. Therefore, the decision was made to concentrate on a generic approach relying on exogenous internal positive controls. This approach involves the use of an unrelated template to spike every sample. Exogenous positive controls are the most suitable for aquatic animal diagnostic assays (due to the wide range of different species from which samples can be derived) as they rely on separate primers and probe, and one exogenous positive control could be used for all RNA assays and another for all DNA assays. Use of standardised, exogenous,

internal positive controls assays within a laboratory network also facilitates rapid troubleshooting of test failures, training of new staff, and comparative evaluation of new reagents and platforms.

For implementation of endogenous and heterologous internal positive control assays, MS2 phage for RNA targets and T4 phage for DNA targets were evaluated. MS2 and T4 were chosen based on implementation for routine use in clinical medical testing (Ninove *et al.*, 2011) and recommendations from colleagues undertaking molecular testing for terrestrial animal pathogens. However, due to inconsistent supplies of MS2, which would make it difficult for other laboratories interested in using these controls in the future, an alternative RNA phage, referred to as QBeta, was sourced and evaluated. The protocol design was to seed homogenised tissue homogenates prior to extraction with either T4 or QBeta, depending on whether or not the genome of the specific pathogen being tested for was DNA or RNA. Therefore, the phage would act as a control to determine if the extraction process had been run as expected (and nucleic acid was extracted) and provide an indication of the level of any inhibition of the subsequent molecular assay, due to the sample matrix that was extracted.

2.3 Evaluation of T4 and QBeta phages for use as heterologous internal positive controls

For both T4 and QBeta phages, real-time probe-based assays (Table 2) were evaluated and tested according to the standard qPCR and RT-qPCR conditions described in Section 1.3. As analytical sensitivity was determined to be acceptable when the assays were used on 10-fold serial dilutions of quantified phage nucleic acid, additional trials were run to:

1. determine the quantity of phage used to seed tissue to obtain repeatable results with relatively high C_T values (≥30).
2. determine the effect of different host tissues regarding potential PCR inhibition.
3. determine repeatability using samples of known and unknown pathogen status.
4. evaluate the use of phage in routine testing protocols.

Table 2. Primer and probe sequences for the DNA (T4) and RNA (QBeta) heterologous internal positive controls

Primer/Probe	Sequence	Reference
T4F	5'-CCATCCATAGAGAAAATATCAGAACGA	Ninove *et al.* (2011)
T4R	5'- CGCTGGGAAAAGAGGAATTATTTA	
T4probe	5'-**VIC**-ACCAGTAATTTCATCTGCTTCTGATGTGAGGC-**QSY**-3'	
*QBeta Atto F	Sequence not available for distribution	Attostar (www.attostar.com)
*QBeta Atto R	Sequence not available for distribution	
*QBeta Atto Pr	Sequence not available for distribution	

*Sequence is not currently publicly available although primers and probe can be purchased from Attostar

2.3.1 Determine the quantity of phage used to seed tissue to obtain repeatable results with relatively high C_T values (≥30)

Analytical sensitivity testing, using 10-fold dilutions, was used to determine the required dilution and volume of the stock T4 and QBeta phages to generate C_T values of approximately 30 that would be used in subsequent assessment. The effect of host tissues on detection and repeatability of detection were undertaken using the T4 phage. This evaluation protocol was then used for QBeta.

2.3.2 Investigation of the potential inhibition of host tissues on the detection of spiked T4

Representative tissues from prawns, abalone and oysters were used to assess the effect of host nucleic acid on inhibition of detection of the T4 phage. Finfish tissue was not used, as nucleic acid extracted from these tissues (or cell culture where finfish viruses have been isolated) are generally not as inhibitory to PCR as tissues from prawn, abalone or oyster species. The sample tissues used were 500µL of the ethanol-fixed tissue homogenate prepared as negative control material for proficiency testing panels. Tissues were processed and extracted with the extraction protocols using the extraction kits (i.e. QIAamp DNA Mini Kit, QIAamp Viral RNA Mini Kit and MagMAX-96 Viral RNA Isolation Kit) in routine use in AFDL with 5µL of stock T4 added, at dilutions of 1/100, 1/1000 and 1/10000, before the samples were applied to spin columns or added to deep-well plates for bead extractions. Three replicates of each sample containing each dilution of phage were extracted and tested in singlicate using the T4 qPCR primers (Table 2).

In addition, based on previous experiences with specific hosts and pathogens, a number of targeted evaluations were undertaken:

1. T4 was evaluated with a molluscan pathogen using diagnostic samples (gill and mantle) that were submitted to AFDL for a *Bonamia* investigation (i.e. using "real" samples rather than highly processed proficiency testing samples). The samples had 5µl of T4 phage at a dilution of 1/100 added to the stock ATL/Proteinase K digestion buffer. Samples were extracted and tested undiluted and diluted 1/10 using the CSIRO *Bonamia* sp. qPCR, standard 18S qPCR and T4 qPCR.

2. To determine if prawn tissue (muscle) inhibits the T4 qPCR, 5 µl of diluted T4 phage at 1/10, 1/100, 1/1000, 1/10000 was added to two volumes of clarified homogenate from homogenised ethanol-fixed prawn muscle tissue (50 µl and 500 µl) with extraction using the Viral RNA Mini Kit (Qiagen). Using 50 µl of tissue (1/10 dilution of what was extracted previously) would indicate if excess tissue was the cause of PCR inhibition seen with undiluted tissue, while adding a 1/10 dilution of T4 phage would also show if addition of more phage overcame any sensitivity problem with the T4 detection.

3. Duplicate tissues (cuticular epithelium) were processed from a submission of imported commodity prawns, which consisted of six bags with five prawns/bag. Each sample consisted of a pool of tissue collected from each of the five prawns in each bag which was then homogenised by bead-beating. Homogenised tissues from one of each duplicate sample collected was seeded with T4 (5 µL of a 1/100 dilution) and all samples were processed and tested for WSSV according to the standard AFDL protocol, using the CSIRO WSSV qPCR. Each nucleic acid extraction was tested undiluted and diluted 1/10.

Similar experiments were undertaken with QBeta. Homogenates generated from a typical submission of imported commodity prawns were seeded with three dilutions of QBeta prior to extraction with the MME96 bead extraction system (Applied Biosystems) and nucleic acids tested undiluted and diluted 1/10 with the QBeta RT-qPCR. QBeta diluted in water was run as a positive control.

The use of T4 was further assessed during preliminary investigation into the use of swabs, as an alternative sample to cuticular epithelium, and comparison with sampling tissue biopsies, gills and pleopods for detection of WSSV. This work is being undertaken as part of an ongoing DAWE-funded project "Pool-level sensitivity for aquatic animal pathogens of national and trade significance". A submission had been tested

using cuticular epithelium as the tissue type sampled and tested using the CSIRO WSSV qPCR. The prawns in this submission were re-sampled using swabbing as the sampling method, with T4 added to the extraction buffer. The swabbing protocol was kindly provided by EMAI (Peter Kirkland, pers.com.) and as it is yet to be published cannot be detailed in this report.

Further evidence of the usefulness of T4 as an exogenous, heterologous internal positive control was obtained during the WSSV Emergency Response in 2017. A range of atypical samples of high importance were submitted (Submission 5; Table 16) where the performance of currently available housekeeping gene assays (e.g. 18S, Decapod PCR, other) was unknown and not possible to validate due to short expected test turn-around times. Prior to extraction, samples were spiked with 5 µL of a 1/100 dilution of T4 and extracted and tested using the CSIRO WSSV qPCR, OIE WSSV qPCR, 18S qPCR and T4 qPCR according to standard procedures. With this testing, T4 was added to the extraction kit lysis buffer, with the negative extraction control (containing T4) acting as a T4 positive control and providing the expected T4 C_T value used to assess potential inhibition of extracted tissues samples.

Objective 4. Technology transferred and adopted by participating laboratories

3.1 Distribution of plasmid positive controls

Aquatic animal diagnostic laboratories, both within Australia (e.g. state government and commercial laboratories) as well as overseas (e.g. national diagnostic laboratories) were made aware of this project and the availability of the various control reagents, made on request. The controls were also offered to laboratories requesting positive control material from AFDL in its roles as an OIE Reference Laboratory for infection with YHV1, AbHV, EHNV and Ranavirus. The controls were also provided to collaborative projects where test implementation was a component.

Requests for reagents were actioned as soon as possible with a Materials Transfer Agreement (MTA) between the requesting institute and CSIRO ACDP drafted and executed prior to transfer of materials.

Conditions detailed in the MTA were as follows:

- CSIRO has agreed to transfer positive control material, including non-infectious plasmid controls, ethanol-fixed tissues and tissue culture supernatants
- Materials are to be used by the laboratory to assist in establishing molecular tests.
- The recipient agrees:
  (1) to provide CSIRO with methods and results using these positive controls to ensure that they are performing as expected
  (2) materials are not to be transferred to any Third Parties without approval from CSIRO
  (3) a draft of any publication using these materials be provided to CSIRO for review prior to submission for publication.

The positive control plasmids were provided with documentation describing the number and volume of vials being transferred, the specific assay the controls were to be used for and the expected C_T value for each of the two concentrations of positive control provided.

3.2 Provision of advice regarding implementation of protocols and troubleshooting based on feedback from participating laboratories

On request, CSIRO provided advice regarding implementation of protocols and troubleshooting on feedback from participating laboratories, particularly when laboratories did not produce expected results when using the controls.

Results and Discussion

Objectives 1 and 2: Produce quantified synthetic RNA and DNA positive control material for conventional and real-time RT-PCR assays, available on request

1.1 Determination of analytical sensitivity (ASe)

Limits of detection for the real-time assays varied from 2 to 200 copies regardless of whether the plasmid was diluted in water or host DNA, with a mode of 20 and 2 copies, respectively (Table 3). More often than not, dilution of plasmid in host DNA seemed to lead to a decrease in the limit of detection, possibly due to preferential non-specific binding of host DNA to the tube. However, where there were differences in ASe between plasmids diluted in water and host DNA, the difference was never greater than one 10-fold dilution. The ASe of the transcribed synthetic RNA for the ISAV EUNA8 and USyd NNV RT-qPCR assays was 10-fold higher than the ASe of the plasmids for these assays.

The ASe for the plasmids targeted by the conventional PCR assays was a lot more variable than that for real-time PCR assays (Table 4). This is most likely due to the greater variation in primer design parameters, cycle number and amplicon length for these assays and the lower sensitivity of the indicator system used for conventional PCR assays (i.e. agarose gel electrophoresis compared to fluorescence measurement). As expected, addition of a nested PCR significantly increased assay sensitivity.

Table 3. Analytical sensitivity (ASe) of plasmids developed for real-time PCR assays targeting DNA pathogens. Limit of detection endpoint is boxed and shaded grey.

Plasmid diluted in water	C_T value											
Plasmid Copies	AbHV ORF49	AbHV ORF66	AbHV ORF77	WSSV CSIRO	WSSV OIE	OsHV-1 Martenot	OsHV-1 EMAI	Megalo-cytivirus CSIRO	AHPND OIE	*Bonamia* spp. CSIRO	*Perkinsus* spp. Gauthier	*Perkinsus olseni* AFDL
200,000,000	11.85	11.04	11.42	5.68	10.48	9.19	10.75	10.33	11.35	7.22	8.19	6.40
20,000,000	15.64	14.9	15.64	7.55	13.49	14.47	14.99	14.87	14.71	11.48	12.37	10.14
2,000,000	19.01	18.21	18.84	12.21	17.11	17.21	18.54	18.81	18.39	15.91	16.75	14.79
200,000	22.48	21.5	22.58	15.78	20.66	20.47	22.02	21.98	21.39	18.78	19.97	18.44
20,000	25.44	25.3	26.04	19.49	24.31	24.68	24.94	25.96	24.78	22.54	23.68	22.01
2,000	29.52	28.73	30.21	23.15	27.45	27.82	28.84	29.28	29.48	25.99	27.30	25.72
200	34.39	32.99	34.83	27.3	31.97	31.44	34.6	33.62	33.11	29.40	31.12	29.58
20	Neg	37.08	38.61	30.82	35.6	33.95	36.11	37.11	36.60	32.82	34.86	33.10
2	Neg	Neg	Neg	34.51	39.02	36.8	Neg	Neg	Neg	35.87	38.21	37.07
0.2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Plasmid diluted in host DNA	C_T value											
Plasmid Copies	AbHV ORF49	AbHV ORF66	AbHV ORF77	WSSV CSIRO	WSSV OIE	OsHV-1 Martenot	OsHV-1 EMAI	Megalo-cytivirus CSIRO	AHPND OIE	*Bonamia* spp. CSIRO	*Perkinsus* spp. Gauthier	*Perkinsus olseni* AFDL
200,000,000	10.39	10.18	11.07	6.94	10.85	8.62	10.76	10.07	8.54	7.45	8.46	5.88
20,000,000	14.73	13.57	13.57	11.11	14.28	13.56	14.42	14.06	13.10	11.27	12.35	10.11
2,000,000	18.2	17.14	18.26	15.84	17.47	16.82	17.91	17.61	16.38	14.62	15.84	13.93
200,000	21.71	20.36	22.19	18.3	21.09	20.3	20.52	20.72	19.56	17.87	19.18	17.49
20,000	24.96	24.16	26.54	22.91	24.74	23.6	24.9	24.38	22.87	21.21	22.68	21.09
2,000	28.39	28.49	29.6	26.86	28.18	26.94	27.27	27.81	26.23	24.52	26.06	24.76
200	32.54	31.45	32.81	30.31	31.61	30.76	30.96	31.66	29.53	27.75	29.47	28.34
20	34.35	36.54	38.18	34.13	35.87	33.79	35.78	34.73	32.93	30.98	32.97	32.02
2	Neg	38.01	39.31	Neg	37.96	37.13	Neg	38.13	35.92	33.93	36.36	35.69
0.2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	34.66	39.43	39.86

Table 3 (cont.). Analytical sensitivity (ASe) of plasmids developed for real-time PCR assays targeting RNA pathogens. Limit of detection endpoint is boxed and shaded grey. For the ISAV EUNA8 and NNV USyd assays synthetic RNA was transcribed and tested to compare with plasmid DNA.

Plasmid diluted in water	C_T value										Synthetic RNA	
Plasmid Copies	VHSV Jonstrup	VHSV Garver	TSV OIE	TSRV CSIRO	SVCV Zhang	POMV CSIRO	YHV1 CSIRO	ISAV EUNA7	ISAV EUNA8	NNV USyd	ISAV EUNA8	NNV USyd
200,000,000	NA	6.33	9.89	8.79	8.79	6.46	7.24	11.25	7.54	8.94	16.54	15.67
20,000,000	NA	12.83	14.05	13.98	13.98	13.53	12.67	15.53	13.49	13.87	19.82	18.89
2,000,000	8.99	17.01	17.51	17.43	17.43	16.58	15.89	19.11	16.89	16.66	24	22.67
200,000	14.58	18.24	21	20.69	20.69	19.64	19.47	22.39	20.38	20.03	27.13	25.75
20,000	18.03	22.06	24.67	24.9	24.9	22.51	22.86	26.07	23.97	23.31	30.92	29.18
2,000	1.41	26.24	28.17	28.49	28.49	27.94	27.48	30.04	27.46	26.37	34.15	33.1
200	25.16	28.76	31.91	31.2	31.2	31.42	32.78	33.71	31.4	30.41	37.73	37.11
20	28.64	30.71	34.86	36.72	36.72	35.38	35.84	37.68	35.5	35.54	Neg	Neg
2	31.64	34.27	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
0.2	Neg	36.27	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Plasmid diluted in host DNA	C_T value										Synthetic RNA	
Plasmid Copies	VHSV Jonstrup	VHSV Garver	TSV OIE	TSRV CSIRO	SVCV Zhang	POMV CSIRO	YHV1 CSIRO	ISAV EUNA7	ISAV EUNA8	NNV USyd	ISAV EUNA8	NNV USyd
200,000,000	NA	8.20	10.27	8.37	8.37	7.04	7.72	10.98	10.91	8.43	15.33	14.22
20,000,000	NA	12.78	13.73	12.83	12.83	11.05	11.95	14.53	14.12	12.3	18.58	17.55
2,000,000	12.60	15.87	17.58	16.53	16.53	14.91	15.75	18.51	17.75	15.58	21.47	20.81
200,000	15.18	19.19	20.49	19.78	19.78	18.87	19.45	21.7	21.66	18.87	24.63	23.77
20,000	18.32	22.42	24.32	23.89	23.89	22.06	23.35	25.13	24.82	22.22	27.49	26.75
2,000	23.26	25.46	27.03	27.31	27.31	25.97	27.5	28.48	28.74	25.34	30.08	29.97
200	26.53	27.85	29.95	29.98	29.98	30.1	31.06	32.35	32.60	28.26	34.17	34.06
20	29.75	30.34	34.81	33.91	33.91	Neg	Neg	36.56	35.23	31.79	Neg	38.00
2	32.67	33.79	37.54	36.09	36.09	Neg	Neg	Neg	Neg	Neg	Neg	Neg
0.2	41.57	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Limit of detection boxed and shaded in grey; Neg = negative

Table 4. Analytical sensitivity (ASe) of plasmids developed for single-step conventional PCR assays.

Plasmid diluted in water										
Plasmid Copies	EHNV MCP1 PCR	EHNV MCP2 PCR	ISAV RT-PCR	TABV RT-PCR	VHSV RT-PCR	TSV RT-PCR	WSSV PCR	YHV1 RT-PCR	NNV RT-PCR	POMV RT-PCR
200,000,000	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
20,000,000	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
2,000,000	POS	POS	POS	POS	POS	POS	POS	wPOS	POS	wPOS
200,000	POS	POS	POS	POS	POS	POS	POS	vwPOS	POS	wPOS
20,000	POS	POS	POS	POS	POS	wPOS	POS	Neg	POS	Neg
2,000	POS	wPOS	POS	wPOS	POS	wPOS	POS	Neg	POS	Neg
200	wPOS	wPOS	wPOS	wPOS	wPOS	Neg	wPOS	Neg	wPOS	Neg
20	Neg	Neg	wPOS	Neg	Neg	Neg	wPOS	Neg	Neg	Neg
2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
0.2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Plasmid diluted in host DNA										
Plasmid Copies	EHNV MCP1 PCR	EHNV MCP2 PCR	ISAV RT-PCR	TABV RT-PCR	VHSV RT-PCR	TSV RT-PCR	WSSV PCR	YHV1 RT-PCR	NNV RT-PCR	POMV RT-PCR
200,000,000	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
20,000,000	POS	POS	POS	POS	POS	POS	POS	POS	POS	POS
2,000,000	POS	POS	POS	POS	POS	POS	POS	wPOS	POS	POS
200,000	POS	POS	POS	POS	POS	POS	POS	wPOS	POS	wPOS
20,000	POS	POS	POS	POS	POS	wPOS	POS	Neg	POS	wPOS
2,000	POS	POS	POS	wPOS	POS	wPOS	POS	Neg	POS	Neg
200	wPOS	wPOS	POS	wPOS	wPOS	Neg	wPOS	Neg	wPOS	Neg
20	wPOS	Neg	POS	Neg	wPOS	Neg	wPOS	Neg	Neg	Neg
2	Neg	Neg	wPOS	Neg	Neg	Neg	Neg	Neg	Neg	Neg
0.2	Neg	Neg	wPOS	Neg	Neg	Neg	Neg	Neg	Neg	Neg

POS=amplicon of the expected size, deemed to be repeatable; vwPOS=very weak positive; wPOS=weak positive. vwPOS and wPOS excluded form ASe determinations due to lack of repeatability; Neg=negative.

Table 4 (cont.). Analytical sensitivity (ASe) of plasmids developed for nested conventional PCR assays.

Plasmid diluted in water				
Plasmid Copies	WSSV nPCR	YHV1 nPCR	NNV nPCR	POMV nPCR
200,000,000	POS	POS	POS	POS
20,000,000	POS	POS	POS	POS
2,000,000	POS	POS	POS	POS
200,000	POS	POS	POS	POS
20,000	POS	POS	POS	POS
2,000	POS	POS	POS	POS
200	POS	POS	POS	POS
20	POS	POS	POS	POS
2	Neg	Neg	POS	POS
0.2	Neg	Neg	Neg	Neg
Plasmid diluted in host DNA				
Plasmid Copies	WSSV nPCR	YHV1 nPCR	NNV nPCR	POMV nPCR
200,000,000	POS	POS	POS	POS
20,000,000	POS	POS	POS	POS
2,000,000	POS	POS	POS	POS
200,000	POS	POS	POS	POS
20,000	POS	POS	POS	POS
2,000	POS	POS	POS	POS
200	POS	POS	POS	POS
20	POS	POS	POS	POS
2	Neg	POS	Neg	POS
0.2	Neg	Neg	Neg	POS

1.2 Preparation of working stocks for distribution and quality assurance testing

For each assay, aliquots were tested for each of the 10^4 and 10^2 copies/µL concentrations with the specific pathogen probe and artificial probe as singleplex and multiplex assays. Both assay formats were tested to assess the effect of multiplexing on assay sensitivity. This was done as a previous evaluation of a multiplexed assay, using two multiplexed pathogen-specific probes in the one master mix demonstrated that multiplexing led to a 100-fold decrease in assay sensitivity compared to using two singleplex assays for the pathogen-specific assays being evaluated. However, multiplexing of the pathogen-specific and artificial-probes in the one real-time assay, evaluated for 18 assays targeting both DNA and RNA pathogens (Table 5) demonstrated that this had no appreciable effect on the expected result for either assay. This would indicate that duplexing by the addition of one additional probe does not introduce the same competition for reagents as combining two qPCRs (i.e. additional primers as well as additional probe) in the same tube.

Table 5. Comparison of testing using singleplex and duplex formats for pathogen-specific and artificial probes.

			Pathogen-specific Probe (Average $C_T \pm$ SD)		Artificial Probe (Average $C_T \pm$ SD)	
Pathogen	**Plasmid**	**Copies**	**Singleplex**	**Dualplex**	**Singleplex**	**Dualplex**
WSSV	CSIRO WSSV qPCR	10^4	23.73 ± 0.35	23.70 ± 0.36	25.89 ± 0.23	26.60 ± 0.41
		10^2	31.95 ± 0.49	30.56 ± 1.35	33.04 ± 0.82	33.00 ± 1.40
WSSV	OIE WSSV qPCR	10^4	25.23 ± 0.14	25.51 ± 0.12	28.48 ± 0.29	27.34 ± 0.23
		10^2	32.75 ± 0.33	33.18 ± 0.23	35.22 ± 0.78	34.79 ± 0.16
OsHV-1	EMAI OsHV-1 qPCR	10^4	25.26 ± 0.40	25.22 ± 0.22	27.54 ± 0.20	28.07 ± 0.12
		10^2	33.41 ± 0.59	33.11 ± 0.55	35.53 ± 0.85	35.70 ± 0.68
OsHV-1	OIE OsHV-1 Martenot qPCR	10^4	23.75 ± 0.08	24.02 ± 0.14	23.61 ± 0.48	24.06 ± 0.03
		10^2	31.34 ± 0.50	31.14 ± 0.37	30.75 ± 0.55	31.14 ± 0.34
Megalocytivirus	CSIRO *Megalocytivirus* qPCR	10^4	25.33 ± 0.29	25.53 ± 0.26	27.53 ± 0.41	27.02 ± 0.30
		10^2	32.49 ± 0.79	33.31 ± 0.39	35.32 ± 0.24	34.54 ± 0.41
AbHV	CSIRO AbHV ORF49 qPCR	10^4	26.03 ± 0.29	26.08 ± 0.47	30.36 ± 0.23	32.85 ± 0.80
		10^2	34.09 ± 0.27	33.79 ± 0.63	38.81 ± 0.42	39.61 ± 0.91
AbHV	OIE AbHV ORF66 qPCR	10^4	24.84 ± 0.33	24.92 ± 0.17	27.03 ± 0.26	28.45 ± 0.39
		10^2	31.76 ± 1.44	32.54 ± 0.46	34.63 ± 1.28	35.69 ± 0.73
AbHV	OIE AbHV ORF77 qPCR	10^4	26.53 ± 0.12	25.94 ± 0.47	28.18 ± 0.47	28.63 ± 0.51
		10^2	34.97 ± 0.35	34.47 ± 0.34	36.25 ± 0.32	36.86 ± 0.57
NNV	ANZSDP NNV RT-qPCR	10^4	23.00 ± 0.12	22.93 ± 0.10	25.34 ± 0.09	25.65 ± 0.18
		10^2	30.08 ± 0.16	30.18 ± 0.75	31.67 ± 0.55	32.22 ± 0.61
ISAV	ISAV EUNA7 RT-qPCR	10^4	24.42 ± 0.11	25.28 ± 0.64	23.52 ± 0.07	23.62 ± 0.02
		10^2	32.28 ± 0.13	32.17 ± 0.13	30.43 ± 0.03	30.52 ± 0.14
ISAV	ISAV EUNA8 RT-qPCR	10^4	25.02 ± 0.03	25.27 ± 0.05	24.79 ± 0.05	24.88 ± 0.05
		10^2	31.63 ± 0.13	31.96 ± 0.22	31.56 ± 0.08	31.67 ± 0.22
VHSV	OIE VHSV Jonstrup RT-qPCR	10^4	22.15 ± 0.15	22.49 ± 0.39	23.82 ± 0.13	23.74 ± 0.45
		10^2	30.26 ± 0.17	30.65 ± 0.09	32.02 ± 0.13	32.03 ±0.18
VHSV	OIE VHSV Garver RT-qPCR	10^4	21.78 ± 0.06	21.69 ± 0.06	21.56 ± 0.07	21.62 ± 0.06
		10^2	28.14 ± 0.13	28.59 ± 0.08	28.35 ± 0.08	28.44 ± 0.07
SVCV	SVCV Zhang RT-qPCR	10^4	21.90 ± 0.16	22.07 ± 0.16	22.17 ± 0.11	22.39 ± 0.05
		10^2	29.24 ± 0.15	29.37 ± 0.12	29.59 ± 0.05	29.66 ± 0.14
TSRV	CSIRO TSRV RT-qPCR	10^4	22.07 ± 0.05	22.57 ± 0.03	22.93 ± 0.06	23.16 ± 0.04
		10^2	29.54 ± 0.05	30.03 ± 0.22	30.43 ± 0.12	30.64 ± 0.17
POMV	AFDL POMV RT-qPCR	10^4	22.34 ± 0.13	22.87 ± 0.19	23.50 ± 0.07	23.68 ± 0.06
		10^2	29.74 ± 0.12	30.08 ± 0.19	30.90 ± 0.28	30.91 ± 0.27
YHV1	AFDL YHV1 RT-qPCR	10^4	23.41 ± 0.04	23.47 ± 0.08	22.66 ± 0.06	22.90 ± 0.04
		10^2	30.74 ± 0.12	30.82 ± 0.34	30.03 ± 0.11	30.31 ± 0.23
AHPND	OIE AHPND qPCR	10^4	23.45 ± 0.08	23.47 ± 0.05	22.64 ± 0.04	22.88 ± 0.04
		10^2	30.18 ± 0.10	30.36 ± 0.16	29.37 ± 0.11	29.84 ± 0.12

During repeatability testing of the real-time PCR plasmids, issues were identified with homogeneity for some plasmids. It was observed that replicates would occasionally produce C_T values well below the expected value (Table 6). Extensive troubleshooting, including detailed discussions with the supplier, indicated that this was most likely caused by aggregation of the plasmid with detection of the aggregates, causing the unexpectedly lower C_T values, being a random event.

Table 6. *Megalocytivirus* qPCR: Results of troubleshooting lower C_T values obtained during repeatability testing.

Megalocytivirus qPCR: Expected C_T values based on initial screen of stocks

Control	C_T1	C_T2	C_T3	C_T4	C_T5	Mean
NQC-1	26.00	25.72	25.4	25.85	25.33	25.66
NQC-2	31.22	33.25	32.4	33.04	32.53	32.49

Results of testing 05/01/2016

Control	C_T1	C_T2	Mean
NQC-1	25.14	25.26	25.20
NQC-2	27.43*	26.79*	27.11*

*expected CT is 32.49

Results of testing 06/01/2016

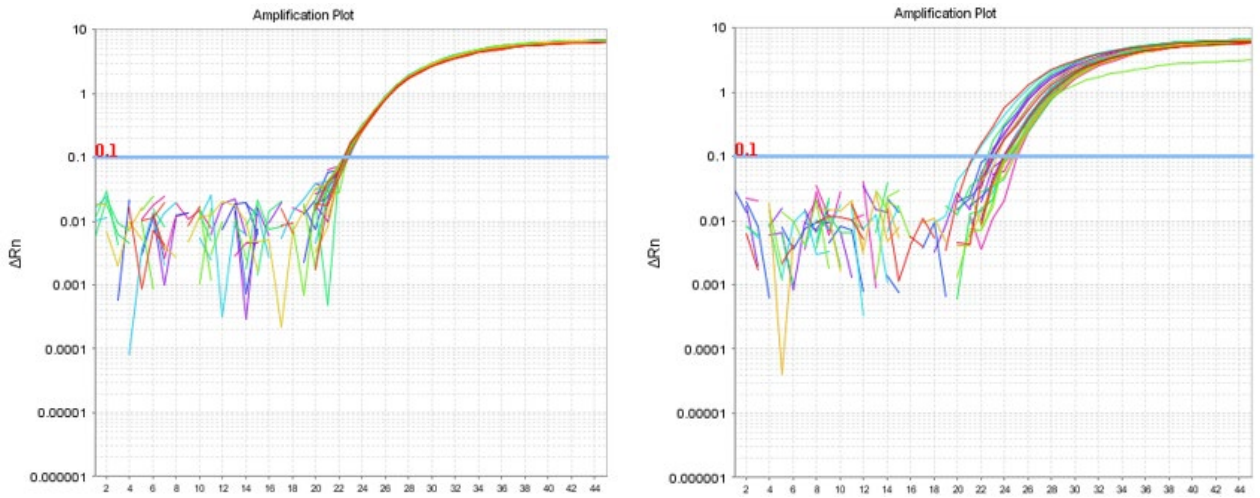
Control	C_T1	C_T2	C_T3	Mean
NQC-1	25.75	25.97	24.92	25.55
NQC-2	33.52	33.23	33.82	33.52

Control	C_T1	C_T2	C_T3	Mean
NQC-1	25.80	25.91	25.64	25.78
NQC-2	33.05	33.44	33.35	33.28

Results of testing NQC-2 on 05/01/2016 resulted in an observed mean C_T of 27.11 which was lower than the expected C_T of 32.49±0.5. Subsequent testing of two additional aliquots on 06/01/2016 produced C_T values (33.52 and 33.28) similar to the expected C_T value of 32.49. While only one example has been provided in Table 6, these unacceptably non-homogenous results were also observed with a number of other plasmids.

This phenomenon has never been observed with plasmids prepared in-house and from discussions with the manufacturer it may be due to the use of commercially-produced plasmids which are then lyophilised for transportation. The manufacturer suggested heating the plasmid preparations at 90°C for 10 minutes. This treatment resolved the issue with subsequent homogeneity testing producing acceptable results (for example, see Figure 4 and Table 7).

Consequently, all aliquots of the 10^4 and 10^2 copies/µL dilutions of all plasmids have been heated to 90°C for 10 minutes and checked for homogeneity. All have passed with examples of typical results (C_T values) of five aliquots tested in ten replicates presented in Table 8. Based on assessment of the performance of the different plasmid positive controls generated during this project, for real-time PCR assays, acceptance criteria for routine use has been established as follows; for the first ten test runs where the plasmid is used, the observed result is compared with the expected result as determined from homogeneity testing undertaken after aliquoting the plasmid. The mean C_T value the is then calculated from the results of the first 10 runs and the acceptance limit is this mean $C_T \pm 2C_T$. A list of the real-time PCR assays with positive control plasmids is provided in Appendix 3.



Heat-treated **Untreated**

Figure 4. Results of troubleshooting lower C_T values obtained during repeatability testing for the YHV-1 plasmid.

Table 7. YHV-1 plasmid: Results of troubleshooting lower C_T values obtained during repeatability testing.

Sample Name	C_T	Sample Name	C_T
Heat-treated 1	22.32	Untreated 1	21.45
Heat-treated 2	22.33	Untreated 2	21.49
Heat-treated 3	22.40	Untreated 3	22.47
Heat-treated 4	22.40	Untreated 4	22.59
Heat-treated 5	22.41	Untreated 5	22.82
Heat-treated 6	22.42	Untreated 6	22.88
Heat-treated 7	22.44	Untreated 7	23.13
Heat-treated 8	22.45	Untreated 8	23.19
Heat-treated 9	22.46	Untreated 9	23.29
Heat-treated 10	22.49	Untreated 10	23.88
Heat-treated 11	22.49	Untreated 11	24.00
Heat-treated 12	22.55	Untreated 12	24.03
Heat-treated 13	22.57	Untreated 13	24.08
Heat-treated 14	22.60	Untreated 14	24.12
Heat-treated 15	22.61	Untreated 15	24.14
Heat-treated 16	22.62	Untreated 16	24.36
Heat-treated 17	22.63	Untreated 17	24.36
Heat-treated 18	22.65	Untreated 18	24.48
Heat-treated 19	22.66	Untreated 19	24.48
Heat-treated 20	22.71	Untreated 20	24.55
Heat-treated 21	22.73	Untreated 21	24.60
Heat-treated 22	22.77	Untreated 22	24.78
Heat-treated 23	22.79	Untreated 23	24.82
Heat-treated 24	22.79	Untreated 24	25.03
Mean	**22.55**	**Mean**	**23.71**
SD	**0.145**	**SD**	**1.009**
Range	**22.32-22.79**	**Range**	**21.45-25.03**

Numerical results from Figure 4 above with results sorted from lowest to highest

Table 8. Representative examples of homogeneity testing of plasmids positive controls. Five aliquots of each plasmid were tested with 10 replicates for each.

CSIRO *Megalocytivirus* qPCR

NQC-1	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	25.30	25.20	25.14	25.24	25.29	25.18	25.23	25.24	25.17	25.17	25.22	0.05
Tube 2	25.31	25.21	25.31	25.29	25.23	25.37	25.31	25.31	25.31	25.34	25.30	0.05
Tube 3	25.42	25.40	25.44	25.49	25.49	25.52	25.46	25.44	25.50	25.43	25.46	0.04
Tube 4	25.43	25.41	25.49	25.67	25.45	25.58	25.51	25.52	25.47	25.44	25.50	0.08
Tube 5	25.35	25.41	25.35	25.42	25.38	25.41	25.43	25.34	25.43	25.29	25.38	0.05

0.1 Threshold — C_T MEAN AVG = 25.37

NQC-2	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	32.36	32.60	32.43	32.39	32.31	32.61	32.41	32.26	32.44	32.44	32.43	0.11
Tube 2	32.01	32.12	32.16	32.15	32.08	32.22	32.26	32.52	32.56	32.44	32.25	0.19
Tube 3	32.57	32.64	32.55	32.63	32.83	32.56	32.61	32.56	32.34	32.66	32.60	0.13
Tube 4	32.61	33.02	32.71	33.04	32.57	32.67	32.91	32.74	32.75	32.76	32.78	0.16
Tube 5	32.21	32.30	32.2	32.58	32.16	32.31	31.98	32.32	32.33	32.67	32.34	0.21

0.1 Threshold — C_T MEAN AVG = 32.48

OIE Martenot OsHV-1 qPCR

NQC-1	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	23.64	23.58	23.54	23.62	23.68	23.55	23.64	23.67	23.60	23.68	23.62	0.05
Tube 2	23.59	23.52	23.44	23.53	23.41	23.54	23.46	23.50	23.62	23.70	23.53	0.09
Tube 3	23.63	23.77	23.56	23.56	23.56	23.57	23.56	23.64	23.68	23.79	23.63	0.09
Tube 4	23.73	23.76	23.66	23.65	23.59	23.57	23.60	23.66	23.76	23.71	23.67	0.07
Tube 5	23.65	23.74	23.75	23.67	23.64	23.62	23.54	23.64	23.71	23.69	23.67	0.06

0.1 Threshold — C_T MEAN AVG = 23.62

NQC-2	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	30.40	30.41	30.31	30.35	30.33	30.18	30.36	30.06	30.34	30.28	30.30	0.11
Tube 2	30.40	30.35	30.53	30.36	30.20	30.72	30.39	30.33	30.22	30.37	30.39	0.15
Tube 3	30.69	30.91	30.60	30.76	30.91	30.57	30.73	30.62	30.69	30.51	30.70	0.13
Tube 4	30.63	30.57	30.55	30.60	30.42	30.32	30.35	30.40	30.80	30.54	30.52	0.15
Tube 5	30.33	30.45	30.39	30.52	30.39	30.40	30.25	30.15	30.44	30.42	30.37	0.11

0.1 Threshold — C_T MEAN AVG = 30.46

EMAI OsHV-1 qPCR

NQC-3	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	23.79	23.86	23.63	23.74	23.84	23.78	23.84	23.90	23.94	23.82	23.81	0.09
Tube 2	24.07	24.02	23.98	24.00	23.96	24.03	24.05	24.02	24.12	24.24	24.05	0.08
Tube 3	24.06	24.04	23.95	23.79	23.94	23.99	23.93	23.95	24.16	24.10	23.99	0.10
Tube 4	24.42	24.54	24.57	24.39	24.42	23.38	24.43	24.33	24.44	24.50	24.34	0.34
Tube 5	24.48	24.39	24.42	24.44	24.51	24.35	24.45	24.52	24.48	24.53	24.46	0.06

0.1 Threshold — C_T MEAN AVG = 24.13

NQC-4	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	31.14	31.08	31.21	31.16	31.04	31.24	30.94	31.11	30.88	31.36	31.02	0.14
Tube 2	30.26	30.42	30.49	30.42	30.54	30.74	30.40	30.56	30.26	30.62	30.47	0.15
Tube 3	31.12	31.30	31.48	31.56	30.97	31.02	31.29	31.38	31.10	31.08	31.23	0.20
Tube 4	30.93	31.22	31.22	31.16	31.03	31.16	31.09	31.23	31.30	31.23	31.16	0.11
Tube 5	31.85	32.05	31.62	31.89	31.78	31.83	31.31	31.58	31.73	31.66	31.73	0.20

0.1 Threshold — C_T MEAN AVG = 31.12

Table 8 (contd.). Representative examples of homogeneity testing of plasmids positive controls. Five aliquots of each plasmid were tested with 10 replicates for each.

CSIRO WSSV qPCR

NQC-1	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	24.14	24.12	24.05	24.09	24.15	24.06	24.00	24.17	24.15	24.08	24.10	0.05
Tube 2	24.08	24.03	24.00	23.99	23.97	24.05	23.96	24.00	24.06	24.08	24.08	0.04
Tube 3	24.09	24.13	24.11	24.12	24.09	24.19	24.11	24.00	24.09	24.11	24.10	0.05
Tube 4	23.79	23.93	23.92	23.90	23.85	23.90	23.87	23.92	23.91	23.89	23.90	0.04
Tube 5	24.19	24.20	24.23	24.24	24.22	24.22	24.27	24.24	24.23	24.18	24.22	0.03

0.1 Threshold — C_T MEAN AVG = 24.08

NQC-2	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	31.25	31.56	31.12	31.18	30.99	30.99	31.48	31.35	31.08	31.31	31.23	0.20
Tube 2	31.80	31.64	31.84	31.33	31.55	31.55	31.53	31.58	31.51	31.51	31.58	0.15
Tube 3	31.45	31.58	31.49	31.93	31.37	31.52	31.56	31.63	31.14	31.40	31.51	0.20
Tube 4	31.80	31.31	31.67	31.53	31.73	31.61	31.51	31.44	31.65	31.41	31.57	0.15
Tube 5	30.85	30.95	30.93	30.83	30.79	30.94	31.08	31.04	31.07	31.11	30.96	0.11

0.1 Threshold — C_T MEAN AVG = 31.37

OIE AHPND Han qPCR

NQC-1	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	23.51	23.47	23.37	23.31	23.47	23.30	23.28	23.40	23.38	23.31	23.38	0.08
Tube 2	23.65	23.43	23.44	23.34	23.25	23.47	23.29	23.34	23.45	23.49	23.42	0.12
Tube 3	23.55	23.22	23.34	23.47	23.45	23.30	23.44	23.36	23.49	23.29	23.39	0.10
Tube 4	23.46	23.34	23.39	23.44	23.37	23.45	23.38	23.35	23.37	23.49	23.40	0.05
Tube 5	23.49	23.58	23.14	NT	23.48	23.47	23.31	23.49	23.47	23.36	23.42	0.13

0.1 Threshold — C_T MEAN AVG = 23.40

NQC-2	C_T 1	C_T 2	C_T 3	C_T 4	C_T 5	C_T 6	C_T 7	C_T 8	C_T 9	C_T 10	MEAN	SD
Tube 1	30.25	30.28	30.42	30.29	30.28	30.28	30.25	30.25	30.20	30.39	30.29	0.07
Tube 2	30.29	30.09	30.14	30.14	30.14	30.30	30.34	30.23	30.15	30.47	30.23	0.12
Tube 3	30.12	29.89	30.13	30.17	30.13	30.25	30.10	30.23	30.06	30.01	30.11	0.11
Tube 4	30.19	29.99	30.15	30.17	30.14	30.09	29.99	29.96	30.24	30.16	30.11	0.10
Tube 5	30.09	30.18	30.10	30.10	30.08	30.16	30.19	29.48	NT	30.19	30.06	0.22

0.1 Threshold — C_T MEAN AVG = 30.16

CSIRO *Bonamia* sp. qPCR

NQC-2	C_T 1	C_T 2	C_T 3	MEAN	SD
Tube 1	19.07	19.01	19.01	19.03	0.04
Tube 2	18.97	18.92	18.98	18.96	0.03
Tube 3	18.95	18.93	18.93	18.94	0.01
Tube 4	18.93	19.12	19.04	19.03	0.09
Tube 5	18.99	18.97	18.96	18.97	0.02

0.1 Threshold — C_T MEAN AVG = 18.99

NQC-2	C_T 1	C_T 2	C_T 3	MEAN	SD
Tube 1	25.95	25.90	25.82	25.89	0.06
Tube 2	25.77	25.87	25.89	25.84	0.07
Tube 3	25.90	25.92	25.86	25.90	0.03
Tube 4	25.89	25.87	25.86	25.88	0.01
Tube 5	25.84	25.92	25.86	25.87	0.04

0.1 Threshold — C_T MEAN AVG = 25.88

AFDL Pols qPCR

NQC-2	C_T 1	C_T 2	C_T 3	MEAN	SD
Tube 1	21.44	21.24	21.33	21.34	0.10
Tube 2	21.27	21.31	21.32	21.30	0.03
Tube 3	21.38	21.31	21.33	21.34	0.03
Tube 4	21.27	21.37	21.38	21.34	0.06
Tube 5	21.25	21.21	21.32	21.26	0.06

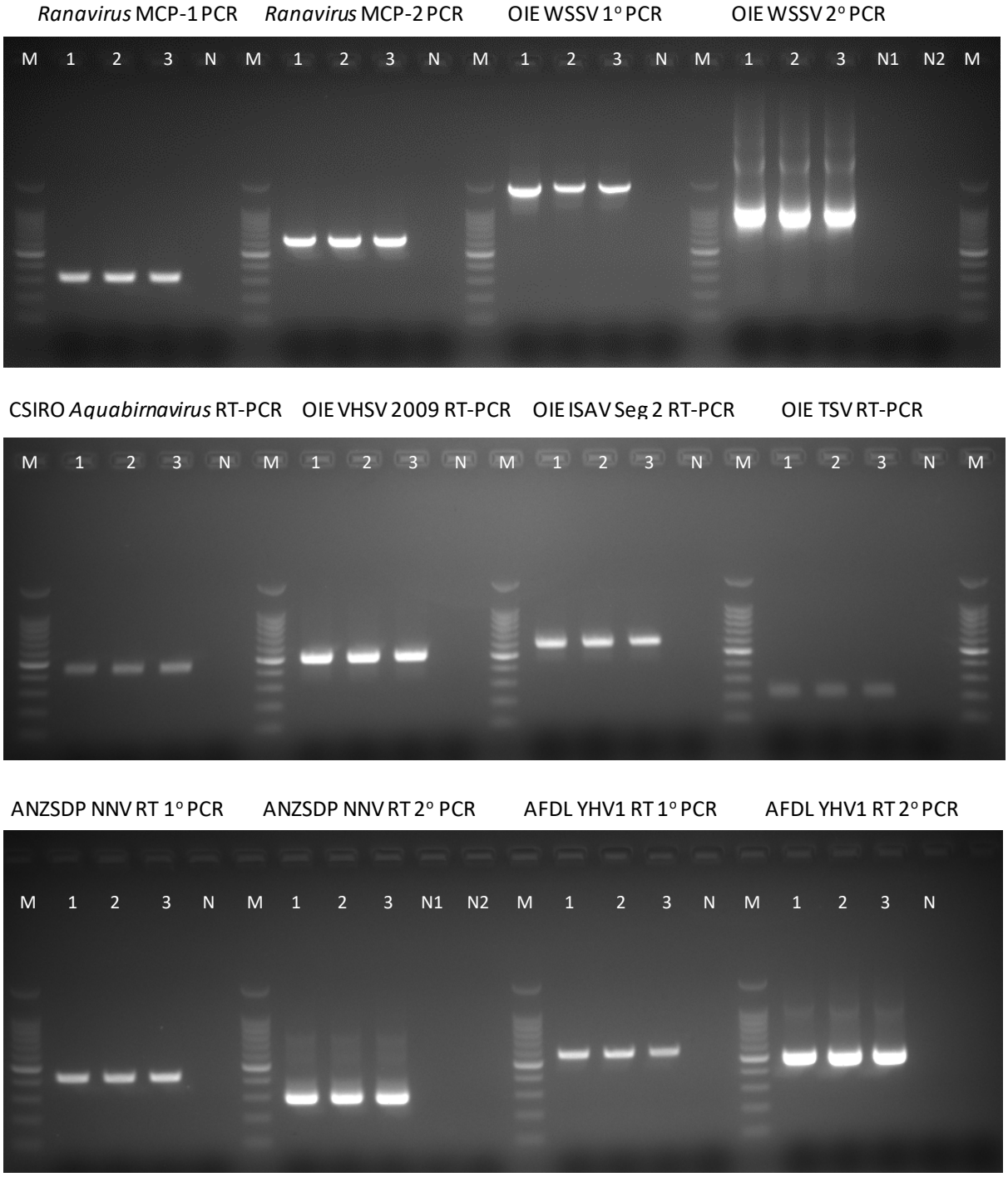
0.1 Threshold — C_T MEAN AVG = 21.32

NQC-2	C_T 1	C_T 2	C_T 3	MEAN	SD
Tube 1	28.75	28.76	28.75	28.75	0.01
Tube 2	28.75	28.79	28.82	28.79	0.04
Tube 3	28.86	28.73	28.71	28.77	0.08
Tube 4	28.80	28.78	28.77	28.78	0.02
Tube 5	28.99	28.70	28.66	28.78	0.18

0.1 Threshold — C_T MEAN AVG = 28.77

There was more variability in the limits of detection of the plasmids generated for the conventional PCR assays, when tested in triplicate so single positive controls, at one 10-fold dilution lower than the limit of

detection have been prepared for these controls (see Figure 5). Dilutions prepared for nested PCR assays were based on end-point dilution results for the primary PCR. One control has been prepared for each of the conventional PCR assays and are in routine use during testing at AFDL. A list of the conventional PCR assays with positive control plasmids is provided in Appendix 4.



samples tested in triplicate indicated by 1, 2 and 3. M = molecular weight marker, N1 = cPCR no template control, N2 = nPCR no template control).

Figure 5. Representative examples of homogeneity testing of real-time plasmid positive controls after end-point dilution to determine the concentration of plasmid for use as a positive control

Objective 3. Optimised universal internal control based on plant viral RNA and DNA and/or species-specific genes for use in molecular assays completed and implemented

2.1 Literature search for internal control assays

Extensive literature searches (using Science Direct, Web of Science) yielded very few assays specifically designed as internal control, housekeeping gene assays for molecular diagnostics tests (Table 9).

A number of housekeeping gene assays have been identified in the Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*), western blue shrimp (*Penaeus stylirostris*) and black tiger prawn (*Penaeus monodon*) for gene expression studies (Morga *et al.*, 2010; Du *et al.*, 2013; Dhar *et al.*, 2009; Leelatanawit *et al.*, 2012). However, these assays have not been specifically designed as housekeeping gene assays for diagnostic testing for specific pathogens. Also, all have been identified based on the tissue tropism of the pathogen, or the organ system being investigated and therefore may not be appropriate for all sample types submitted for testing from specific species. Also, they are universally not useful for diagnostic testing since they are not in a format acceptable to all diagnostic testing laboratories (e.g. SYBR Green rather than TaqMan probe-based assays).

Currently, alternatives to 18S-based internal control housekeeping gene assays do not exist for real-time assays targeting pathogens from molluscs, crustaceans and most non-salmonid finfishes. The alternative to developing host-specific internal control housekeeping gene assays is to seed samples prior to extraction with a standard control. A number of generic approaches were considered to establish more reliable, robust and cost-effective internal positive control strategies, relying on exogenous internal positive controls. This strategy involves the use of an unrelated (exogenous) template to spike every sample. Exogenous positive controls are the most suitable for aquatic animal diagnostic assays (due to the wide range of different species from which samples can be derived) as they rely on separate primers and probe, and one control could be used for all RNA assays and another for all DNA assays. Use of standardised exogenous internal positive control assays within a laboratory network also facilitates troubleshooting of test failures, training of new staff, and comparative evaluation of new reagents and platforms.

One widely-used approach for exogenous positive controls involves the use of bacteriophages, for example, MS2 phage for RNA targets and T4 phage for DNA targets (Dreier *et al.*, 2005; Gerriets *et al.*, 2008; Pecson *et al.*, 2009; Perrott *et al.*, 2009; Ninove *et al.*, 2011; Felder and Wöfel, 2014; McGlynn, 2014). Primers and probes for detection by conventional and real-time assays are readily available and both MS2 and T4 bacteriophages can be readily sourced from commercial suppliers. While a number of companies produce bacteriophage-based systems that can be purchased off-the-shelf, this is not considered desirable as the primer and probe sequences are proprietary and must be purchased from the same supplier. Use of MS2 and T4 will enable stocks to be produced at ACDP, rigorous QC can be undertaken, and the reagents can be stored and supplied to laboratories in the same manner as the positive controls.

Table 9. List of housekeeping gene assays for real-time and conventional PCR assays. Assays in use at AFDL are marked with "★".

Assay target	Assay format	Species	Reference	Comment
★18S (rRNA subunit)	qPCR	Finfish and molluscs (widely used for terrestrial animals)	Applied Biosystems Cat # 4308329	In routine use at AFDL for finfish and molluscan hosts unless a better assay is available, will not detect crustacean DNA, prone to false-positive results in no template controls
★Salmonid ELF1α	RT-qPCR	Atlantic salmon (*Salmo salar*) Rainbow trout (*Oncorhynchus mykiss*) Brown trout (*Salmo trutta*)	Moore *et al*. (2005) Bland *et al*. (2012)	In routine use at AFDL for Atlantic salmon and rainbow trout for RNA virus testing
Salmonid ELF1α	qPCR	Atlantic salmon (*Salmo salar*)	Bland *et al*. (2012)	In-house method with specificity determined *in silico*
Cod ELFα	RT-qPCR	Cod (*Gadus morhua*) Haddock (*Melanogrammus aeglefinus*) Saithe (*Pollachius virens*) Whiting (*Merlangius merlangius*) Norway trout (*Trisopterus esmakii*)	Das *et al*. (2007) in Bland *et al*. (2012) (review)	
Carp ELF1α	RT-qPCR	Carp (Family *Cyprinidae*) Eel (*Anguilla anguilla*) Roach (*Rutilis rutilis*)	Bland *et al*. (2012)	In-house method with specificity determined *in silico*
Carp ELF1α	qPCR	Carp (Family *Cyprinidae*)	Bland *et al*. (2012)	In-house method with specificity determined *in silico*
Herring ELF1α	RT-qPCR	Herring (*Clupea harengus*)	Matejusova *et al*. (2010)	
★CSIRO Shrimp EF1	qPCR	*Penaeus* spp. *Fenneropenaeus* spp. *Litopenaeus* spp. *Melicertus* spp.	Cowley et al (2018)	Routinely used for high-throughput testing of commercially cultured prawn species (i.e. *P. monodon* and *P. merguiensis*).
★OIE Decapod PCR	PCR	Almost all decapod species	Lo *et al*. (1996) OIE (2012a)	In routine use at ACDP for crustaceans
★Decapod Cox1	PCR	Almost all decapod species, including Western King prawn (*Melicertus latisulcatus*) Freshwater crayfish (*Macrobrachium rosenbergii*) Yabby (*Cherax destructor*)	Quan *et al*. (2004)	Alternative assay used at ACDP if the OIE Decapod PCR fails
★ Finfish Cox1	PCR	All finfish species	Ward *et al*. (2005)	In routine use at AFDL for finfish for DNA virus testing

2.2 Preliminary evaluation of endogenous and heterologous internal positive control assays

Given the number of different host species from which samples are obtained it is cost- and time-prohibitive to develop, evaluate and implement assays for each and every host species. Additionally, there is an unrealistic cost associated with maintenance of primers and probe for assays that are used infrequently, and which would require regular check-testing as part of a laboratory quality system. Therefore, the decision was made to concentrate on a generic approach relying on exogenous internal positive controls. This approach involves the use of an unrelated template to spike every sample. Exogenous positive controls are the most suitable for aquatic animal diagnostic assays (due to the wide range of different species from which samples can be derived) as they rely on separate primers and probe, and one control could be used for all RNA assays and another for all DNA assays. Use of standardised, exogenous, internal positive controls assays within a laboratory network also facilitates rapid troubleshooting of test failures, training of new staff, and comparative evaluation of new reagents and platforms.

MS2 and T4 were chosen based on implementation for routine use in clinical medical testing (Ninove *et al.*, 2011) and recommendations from colleagues undertaking molecular testing for terrestrial animal pathogens. Commercial products, *viz*. VetMAX™ Xeno™ Internal Positive Control (IPC) DNA and VetMAX™ Xeno™ Internal Positive Control (IPC) RNA (Life Technologies), are available as alternatives in the event that the MS2 and/or T4 assays do not perform as expected. These assays are in use in a number of state veterinary diagnostic laboratories. However, they are considerably more expensive per sample than the MS2 and T4 controls (approx. $1 per Xeno™ Internal Positive Control sample compared to less than <$0.01 for phage for the spike, excluding primer/probe cost). However, due to preliminary results using MS2 and the unreliable source for this phage, it was decided to evaluate the QBeta phage as an internal control for RNA viruses.

2.3 Evaluation of T4 and QBeta phages for use as heterologous internal positive controls

2.3.1 Determine the quantity of phage used to seed tissue to obtain repeatable results with relatively high C_T values (≥30)

Based on analytical sensitivity testing, addition of 5 µL to the lysis buffer of either T4 (stock diluted 1/100) or QBeta (stock diluted 1/10,000) produced C_T values of approximately 30. Therefore, addition of 5 µL of T4 (stock diluted 1/100) was used in further investigations into the effect of host tissues on detection and repeatability studies. Once evaluation of T4 was complete, the process was repeated with QBeta.

2.3.2 Investigation of the potential inhibition of host tissues on the detection of spiked T4

Using representative tissues from prawns, abalone and oysters it was confirmed the 1/100 dilution of T4 repeatably generated expected C_T values and was appropriate for all different tissue samples, as described in the Methods, depending on the extraction protocol used. The results also identified prawn as the host from which nucleic acid is most likely to inhibit real-time PCR detection (Table 10) particularly when extracted using the QIAamp Viral RNA Mini Kit (Qiagen) or magnetic beads (Applied Biosystems) where tissues are digested using ATL/PK.

Table 10. Effect on C_T value of nucleic acid extracted from different hosts on detection of T4

DNA mini kit C_T value									
	1/100 T4 Phage Dilution			1/1000 T4 Phage Dilution			1/10,000 T4 Phage Dilution		
Prawn Tissue	32.65	33.44	34.39	37.62	37.80	36.00	40.98	39.03	39.72
Abalone Tissue	29.58	29.17	29.87	34.91	33.52	34.58	36.20	39.38	36.78
Oyster Tissue	31.19	31.61	31.15	36.20	36.28	35.85	37.96	38.06	Neg
Viral RNA mini kit C_T value									
	1/100 T4 Phage Dilution			1/1000 T4 Phage Dilution			1/10,000 T4 Phage Dilution		
Prawn Tissue	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Abalone Tissue	32.06	31.97	32.34	36.98	36.71	36.57	39.67	Neg	Neg
Oyster Tissue	31.91	32.26	31.74	35.95	36.41	36.24	39.61	41.02	37.83
MME96 – ATL/PK digestion C_T value									
	1/100 T4 Phage Dilution			1/1000 T4 Phage Dilution			1/10,000 T4 Phage Dilution		
Prawn Tissue	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Abalone Tissue	29.73	29.31	29.33	33.47	33.45	33.32	36.11	36.34	35.88
Oyster Tissue	34.80	Neg	35.75	36.80	38.15	35.82	Neg	43.18	41.33
MME96 – AVL buffer C_T value									
	1/100 T4 Phage Dilution			1/1000 T4 Phage Dilution			1/10,000 T4 Phage Dilution		
Prawn Tissue	30.43	30.40	30.24	34.52	34.94	35.16	38.02	38.03	38.91
Abalone Tissue	29.91	30.30	30.11	35.04	34.68	34.12	35.82	37.10	39.15
Oyster Tissue	30.55	30.47	30.45	35.31	35.11	35.79	37.49	37.99	37.51
T4 POS control	30.33			35.06			36.58		

Neg=negative

Use of T4 was subsequently evaluated when testing a diagnostic submission for a mollusc pathogen, with 16 samples submitted for a *Bonamia* investigation (Submission 1; Table 11). Results for the 18S qPCR were as expected with both dilutions testing positive for all samples (Table 11). Both dilutions tested for all samples also tested positive for the T4 qPCR with these results more consistent, based on comparison with the expected T4 result from the positive extraction control, than the 18S results, where there is additional variability due to variability in the amount of input tissue. The effect of inhibition is also more easily observed for T4 due to a known amount consistently added, with expected reference C_T results obtained for the T4 extraction control. This contrasts with the 18S results, where upper acceptable limits still need to be determined and, due to the ubiquitous nature of 18S, negative extraction controls (NEC) are more commonly returning positive test results. In addition, it is difficult to observe a C_T change in the 18S diluted samples, providing little evidence that inhibitors are not present. The results indicate that T4 is a very promising alternative to 18S for real-time PCR assays in molluscs.

Table 11. Comparison of 18S qPCR and T4 qPCR results for samples submitted for *Bonamia* sp. testing.

Sample	CSIRO *Bonamia* qPCR		18S qPCR		T4 qPCR	
	Undiluted C_T value	1/10 C_T value	Undiluted C_T value	1/10 C_T value	Undiluted C_T value	1/10 C_T value
Submission 1-01	20.44	23.32	13.16	14.09	30.71	33.66
Submission 1-02	29.58	31.75	15.91	14.84	31.84	33.07
Submission 1-03	28.87	31.42	16.12	16.27	31.02	32.98
Submission 1-04	25.41	28.26	15.52	15.59	31.71	33.13
Submission 1-05	27.29	30.45	13.93	16.27	30.47	33.58
Submission 1-06	29.93	33.03	14.61	14.88	30.88	33.58
Submission 1-07	28.14	30.68	16.71	15.89	31.69	33.22
Submission 1-08	29.49	31.83	15.66	15.61	30.07	32.85
Submission 1-09	33.53	Neg, 38.60	10.50	15.57	29.87	32.55
Submission 1-10	Neg, 38.72	Neg	7.73	15.03	29.95	32.65
Submission 1-11	Neg, 38.72	Neg	15.12	14.23	30.07	31.71
Submission 1-12	Neg	Neg	14.87	16.18	29.33	33.38
Submission 1-13	Neg	Neg	15.04	15.15	30.04	34.08
Submission 1-14	39.32	Neg	21.47	15.90	31.20	32.25
Submission 1-15	Neg, 37.07	Neg	14.82	15.27	29.85	31.57
Submission 1-16	35.33	35.24	20.85	15.27	31.58	32.05
NQC-1	19.22		$\bar{x}$ = 15.13 SD = 3.26	$\bar{x}$ = 15.38 SD = 0.66	$\bar{x}$ = 30.64 SD = 0.80	$\bar{x}$ = 32.90 SD = 0.72
NQC-2	26.21					
PEC	Neg	Neg	36.90	Neg	29.89	33.06
NTC	Neg	Neg	Neg	Neg	Neg	Neg
NEC	Neg	Neg	36.68	40.10	Neg	Neg

PEC = Positive Extraction Control; NTC = No Template Control; NEC = Negative Extraction Control; Neg=negative

Using prawn tissue, results (Table 12) indicated that use of excess tissue during the extraction of prawn samples can inhibit the assay, resulting in false-negative test results. Given the impracticality of determining the weight of individual tissue samples being extracted, due either to high sample numbers and/or small amount of the tissue that is extracted, testing samples undiluted and diluted 1/10 is a realistic approach. Further evidence of the value of seeding with T4 and using a real-time PCR to detect the internal positive control was that the current alternative, the OIE Decapod PCR, produced positive results for both undiluted and nucleic acid diluted 1/10 samples. The results indicated that seeding with 5 µL of T4 diluted 1/100 was an appropriate alternate approach, so further evaluation involving extraction and testing of samples from routine submissions for a variety of pathogens was undertaken.

Table 12. Effect on C_T value of reducing the volume of tissue extracted and diluting extracted nucleic acid on detection of T4 phage.

Sample	1/10 T4 Phage Dilution	1/100 T4 Phage Dilution	1/1000 T4 Phage Dilution	1/10000 T4 Phage Dilution
500µl Prawn tissue	Neg, Neg	Neg, Neg	Neg, Neg	Neg, Neg
50µl Prawn tissue	25.39, 25.52	30.94, 31.03	36.14, 37.66	40.98, 38.11
Retesting of diluted samples from Table 10				
Sample			**Undiluted**	**1/10 Dilution**
Viral RNA Mini Kit: 500µl Prawn + T4 diluted 1/100			Neg	34.65
Viral RNA Mini Kit: 500µl Prawn + T4 diluted 1/100			Neg	34.74
Viral RNA Mini Kit: 500µl Prawn + T4 diluted 1/100			Neg	34.30
MME96 ATL/PK: 500µl Prawn + T4 diluted 1/100			Neg	34.51
MME96 ATL/PK: 500µl Prawn + T4 diluted 1/100			Neg	35.27
MME96 ATL/PK: 500µl Prawn + T4 diluted 1/100			Neg	34.38
Retest Viral RNA Mini Kit: 500µl Prawn + T4 diluted 1/100			Neg	34.86
Re-extraction Viral RNA Mini Kit: 500µl Prawn + T4 diluted 1/10			Neg	29.15
Re-extraction Viral RNA Mini Kit: 500µl Prawn +T4 diluted 1/100			Neg	34.57

Samples were initially tested in duplicate then in singlicate for retesting. Neg=negative

Using real diagnostic samples (imported commodity prawns), only 3/6 samples were positive using the CSIRO WSSV qPCR when tested undiluted, however all samples were test-positive when diluted 1/10 (Table 13). Similarly, only 1 sample was positive by the T4 qPCR when tested undiluted, while all were test-positive with consistent C_T values (34.12 to 34.58) when tested diluted 1/10. The OIE Decapod PCR was positive for all samples at both dilutions. Reliance on the OIE Decapod PCR assay, with only undiluted samples, would have resulted in 50% of results being false negatives. Addition of T4 to each sample prior to extraction did not affect the sensitivity of the WSSV qPCR.

Table 13. Evaluation of seeding on C_T value with T4 using samples representative of typical submission of prawns for WSSV testing.

Sample	WSSV qPCR		T4 qPCR		OIE Decapod cPCR	
	Undiluted	1:10 Dilution	Undiluted	1:10 Dilution	Undiluted	1:10 Dilution
Submission 2-03	Neg	34.54	Neg	Neg	POS	POS
Submission 2-03 + T4	42.67	34.33	Neg	34.58	POS	POS
Submission 2-04	37.66	22.91	Neg	Neg	POS	POS
Submission 2-04 + T4	28.70	23.01	Neg	34.35	POS	POS
Submission 2-05	Neg	31.36	Neg	Neg	POS	POS
Submission 2-05 + T4	Neg	31.59	Neg	34.55	POS	POS
Submission 2-09	34.30	31.23	Neg	Neg	POS	POS
Submission 2-09 + T4	30.32	31.59	35.32	34.12	POS	POS
Submission 2-10	Neg	25.91	Neg	Neg	POS	POS
Submission 2-10 + T4	Neg	25.74	Neg	34.62	POS	POS
Submission 2-13	Neg	29.73	Neg	Neg	POS	POS
Submission 2-13 + T4	Neg	29.63	Neg	34.54	POS	POS
NEC	Neg	Neg	Neg	Neg	Neg	Neg
T4 POS control (1/100 dilution)	N/A	N/A	28.23	31.94	N/A	N/A

Neg=negative; N/A=not applicable; NEC = Negative Extraction Control

More consistent results were also obtained when nucleic acid extracts were tested undiluted and diluted 1/10 when QBeta was seeded into tissue homogenates prior to extraction. Homogenates generated from a typical submission of imported commodity prawns (Submission 3; Table 14) were seeded with three dilutions of QBeta prior to extraction with the MME96 bead extraction system and tested undiluted and diluted 1/10 with the QBeta RT-qPCR (Table 14). The most consistent results, based on comparison with QBeta diluted in water, were obtained from testing samples diluted 1/10. Inhibition, based on increased standard deviation (although minor), was observed when nucleic acid was tested undiluted. QBeta, diluted 10^{-3} and 10^{-4}, provided repeatable results with C_T values >30. These dilutions will be used for further evaluation with diagnostic samples submitted in the future.

Table 14. Evaluation of seeding on C_T value with QBeta using samples representative of typical submission of prawns.

Sample	QBeta diluted 10^{-3}		QBeta diluted 10^{-4}		QBeta diluted 10^{-5}	
	Undiluted	1/10	Undiluted	1/10	Undiluted	1/10
QBeta in water	27.03	30.38	30.29	33.96	33.71	37.47
Submission 3-01	29.65	30.37	32.82	33.05	37.79	37.17
Submission 3-02	29.53	30.72	32.72	33.83	35.80	38.33
Submission 3-03	29.15	30.31	32.92	33.43	35.44	38.82
Submission 3-04	29.40	30.21	32.99	33.45	35.46	37.25
Submission 3-05	29.29	30.18	32.53	33.78	35.95	37.20
Submission 3-06	29.23	30.31	32.59	33.80	37.69	37.19
Submission 3-07	28.56	30.22	31.68	34.72	34.00	37.32
Submission 3-08	27.89	30.22	31.06	34.06	34.61	38.27
	$\bar{x}$ = 28.86 SD = 0.88	$\bar{x}$ = 30.32 SD = 0.17	$\bar{x}$ = 32.18 SD = 0.95	$\bar{x}$ = 33.97 SD = 0.47	$\bar{x}$ = 35.61 SD = 1.44	$\bar{x}$ = 37.67 SD = 0.63
NTC	All Negative (>45.0)		All Negative (>45.0)			
NEC	All Negative (>45.0)		All Negative (>45.0)			

0.1 Threshold

The use of T4 during preliminary investigation into the use of swabs as an alternative sample to cuticular epithelium when testing imported commodity prawns for WSSV (Submission 4; Table 15) demonstrated that CSIRO WSSV qPCR results obtained with swabs were consistent with the original results using cuticular epithelium and the T4 results showed very good repeatability (Table 15).

Table 15. Evaluation of seeding on C_T value with T4 during comparison of cuticular epithelium and swabs for a typical submission for WSSV testing.

Sample	Cuticular Epithelium CSIRO WSSV qPCR		Swab - 2hrs CSIRO WSSV qPCR		Swab - O/N CSIRO WSSV qPCR		Swab - 2hrs T4 Phage qPCR		Swab - O/N T4 Phage qPCR	
	Undiluted	1/10	Undiluted	1/10	Undiluted	1/10	Undiluted	1/10	Undiluted	1/10
Submission 4-03	39.92	34.85	33.22	35.69	32.96	38.03	28.85	32.72	28.65	32.35
Submission 4-04	29.54	22.56	24.52	28.12	23.20	26.87	28.92	32.55	28.61	32.32
Submission 4-05	39.26	31.47	30.44	33.66	29.69	33.51	28.82	32.71	28.72	32.14
Submission 4-09	34.24	30.96	30.90	34.86	30.47	34.63	28.58	32.55	28.84	32.29
Submission 4-10	29.74	25.78	25.28	29.09	23.62	27.13	29.07	32.71	28.94	32.32
Submission 4-13	34.40	29.87	29.34	32.64	28.35	32.41	28.68	32.12	28.74	32.40
PEC (T4)	N/A	N/A	N/A	N/A	N/A	N/A	28.53	32.28	28.53	32.28
NEC	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
NQC-1	24.15		24.69		24.56		N/A		N/A	
NQC-2	31.19		31.42		31.56		N/A		N/A	

0.1 Threshold; Neg=negative; O/N = overnight

Use of T4 was then evaluated using a range of atypical samples of high importance (Submission 5; Figure 7). Results demonstrated that the use of T4 allowed the levels of any PCR inhibition to be more reliably determined, enabling greater confidence in classification of samples as positive, negative or unsuitable (Submission 5; Table 16). This was particularly important where 18S results were less consistent and unreliable due to the unknown sample type. Failure of the T4 assays, in conjunction with negative 18S results, for Samples 5-01, 5-06 and 5-14 enabled reporting of the test result as invalid due to the inability to classify the result as definitively negative. Without concurrent use of T4 it would not have been possible to report unambiguously test results for these samples.

Figure 6. Examples of atypical samples received, of unknown or poorly described sample material

Table 16. Evaluation of seeding on C_T value with T4 for a submission of atypical samples for WSSV testing.

Sample	Sample Type	CSIRO WSSV qPCR Mean C_T		OIE WSSV qPCR Mean C_T		18S Standard qPCR Mean C_T		T4 Phage qPCR Mean C_T		Result
		Neat	1/10	Neat	1/10	Neat	1/10	Neat	1/10	
Submission 5-01	Animal	Neg	Neg	Neg	Neg	Neg	44.96	Neg	Neg	INVALID
Submission 5-02	Animal	Neg	Neg	Neg	Neg	16.72	19.11	27.05	29.93	**Negative**
Submission 5-03	Unknown	Neg	Neg	Neg	Neg	Neg	20.64	25.23	28.42	**Negative**
Submission 5-04	Plant	Neg	Neg	Neg	Neg	16.84	20.34	25.62	28.77	**Negative**
Submission 5-05	Plant	Neg	Neg	Neg	Neg	Neg	16.38	24.78	27.58	**Negative**
Submission 5-06	Unknown	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	INVALID
Submission 5-07	Plant	Neg	Neg	Neg	Neg	16.48	19.83	26.20	29.23	**Negative**
Submission 5-08	Unknown	Neg	Neg	Neg	Neg	Neg	20.09	24.69	27.67	**Negative**
Submission 5-09	Animal	29.32	32.2	30.01	34.00	20.26	19.96	28.56	30.27	POSITIVE
Submission 5-10	Plant	Neg	Neg	Neg	Neg	17.56	20.90	26.01	29.12	**Negative**
Submission 5-11	Plant	Neg	Neg	Neg	Neg	15.99	19.10	26.31	29.25	**Negative**
Submission 5-12	Plant	Neg	Neg	Neg	Neg	Neg	16.63	24.82	27.91	**Negative**
Submission 5-13	Animal	Neg	Neg	Neg	Neg	16.65	18.84	27.40	29.94	**Negative**
Submission 5-14	Animal	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	INVALID
Submission 5-15	Unknown	Neg	Neg	Neg	Neg	37.49	42.23	25.67	28.48	**Negative**
NEC	N/A	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Negative
PEC (T4)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	24.17	27.17	Positive

Neg=negative; PEC = Positive Extraction Control; NEC = Negative Extraction Control

Objective 4. Technology transferred and adopted by participating laboratories

3.1 Distribution of positive control plasmids

Positive control plasmids have been distributed nationally to at least nine university, commercial and state government laboratories within Australia and New Zealand. Plasmid positive controls are provided to:

- support ongoing comparative performance of laboratory testing for OsHV-1 and WSSV as part of the national LEADDR network.
- assist with technology transfer of newly developed diagnostic tests.
- assist with implementation of established assays to increase laboratory capability.
- reduce laboratories reliance on genomic material.
- contribute to collaborative projects with national partners

Plasmids have been (and still are) provided free-of-charge to most laboratories as assistance with implementation of improved diagnostic capability is seen as in the national interest or a direct contribution to achieving the aims of a collaborative research project. Clients have only been charged for plasmids of they are a commercial company and the use of for commercial benefit. Plasmid positive controls, including those currently prepared and those that will be prepared in the future, will continue to be provided when requested, with the caveat that there may be delays in supplying non-essential materials in the event of a national disease emergency response.

Either by direct request, or as part of an established collaborative project, plasmids have also been distributed government laboratories, or laboratories undertaking testing at the request of the government, in Brazil, Canada, Chile, Denmark France, India, Indonesia, Japan, Malaysia, Mexico, South Africa, South Korea, Sri Lanka, Taiwan, Thailand and Vietnam. Plasmid positive controls are provided to these international laboratories to:

- support the activities of the OIE Collaborating Centre for Laboratory Capacity Building
- support the activities of the OIE Reference Laboratories for infection with EHNV, AbHV, Ranavirus and YHV1.
- support the activities of the OIE Regional Collaboration Framework on Aquatic Animal Health in Asia and the Pacific
- assist with establish national testing capability for new, emerging and OIE-listed pathogens.
- contribute to collaborate projects with international partners

Plasmids have generally been (and still are) provided free-of-charge as assistance with implementation of improved diagnostic capability is seen as a good global citizen activity or a direct contribution to achieving the aims of a collaborative research project.

3.2 Provision of advice regarding implementation of protocols and troubleshooting based on feedback from participating laboratories

Feedback from laboratories who have received the positive control plasmids was actively sought and results have been satisfactory and there have been no issues with implementation. Performance of several of the plasmids will continue to be monitored through monthly submission of NQC results to the LEADDR network. Delays in distribution of information relating to the use of the positive control probe and T4 and QBeta internal controls occurred due to emergency response requirements of the white spot syndrome virus incursion in Queensland in 2016 and delays to existing project work.

3.3 Implementation of the plasmid and internal positive controls at the ACDP Fish Diseases Laboratory

The plasmid positive controls are in routine use at AFDL and they are designed, prepared, quality assured and used for all new assays that are implemented. Their utility became apparent during the confirmatory, emergency response and surveillance testing resulting from the incursion of WSSV in prawn farms along the Logan River in Queensland in 2016/17.

Testing for WSSV using the CSIRO qPCR, (559 plates run over an 11-month period by 13 different operators) and the OIE qPCR (293 plates run over a 15-month period by 10 different operators) demonstrated the repeatability of the positive control plasmids and enabled accurate monitoring of the performance of the assays, where different operators and batches of primer, probe and master mix were used. The use of the plasmid positive controls also eliminated the need to continuously produce positive control material from clinical material, where batch-to-batch variation would not have allowed such an accurate assessment of test performance over such a large number of test runs, and would have required essential staff to be removed from testing samples to produce and evaluate new preparations of positive control material.

Use of the plasmid positive controls, artificial probe and T4 internal control was implemented for the following projects:

1. *Xenohaliotis californiensis* qPCR testing of 276 abalone samples at ACDP during the DAWE-funded Southern Surveillance – Abalone project.

2. Evaluation of 600 nucleic acids derived from prawn tissues and swabs (tested individually and as pools) for WSSV as part of the DAWE-funded Pool-Level Sensitivity project.

All reagents performed as expected, with limited modification of existing paperwork or work practices required. While all positive control samples generated a signal for the specific target and artificial probe, no test samples returned positive results for the artificial probe, indicating there was no contamination of sample nucleic acid with positive control material. Use of T4 demonstrated the utility of this phage as an internal positive control by enabling greater confidence in lack of inhibition to qPCR tests, compared to other controls such as 18S.

Conclusion

This project has resulted in the production of a bank of quality-assured, non-infectious, quantifiable, molecular test controls that can be provided to any diagnostic laboratory in a ready-to-use form to assist them with the implementation of specific aquatic animal disease diagnostic tests. In addition, these controls will be useful in the diagnostic laboratory quality systems to demonstrate laboratory competency.

Thirty-two positive control plasmids (22 for real-time assays and 10 for conventional assays) have been prepared and are in routine use. A further 10 plasmid positive controls (8 for real-time assays and 2 for conventional assays) are undergoing final quality checks prior to release for routine use. Therefore, a total of 42 plasmid positive controls for 25 different pathogens have been generated as a result of this project.

Their most important use is as positive controls during diagnostic testing. Because these controls are distinguishable from the pathogens' genomic nucleic acid, they will assist in identification of cross-contamination between the positive control samples and the diagnostic samples and thus will mitigate against the reporting of false-positive results that occur due to contamination of test samples with positive controls.

In addition, T4 and QBeta phages have been evaluated as heterologous internal positive controls for DNA and RNA targets, respectively, for use in establishing that generic aspects of PCR testing (e.g. nucleic acid extraction and absence of PCR inhibitors) are performing as expected. Implementation of the use of the T4 and QBeta phages as internal positive controls has improved the quality of molecular testing, through more sensitive assessment of the effect of PCR inhibitors and confidence in results generated when testing atypical samples (i.e. plankton, dirt, feed).

Implications

This project is directly related to the FRDC Strategic Priority Area "Program 1: Environment, Theme 1: Biosecurity and aquatic animal health" by developing capability, systems and technologies to enable rapid identification of false-positive test results caused by contamination with positive control material. Use of these reagents and procedures will enhance protection of Australia's aquatic animal industries.

Quality assured positive control material is critical to demonstrate an assay has performed as expected. Similarly, testing for internal positive control material demonstrates extraction procedures have produced template of acceptable quality, free of PCR inhibitors. Both types of controls are particularly important where the samples are being tested to demonstrate freedom from disease (i.e. true negative results). Moreover, use of these controls in diagnostic testing will assist diagnostic laboratories to evaluate their current methods and, in turn, provide laboratories, regulators and trade partners with enhanced confidence in Australia's diagnostic laboratory competence.

Recommendations

The protocols developed during this project are in routine use in the ACDP Fish Diseases Laboratory whenever new tests are implemented and these are made available to laboratories wanting to implement test capability. Occasionally, it can be difficult to source fixed or infectious material for exotic pathogens for use in test evaluation studies to establish diagnostic capability, particularly with diseases that are emerging. In these circumstances, the establishment of plasmid positive controls provides confidence an assay is at least detecting the target it is designed to detect and allows technology transfer to state government laboratories to enhance Australia's diagnostic laboratory capability. The plasmids have also enabled the ASe to be determined for each of the assays which is a requirement for assay validation.

Further development

The project has established protocols for the design, evaluation and standardisation of plasmid positive controls and phage-based internal control assays. While most laboratories are using the plasmid positive controls, uptake of the use of the synthetic probe and implementation of the T4 and QBeta internal control assays has been more limited and will occur as part of the extension when this Final Report has been accepted. By demonstrating the value of the routine use of these tools through scientific presentations and publications, it is anticipated that uptake will increase.

While it was originally planned to develop synthetic RNA positive controls, evidence of incomplete removal of plasmid DNA from the transcribed RNA following DNAse treatment raised concerns with the consistency of producing an RNA population free of plasmid DNA for subsequent analysis. This would essentially defeat the purpose of producing RNA as the RT-PCR assay would still be detecting residual plasmid DNA coding for the same target. Use of QBeta as an internal control provides evidence that the master mix for each QBeta RT-qPCR assay has been prepared correctly. Use of commercial master mixes where the reverse transcriptase and polymerase enzymes are in the same component tube also acts as an indicator that the master mix has been prepared correctly. Even when the target pathogen is an RNA virus and a DNA plasmid is used as the positive control, failure to prepare the master mix correctly would result in the failure of the assay to detect the DNA plasmid positive control.

Extension and Adoption

Diagnostic laboratories within Australia and New Zealand are well-aware of this project through the Sub-committee for Aquatic Animal Health (SCAAH) and have been encouraged to avail themselves of the reagents relevant to their diagnostic activities. In addition, project staff have made presentations at various national and international conferences and workshops (see below) that include information concerning these reagents. An electronic copy of the Final Report will be provided after direct contact with relevant staff in diagnostic laboratories.

Project coverage

Cummins DM. 2015. PCR control development: Positive controls plasmids and internal extraction controls. DAFF/FRDC Aquatic Animal Health Training Scheme - Aquatic Animal Health Technical Forum and Skills Training Workshop, James Cook University, Townsville, Queensland, 17-19 June 2015.

Cummins DM, Moody NJG, Gudkovs N, Jones BJ, Crane MStJ. 2015. A tale of two projects: The development of a Taqman PCR assay for the detection of *Perkinsus olseni* & positive control strategies to reduce the reliance on genomic material. Third FRDC Australasian Scientific Conference on Aquatic Animal Health, The Pullman Reef Hotel, Cairns, Queensland, 6-10 July 2015.

Cummins DM, Slater J, Mohr PG, Hoad J, Williams LM, Moody NJG, Crane MStJ. 2016. Development of PCR plasmid controls & exogenous extraction/amplification controls. Twelfth Annual Meeting of the Australian Association of veterinary Laboratory Diagnosticians, Sage Resort, Darwin, Northern Territory, 23-24 November 2016.

Project materials developed

Thirty-two plasmid positive controls (22 for real-time assays and 10 for conventional assays) have been prepared and in in routine used. A further 10 plasmid positive controls (8 for real-time assays and 2 for conventional assays) are undergoing final quality checks. Therefore, a total of 42 plasmid positive controls have been generated as a result of this project.

See Appendices 3 and 4 for the full lists of positive controls produced for real-time and conventional PCR diagnostic tests.

Requests for reagents are actioned as soon as possible with a Materials Transfer Agreement (MTA) between the requesting institute and CSIRO ACDP drafted and executed prior to transfer of materials with conditions detailed in the MTA as follows:

- CSIRO has agreed to transfer positive control material, including non-infectious plasmid controls, ethanol-fixed tissues, and cell culture supernatants
- Materials are to be used by the laboratory to assist in establishing molecular tests.
- The recipient agrees:

  (1) to provide CSIRO with methods and results using these positive controls to ensure that they are performing as expected

  (2) materials are not to be transferred to any Third Parties without approval from CSIRO

  (3) a draft of any publication using these materials be provided to CSIRO for review prior to submission for publication.

Aside from the three requirements of the standard MTA agreement, no additional restrictions are placed on the use of the plasmids. The design of the plasmids is not protected intellectual property; the complete details of the design of the plasmids (Appendix 1 and 2) are in this report so are in the public domain.

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Appendices

Appendix 1. Plasmid construct details and references for real-time PCR assays.

Appendix 2. Plasmid construct details and references for conventional PCR assays.

Appendix 3. List of pathogens and molecular assays and associated positive control plasmids for real-time PCR assays.

Appendix 4. List of pathogens and molecular assays and associated positive control plasmids for conventional PCR assays.

Appendix 5: Details provided with the plasmid positive controls.

Appendix 6. List of researchers and project staff

Appendix 1. Plasmid construct details and references for real-time PCR assays. Universal probe insert is highlighted in yellow.

Plasmid	Pathogen	Assay	Plasmid Name	Reference
1	WSSV	CSIRO WSSV qPCR	pWSSV_CSIRO_qPCR	East et al. (2004)
CCGACGCCAAGGGAACTGTCGCTTCAGCCATGCCAGCCGTCTTCCAGACCGTCTAGCATCCAGTGCAACCGATGGAAACGGTAACGAATCTGAA				
2	WSSV	OIE WSSV qPCR	pWSSV_OIE_qPCR	Durand and Lightner (2002)
TGGTCCCGTCCTCATCTCAGAAGCCATGAAGAATGCCGTCTATCACACACACCGTCTAGCATCCAGTCTAATTTCCGGCAAGGCAGC				
3	OsHV-1	EMAI OsHV-1 qPCR	pOsHV-1_EMAI_qPCR	Jenkins et al (2013)
TACATCAAACCCACTTTTCCTATGATTATACCGTCTAGCATCCAGTTAGCAGTGATCTTAGCGGTGTTTTCATGAGTGATAATAAAAATCGTGGTGGATAAAACG				
4	OsHV-1	OIE OsHV-1 Martenot qPCR	pOsHV-1_Martenot_qPCR	Martenot et al. (2010)
GTCGCATCTTTGGATTTAACAATTGCCCCTGTCATCTTGAGGTATAGACAATCGCCAGAAAATTTCCCACCGTCTAGCATCCAGTATGTTCAGTCCATGGGTTGTCAGTCGGATCCCAGT				
5	Megalocytivirus	CSIRO Megalocytivirus qPCR	pMegalocytivirus_CSIRO_qPCR	Mohr et al. (2015)
TGACCAGCGAGTTCCTTGACTTTTGGAACGCCTGCATGATGCCTGGCAGCAAACAATCTGACCGTCTAGCATCCAGTGCTACAACAAGATGATTGGCATGCGCAGCGACCTGGTGGGCGGTATCACCAACGGTCAGACTATG				
6	AbHV	CSIRO AbHV ORF49 qPCR	pAbHV_ORF49_qPCR	Corbeil et al. (2010)
AACCCACACCCAATTTTTGAGTGTAGGCGAATACATTTGCTTTCTTACCGCTTTCAATCTGATCCGTGGTTTCTTTAGTCGTTTTGAACCGTCTAGCATCCAGTGAATCTGTTTGCATAAAGGAACAACAAACTTGCCTTGGG				
7	AbHV	OIE AbHV ORF66 qPCR	pAbHV_ORF66_qPCR	Caraguel et al. (2019)
TCCCGGACACCAGTAAGAACTTGTCCCTGCTCGCGCTGTAAATCACCTTGGAATCTTCCATGGCCGTCGAGATGTCCATGGGTGAGTAGCACGTGCTGTTCTCACCGTCTAGCATCCAGTGCTGTGTTTGATAACTTCCAGTCTCATACGCATAGCAGCCTTG				
8	AbHV	OIE AbHV ORF77 qPCR	pAbHV_ORF77_qPCR	Caraguel et al. (2019)
CAACCACTTGTTCGGGTTCTCGACGACGAGTGTACATCCGTACGCGGGATCTTCGTAAAGTTCAAACTCGTGAGTCGTAACGGCCCTTCCTACTTCGATCAAGACAGACCTGTCCTTCAGAGTACCGTCTAGCATCCAGTGGCGTAAGGATCCCAAATCGAGATCACCAACCTGTCTTCGTCGTCAAACTCCGCATTAATCACCCTG				
9	NNV	ANZSDP NNV RT-qPCR	pNNV_Usyd_RT-qPCR	Hick P and Whittington (2010); Moody and Crane (2015)

ACGTTGCTGGCTTCCTGCCTGATCCAACTGACAACGATCACACCTACCGTCTAGCATCCAGTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAATGGTGGGAAAGCAGAACAGTCCGACC				
10	ISAV	ISAV EUNA7 RT-qPCR	pISAV_EUNA7_RT-qPCR	Snow et al. (2006)
AGCTGGATCCAGGGTTGTATCCATGGTTGAAATGGACAGAGACGGCGTATCATTCATCTACGAGAAGCCTAGCATCTAACCGTCTAGCATCCAGTCCATAGTGATGGGTGCACTGGGACAGCATCGAGGGTCTGGAGACGGGATCACAATGAGAGAGCTGGAGTTGAGCTTAGGGCTGGACTTCACTTCA				
11	ISAV	ISAV EUNA8 RT-qPCR	pISAV_EUNA8_RT-qPCR	Snow et al. (2006.
ACAGGCGTCTACACAGCAGGATGCAGATGTATGCTCTAGGAACCGTCTAGCATCCAGTGCGAGTTCGAAAGCCCTGGAAACTTTAGAAAAGGCCATCGTCGCTGCAGTTCATCGACTTCCGGCATCCTGCACGAGCAT				
12	VHSV	OIE VHSV Jonstrup RT-qPCR	pVHSV_OIE_Jonstrup_RT-qPCR	Jonstrup et al. (2012)
GCCATCAGGAAACTCGCAGGATGTGTGCGTCCCTCAGACCGTCTAGCATCCAGTGTCAGCAGAAGATCACCAAGGCCCTCTATGCATTCATCCTGACTGAGATCGCAGACCCCACCAC				
13	VHSV	OIE VHSV Garver RT-qPCR	pVHSV_OIE_Garver_RT-qPCR	Garver et al. (2011.
ATCCAGTTGATGAGGCAGGTGTCGGAGGCGAAGTCCATACCGTCTAGCATCCAGTCCAAGAGCGCTACGCCATCATGATGAGTCGGATGCTGGGAGAGTCCTACTACAAGTCGTATG				
14	SVCV	SVCV Zhang RT-qPCR	pSVCV_Zhang_RT-qPCR	Zhang et al (2009)
CAGGAGAATCATTCAAAGGATTGCATCAGGGACTGATGAAGATCTGGGGTTTCCCCCTCAAAGTTGCGGATGGGCATCTGTCACAACGGTGTCAAAACCGTCTAGCATCCAGTTACTAATTATAGAGTAGTACCCCATTCTGTTCATTTAGAGCCATATGGAGGACATT				
15	TSV	OIE TSV RT-qPCR	pTSV_OIE_RT-qPCR	Tang et al. (2004)
GATGGGTGCTTGGGCACCAAACGACATTCCACACCGTCTAGCATCCAGTAGACAGCACTGACGCACAATATTCGAGCATCACAGTGTGTCCAGTTTAAGCTCCCCACAGAAAA				
16	TSRV	CSIRO TSRV RT-qPCR	pTSRV_CSIRO_qRT-qPCR	Zainathan et al. (2014)
CATGGAGATGATCGAACCCGTCGTGTCTAAGGGAGCCCGAGCCATCTGGGCGCATGCCATGATACCGTCTAGCATCCAGTGAACCAGAGTTGTGACAAGCTGAGCACCGCGTGTCGAC				
17	POMV	AFDL POMV RT-qPCR	pPOMV_AFDL_RT-qPCR	Mohr et al. (2020)
CTTGAGAGCATCAGAAGGGACGGTGGAAGCTCACATAGACAGAACCGTCTAGCATCCAGTGCTTATCACTTGATTCAAGCCCAAGGAACCGCAAAAGCATCCATCCACAGAAAGACCCAATCAAGAGTTGCGAGACCGATTGTCCTG				
18	YHV1	AFDL YHV1 RT-qPCR	pYHV_AFDL_RT-qPCR	AFDL Unpublished

GTTACCCACCAGTCTACAGTGCTCTGATCTCCATTCAGAAATTCAGCACCTGGGCTCGTCTCATGTGTCATGATATTCTCAAGCGAGTTTTCAATCACTGTCGTGACTGTGACACCGTCTAGCATCCAGTCATCTCAACTGCAAGATCTCACGACAACTCATGCGCTTCAAGAATCCACTCTCCAA				
19	AHPND PirA	Han AHPND qPCR	pAHPND_Han_qPCR	Han et al. (2015)
TTGGACTGTCGAACCAAACGGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGGAAGTCGGTCGTAGTGTAGACATTGAGAATACGGGACGTGGGGAGACCGTCTAGCATCCAGTCTTACCATTCAATACCAATGGGGTGC				
20	Bonamia	CSIRO *Bonamia* sp. qPCR	pBonamia_CSIRO_qPCR	Corbeil et al. (2006) Note: Annealing temperature lowered to 60°C
CCCTGCCCTTTGTACACACCGCCACCGTCTAGCATCCAGTCGTCGCTTCTACCGATTGAATAATGAGGTGAATTAGGTGGATAAGAGCCGCCTCGGCGCGTTCTTAGAAGCTTTGTGA				
21	Perkinsus	Gauthier *Perkinsus* sp. qPCR	pPerkinsus_Gauthier_qPCR	Gauthier et al. (2006)
TCCGTGAACCAGTAGAAATCTCAACGCATACTGCACAAAGGGGATCTTTCCTCTTTGTACCGTCTAGCATCCAGTACATACATATCAGTGTCGCTCTTCTTCC				
22	Perkinsus	AFDL *Perkinsus olseni* qPCR	pPerkinsusolseni_AFDL_qPCR	Gudkovs et al. (2016)
TCTCGTATTGTAGCCCCTCCGAGAACCGTCTAGCATCCAGTGGAGGACCGCGCCTGTGAGTGTCTTTGGATGCTCGCAAGTCCGACTGTGTTGTGGTGATATCACGTGTTCCTT				
23	NHP	OIE NHP 16S rRNA qPCR	pOIE_NHP_16SrRNA_qPCR	Aranguren et al. (2010)
CGTTCACGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGACCGTCTAGCATCCAGTTTATCTTTTCTTTAAGGCGATGAGC				
24	SDDV	SDDV de Groof qPCR	pSDDV_degroof_qPCR	de Groof et al (2015)
GCTGAAACAACAATTTAGATGTGTAAGGAATCTTGTATTTTTCAATTGACACAGTGTTACAATGACGGCATATGCTAAAATTATCGGACATCATTTTGCATAAAGTACAACCGTCTAGCATCCAGTTACTGGAATTTCATACGCATCGCTCATATCAGTCATGCTTTCTTGTAATGCACTG				
25	CMNV	CMNV Pooljun RT-qPCR	pCMNV_POOLJUN_RT-qPCR	Pooljun et al. (2016)
TCCAAAAGGACCTCCGCAATCTGATTGCATGCGTCAACCTCAGCGTGACCAAGGACCGTCTAGCATCCAGTTCTCCAAGGCCGTGACATCGCTGATGGACAAGCCGACAGTGGTAGCGTACCTAACAGGTCTGCCCCCTCCAACGAAAGTAGACCCCGACGCCAA				
26	TiLV	TiLV Hong Liu RT-qPCR	pTiLV_HongLiu_RT-qPCR	Hong Liu – China, personal communication with OIE TiLV electronic *ad Hoc* Group.
ATGACTGTTTGAAGAATAAGTGGATTGCCTTTGAACCGTCTAGCATCCAGTGCTGGAAGGCCAGCCGCGGAAATTTCCAAAGGCAACAGTTCGTTGCATTT				
27	EHP	EHP Liu qPCR	pEHP_Liu_qPCR	Liu et al. (2018)

AGTAAACTATGCCGACAATGCTGGGTGTTGCGAGAGCGATGCTTGGTGTGGGAGAAATCTTAGTTTTACCGTCTAGCATCCAGTCGGGCTCTGGGGATAGTACGCTCGCAAGGGTGAAACTTAAAGCGAAATTGACGGAAGGACACTACCAGGAGTGGATTGTGCTGCTTAATT				
28	DIV1 (SHIV)	SHIV Qui qPCR	pSHIV_Qui_qPCR	Qiu et al. (2018)
AGGAGAGGGAAATAACGGGAAAACGGTAACTCAAACGTTATTTGAGAAAATGTTGGGAAAGTTTGCAATTAAATTCAACAACCGTCTAGCATCCAGTCATCTCTGATTACGGGTAAAAAGGCAAACATGGGAGCTGCAAGTCCCGAATTGGCCAGGGCGGGAGATGGTGTTAGATGGGCAGTCATGGATGAACCAAATGCTGACG				
29	SAV	OIE SAV RT-qPCR	pOIE_SAV_RT-qPCR	Hodneland and Endresen (2006)
GCGGACCACCCGGCCCTGAACCAGTTCCAGACTGCGTTTCCAGGGTTCGAAGTGGTGGCCAGCAACAGGTCGTCCAAACCGTCTAGCATCCAGTCGACCATGCCGCCGCCAGAGCTTTCTCCCACTTGGCTACCAAGTGGAT				
30	*X. californiensis*	*Xenohaliotis californiensis* Friedman qPCR	pXcal_Friedman_qPCR	Friedman et al. (2014)
AGTTTACTGAAGGCAAGTAGCAGACGGGTGAGTAATGCTTGGAAATCTACTCAGAAGACATGAATAACTATCAGAAATGGTAGCTAATACAAGACCGTCTAGCATCCAGTATAATACCCTGAGGGGGAAAGGTTTATTCCGCTTTTGAATGAGTCCAAGTTAGA				

Appendix 2. Plasmid construct details and references for conventional PCR assays. Universal probe insert is highlighted in blue.

Plasmid	Pathogen	Assay	Plasmid Name	Reference
31	EHNV	OIE EHNV PCR	pEHNV_OIE_PCR	Marsh et al. (2002)
AACCCGGCTTTCGGGCAGCAGTTTTCGGTCGGCGTTCCCAGGTCGGGGGATTACATCCTCAACGCCTGGTTGGTGCTCAAGACCCCCGAGGTCAAGCTCCTGGCCGCAAACCAGCTGGGAGA CAACCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACCAGCGCCCAGTCCTTTAACACGGCATACCTGGACGCCTGGAGCGAGT ACACCATGCCAGAGGCCAAGCGCATAGGCTACTATAACATGATAGGCAACACCAGCGATCTCATCAACCCCGCCCCGGCCACAGGCCAGAACGGAGCCAGGGTCCTCCCGGCCAAGAACCTG GTTCTTCCCCTCCCATTCTTCTTCTCCAGAGACAGCGGCCTGGCCCTGCCAGTCGTCTCCCTCCCCTACAACGAGATCAGGATAACAGTCAAGCTGAGGGCCATCCAGGACCTCCTGATCCTCCA GCACAACACCACAGGGGCAATCAGCCCCATCGTGGCCGCCGACCTCGAGGGAGGTCTCCCCGACACCGTCGAGGCCAACGTCTACATGACCGTCGCCCTCATCACCGGGGACGAGAGGCAG GCCATGAGCAGCACAGTCAGGGACATGGTCGTGGAGCAGGTGCAGGCCGCCCCAGTCCACATGGTCAACCCCAGGAACGCGGCCACCTTCCACACCGACATGCGGTTCTCACACGCAGTCAA GGCCCTGATGTTTATGGTGCAGAACGTCACACACCCTTCCGTCGGCTCCAATTACACCTGCGCCACTCCCGTCGTGGGAGTCGACAACACGGTCCTGGAGCCAGCCCTGGCGGTGGATCCCAC TAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACTGGTGCAGCCCTGGTACTATGCCACCTCCATCCCAGTCAGCACCGGGCACCACC TCTACTCTTATGCCCTCAGCCTGCAGGACCCCCACCCATCCGGATCCACCAATTACGGCAGACTGACCAACGCCAGCCTTAACGTCACCCTGTCCGCTGAGGCCACCACGGCCTCCGCAGGAGG CGGAGGCGACAACTCTGGGTACACCACCGCCCAAAAGTACGCCCTCATCGTTCTGGCCATCAACCACAACATTATCCGCATCATGAACGGCTCGATGG				
32	WSSV	OIE WSSV nPCR	pWSSV_OIE_nPCR	Lo et al. (1996)
ACTACTAACTTCAGCCTATCTAGTAAAACAAGCTAAAAGATTCGACGGAGTTGACCCAGCCTTCCCTGCCGCCCTCACCTGCGCTTCTCACCTCATGCTTTCTTCCATGGATTCCCATACAAAGTC ATCTTTCATGGACAACATCAAATTGCACATGACTGATACTCAATGCTTCTTCAAGAACATTGAACGATTTGAGAAATTCTTGGGAAGATATGGGGACGAATACGCCATGTCCCACAAGCAAAAT TGTAACTGCCCCTTCCATCTCCACCACACTTTTACTCCCTCAGATAACGAGCATCTGGTATCCTCTTTCGCATTCGCCCGCCCAGAAGTCTCCATGGAAGAAATTAGAGCCACACCCTATCAGGC CAACAAGCTTATTAGTGACAAACATTACGTGATGAACATGTCCAAGATCGATTCTAGAGTAACAGGATCTTCCCTCCTTAAGAAGGTTAGCGAATGGACTGAAATGAGAATGAACTCCAACTT TAATGGAACATTTGAACCATCAAGACTCGCCCTCTCCAACTCTGGCATGACAACGGCAGGAGTCAACCTCGACGTTATTGTCAAACCAAATAATGCAAGAAGTGTACTAGGAATATTGGAATG TCATCGCCAGCACGTGTGCACCGCCGACGCCAAGGGAACTGTCGCTTCAGCCATGCCAGCCGTCTTCCACCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACC ACTAGCAAGCATGATAGACGAGCACAATGAACGCTCAAACTGTCGTGTTTGCTAATGTTTTGGAACAACTTATCGCCGATCTTGGAAAGGTTATCGTGAACGAACTGGCCGGCACCATCGCTG AATCTGTACCAGAAAGCGTATATGAAAACACCAAGGAAATGATTGATAGACTAGGCTCTGACGACCTCTTCAAATCTAATAATAATGGAGGAGTAGAATCAATGGATTATGAAGATAGCGAA ACAACATCCAACAATGGTCCCGTCCTCATCTCAGAAGCCATGAAGAATGCCGTCTATCACACACTAATTTCCGGCAAGGCAGCTCGCCCGGAAAATGTACCATTCGCCTCATGCGCCAGCGGCC CTCTCGCCTTTGATTTCCTTCTGTCAAAGGGAGATACATTCGAAGAAAAGAACGCCGAACAAGGTGCAGCAGCTGCCGTATCCTCTACCTATTCTTCCTCTTCTAACACTACTCTTCGTAAGCAT TTGGCTCGAGTTTTCGAAGCCATCTCTAAGCAAGTAACTGATGCTGAATTCAAGGATATCCTCAACGATATCGAACGTAATATTTCTTCTGACTATACTAACTGTCCACCAAATACTAACCAAAA TGCCTTTGCTCTAGCTATCAAGAGAGAATTCAGCAGAATTGTTTCCTTCTTAACCATTCTTCGTAAGAACATTACACCCGCATTA				
33	POMV	AFDL POMV RT-nPCR	pPOMV_AFDL_RT-nPCR	Mohr et al. (2020)
AAGAAAATTGAAGCAATGGGAAGAGAGACGCTCAAACAGAGGATGATGGAAGAATATTACAAAAGCATAAAAACTGATGTAAAAGAACTGGCAAAAGGACGGGCAACGTATGAGATGATA GGAGACTCCTCTCTCCAAGCAAGGGATTGCCTGAGAGTGTGGATGAGAGCAACAGGGTTCAAAGGAAAGACACTTGGGGACCTAATATCTCATCACAATGAAATAAGGCTAAACAATGGGA AAATGAAGGGGGAATGCAGAACGATCGAATGGAGCGACAAGCACAAAAGGAAAGTGCAAACCAAGAAGCCTTTCATATTGGAATGTAGTGAATATGTAAGAAGAGGCTACAAACTGCTGG CTTTTCTAAAATCGGAAGAGAGGACAAAGAATGAGCCAAGAGCGATCTTCAGTGCCAGCATAATCTGGAGGGCATATGTCTACATCCTGGAAAAGGTGTTTGCTACAGTAAATTCGCAAGAT GCACCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACCTGACACAAGCACCGGCCGCGGTGAGCCTGACAGGAGACAACAGCA				

AATTCAACGAGACACAAAGCCCCGAATCATGCATCTTGTTCATATTGAAAATGGACCTGCCAAAAGAAATGACAGAAGTTCTCGTTTTCGCTCTTCATCAGTTCACACAGAAGAGAGTTGCATG TATGAGAGGAATAAGAAGAGAAACCACGGACTCAAGCGTGATGGTCAACCAAGCTGAGTTACTTGAGGTGCTTCACGTGATGTCGGGCGAAGTCAAGGAACGCATCCAAGCAGTAACAATT GACGTGGATGGATCTTTCATCTGTCCAAGAGGGATGTTGATGGGTATGGCTAACTACGCTTTTACCACAATGGCAACAATGGCGTCTTCTTTCTGTTTCACAGAGGGAACTGTGTTGACTCATC AGAGCTCCGATGATTTCGTCACCTTTGCGGCTGGAAGAGACGTTCAGCACGCCTTGGGAAGGCTTGAGATGGCTTTGAAGGCATCCAAAATTGCGGGGCTGAACTGTTCAGAAAAGAAGTCA TACTTCTGTCAAGGCCTTACTTGGGAGTTCACTTCCTTGTTCATAAACAAAGGCAAGCACATCCCGAATCTTGGGAACATCGAATACAGCACCACTCCTCAGGGACTCGGGCCTGCAACAGATC TCTTCAACATTGGGAAAATTGCAAACAACGCCAGACTCAGAGGCAATTCATCTATGACTTGTTCCAGAGTCATTTGTGAC				
34	YHV1	AFDL YHV1 RT-nPCR	pYHV1_AFDL_RT-nPCR	AFDL unpublished
TTCACTGACCTTGACATTCAAATCCCCATCTACACTGTCCACACATCGCAAGGCAGAACATTTGACCGTGGAATCGTCGTCAGCTACCGCAACACCGCGTTCACAAAGGATCCAAACATTGTGA ATGTAGCCGTCAGTCGTTTCCGCTTCCAGTGTATCTGCATGCATCAGGGCAATCCATACTACGCTAAACTTCCATACTACAACACATCACAAATCTACTTTGAGAAATCTACTCCACTAGCAAGC ATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACTACCACACCCTTGAAAATCGCTATCAGAGTGAAAAGGCAAAGTATCTTGGCAAGAAATTAAT CCTCCACAACAATCCATTTGAAACTCTCAAAGAAGCCAAGAAAGTGTTCACACGCGAAGATAACCTCAGATGGGCCAAAGTATCAGCAGAAGTAATGACTCGCTTGCTGTTCGAGAAATTCAA CAATCCCGATCTAGCTAAGCATCTCATCAACACTGGCAAAAGTCATCTAGTCGAAAACACCAAGCATCCTATCTGGGGCGGCAA				
35	ISAV	OIE ISAV Seg2 RT-PCR	pISAV_OIE_Seg2_RT-PCR	Kibenge et al. (2000)
CTTAAGGAAGTCAATGGAAGAAGCATTCGAGAAGTCTATGAACGAAGAATTTGTCGTACTGAACAAAGGGAAATCGGCCAATGACATTATCTCCGACACAAACGCCATGTGTAAATTCTGCG TTAAAAACTGGATAGTAGCAACAGGTTTCAGAGGAAGAACCATGTCAGACCTGATTGAACACCATTTCAGGTGTATGCAGGGGAAACAAGAAGTGAAAGGATACATATGGAAACATAAATA CAACGAGAGACTTAAGAGAAAACAACTAACCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACACAGCGAAAGAACTAAGCTG GAGCCGAGAGCAGTGTTCACAGCTGGAGTACCTTGGAGGGCTTTCATCTTCGTTCTAGAACAGACAATGCTCGTCGTGAACAAGTTGGACCCAAACTCAGTCATCTGGATGGGAAGCGATGC CAAAATCAACACGACAAACTCCAGGATAAAGGAAATCGGAATGAAGAACCAAGGGCAAACACTGGTAACGCTCACTGGAGACAACTCTAAGTACAATGAGAGCATGTGCCCAGAGGTGATG TAGATTTTCCT				
36	TSV	OIE TSV RT-PCR	pTSV_OIE_RT-PCR	Nunan et al. (1998)
CTTTGGGCAAAGTAGACAGCCGCGCTTGCGTGGTGGGACTTAATTAATGCCTGCTAACCCAGTTGAAATTGATAATTTTGATACAACAACCACTAGCAAGCATGATAGACATGGCAGACGAAG CCACAGCAGCACACCTACCACTAGCAAGCATGATAGACCAATCACTAATCAGAATGTAGTGCTGTCTAAGAATGTAACAGATAACCTGTTTGAAGTCCAGGACCAAGCTCTCATTGAATCTCTC TC				
37	NNV	ANZSDP NNV RT-nPCR	pNNV_ANZSDP_RT-nPCR	Moody et al. (2009); Moody and Crane (2015)
TGTGGTCAACGTGTCAGTCATGTGTCGCTGGAGTGTTCGATTGAGCGTTCCATCTCTTGAGACACCTGAAGAGACTACCGCTCCCATCATGACACAAGGTTCCCTGTACAACGATTCCCTTTCC ACAAATGACTTCAAGTCCATCCTCCTAGGATCCACACCACTGGACATTGCCCCTGATGGAGCAGTCTTCCAGCTGGACCGTCCGCCCACTAGCAAGCATGATAGACATGGCAGACGAAGCCAC AGCAGCACACCTACCACTAGCAAGCATGATAGACGAAATGCTGGCACACCTGCAGGCTGGTTTCGCTGGGGCATCTGGGACAACTTTAACAAGACGTTCACAGATGGCGTTGCCTACTACTCT GATGAGCAGCCCCGTCAAATCCTGCTGCCTGTTGGCACTGTCTTCACCCGTGTTGACTCGGAAAACTAA				
38	TABV	CSIRO TABV RT-PCR	pTABV_CSIRO_RT-PCR	Davies et al. (2010)

TCTCTAACAACGAACCCTCAGGACAAGGTCAACAACCAGCTGGTGACCAAAGGAGTCACAGTCCTGAACCTACCAACCGGGTTCGACAAGCCATACGTCCGACTAGAGGACGAGACACCCCA GGGTCTCCAATCGATGAACGGGGCGAAGATGAGGTGCACAGCTGCGATTGCACCGAGGAGGTACGAGATCGACCCCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGCAGCAC ACCTACCACTAGCAAGCATGATAGACTCGTCAACTCAACAACAGTGACGGGAGACATCAACTTCAGTCTGGCGGACAACCCCCCAACGGAGACCAAGTTCGACTTCCAACTGGACTTCATGGG ACTTGACAACGACGTCCCTGTGGTAACGGTGGTCAGCTCAGTGCTGGCAACAGCCGACAACTACAGAGGAGTACTATGCCAAGATGATCCTGTGCATCCCGAC				
39	VHSV	OIE VHSV 2009 RT-PCR	pVHSV_OIE_2009_RT-PCR	Snow et al. (2004)
TGACAAGGCATGGAAGGAGGAATTCGTGAAGCGTTTTCAGGCCTGAATGATGTTAGGATTGACCCCACCGGTGGAGAGGGACGGGTACTTGTACCTGGTGAAGTGGAGCTCGTCGTGTATG TCGGTGGATTTGGTGAGGAAGATAGGAAGGTGATTGTGGATGCACTCTCCGCACTCGGGGGACCCCAGACTGTACAGGCGTTGTCCGTGCTTCTCTCCTATGTACTCCCCACTAGCAAGCATG ATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACCAGCCAGGGCCACGAGCATCGAGGCAGGAATCATGATGCCCATGAGAGAACTGGCCCTGACTGT CAATGACGACAACCTCATGGAAATCGTTAAGGGGACCTTGATGACATGCTCCCTTCTGACCAAGTACTCGGTGGACAAGATGATCAAGTACATCACCAAGAAACTCGGGGAGCTGGCAGACA CCCAGGGAGTTGGGGAACTGCAGCACTTCACCGCTGACAAGGC				
40	AbHV	OIE AbHV 1617 PCR	pAbHV 1617 PCR	In-house development (cloned PCR amplicon)
This construct is not a synthetic construct and was developed by cloning a PCR amplicon. The amplicon sequence does not contain the “contamination control sequence”.				
41	CMNV	Covert Mortality Nodavirus RT-nPCR	pCMNV_ZHANG_RT-nPCR	Zhang et al. (2014)
ATCGGACCAAAATACGGCGATGACGGCTTGAGCCACAACCGAGTCAAACCTTTCATCAACAAAGTAGCGAACGCACTAGGCCACTAGCAAGCATGATAGACATGGCAGACGAAGCCACAGC AGCACACCTACCACTAGCAAGCATGATAGACTTTTAACACAAACACTTCCATTACGGATCCCCTTCGCTGTTTACGCAAAATCCATCTCACCGCCCGGAACCCTTCCGTCCCTTTAGCAGACGCA TGTTGCGATCGAGTTGAAGGCTATCTCGTGACAGATGCCCTTACACCATTGGTGGGTGATTACTGTCGCTCTATGATTCGATTGTATGGTGGAGCTGCGTCAAGTCAATTGGTAAGACTGAAG CGCAAAACCAGCAATTCCGAGAAGCCCAATTGGTTGACGAATGATGGTTCCTGGCCCCAAAATGCCGCCGATAAAGATGCTATGTTCAACGTCCTTTGTGCCCGCACTCAAATTCATCCCGAG ACGGTAAATAGCCTTATCGAACGCCTGGCCAACATAACTACACCTTTTGACCCTATAATTACTGAGTTTAATGATGCCGCAAGTAACAGTAATACCATCGGCGTTGATGGTCCAGTAGGGTCTG TGGGCACTTCGTTTGAAAGAC				
42	DIV1 (SHIV)	SHIV Qui qPCR	pSHIV_QIU_nPCR	Qiu et al. (2017)
GGGCGGGAGATGGTGTTAGATGGGCAGTCATGGATGAACCAAATGCTGACGAAATCATCAGTTCGGGAACGTTAAAGGGTCTCACGGGAAACGATTCGTATTGGGCTCGAGATCCACTAGC AAGCATGATAGACATGGCAGACGAAGCCACAGCAGCACACCTACCACTAGCAAGCATGATAGACCCAGCAATCAAGGATGCCGATCAAGCAACGTGGAATCGAATCAGGGTTATTCCATTCG AAAGTACATTCAAACATGAAAACGATTGCCCCGTTGAATTTGAAGAACAAATGAAACAGAAAACATTCCCCATGGATAAAAATTTCACAGAAAAGATTCCCGAAATGGTAAAACCCCTGGCTT GGTATCTTATTCAGAGATGGAAGACTATCAGGAAGTGTGAAATTGTAGAGCCAGAGATTGTAACGGTAGCTACATCTTCGTACCGAAACGA				

Appendix 3. List of pathogens and molecular assays and associated positive control plasmids for real-time PCR assays

Plasmid	Pathogen	Assay	Plasmid Name	Format	NQC-1	NQC-2
					10^4 copies/µL	10^2 copies/µL
1	WSSV	CSIRO WSSV qPCR	pWSSV_CSIRO_qPCR	qPCR	Yes	Yes
2	WSSV	OIE WSSV qPCR	pWSSV_OIE_qPCR	qPCR	Yes	Yes
3	OsHV-1	EMAI OsHV-1 qPCR	pOsHV-1_EMAI_qPCR	qPCR	Yes	Yes
4	OsHV-1	OIE OsHV-1 Martenot qPCR	pOsHV-1_Martenot_qPCR	qPCR	Yes	Yes
5	Megalocytivirus	CSIRO Megalocytivirus qPCR	pMegalocytivirus_CSIRO_qPCR	qPCR	Yes	Yes
6	AbHV	CSIRO AbHV ORF49 qPCR	pAbHV_ORF49_qPCR	qPCR	Yes	Yes
7	AbHV	OIE AbHV ORF66 qPCR	pAbHV_ORF66_qPCR	qPCR	Yes	Yes
8	AbHV	OIE AbHV ORF77 qPCR	pAbHV_ORF77_qPCR	qPCR	Yes	Yes
9	NNV	ANZSDP NNV RT-qPCR	pNNV_Usyd_RT-qPCR	RT-qPCR	Yes	Yes
10	ISAV	ISAV EUNA7 RT-qPCR	pISAV_EUNA7_RT-qPCR	RT-qPCR	Yes	Yes
11	ISAV	ISAV EUNA8 RT-qPCR	pISAV_EUNA8_RT-qPCR	RT-qPCR	Yes	Yes
12	VHSV	OIE VHSV Jonstrup RT-qPCR	pVHSV_OIE_Jonstrup_RT-qPCR	RT-qPCR	Yes	Yes
13	VHSV	OIE VHSV Garver RT-qPCR	pVHSV_OIE_Garver_RT-qPCR	RT-qPCR	Yes	Yes
14	SVCV	SVCV Zhang RT-qPCR	pSVCV_Zhang_RT-qPCR	RT-qPCR	Yes	Yes
15	TSV	OIE TSV RT-qPCR	pTSV_OIE_RT-qPCR	RT-qPCR	Yes	Yes
16	TSRV	CSIRO TSRV RT-qPCR	pTSRV_CSIRO_qRT-qPCR	RT-qPCR	Yes	Yes
17	POMV	AFDL POMV RT-qPCR	pPOMV_AFDL_RT-qPCR	RT-qPCR	Yes	Yes
18	YHV1	AFDL YHV1 RT-qPCR	pYHV_AFDL_RT-qPCR	RT-qPCR	Yes	Yes
19	AHPND PirA	Han AHPND qPCR	pAHPND_Han_qPCR	qPCR	Yes	Yes
20	Bonamia	CSIRO *Bonamia* sp. qPCR	pBonamia_CSIRO_qPCR	qPCR	Yes	Yes
21	Perkinsus	Gauthier *Perkinsus* sp. qPCR	pPerkinsus_Gauthier_qPCR	qPCR	Yes	Yes
22	Perkinsus	AFDL *Perkinsus olseni* qPCR	pPerkinsusolseni_AFDL_qPCR	qPCR	Yes	Yes

Appendix 3 (cont'd). List of pathogens and molecular assays and associated positive control plasmids for real-time PCR assays

Plasmid	Pathogen	Assay	Plasmid Name	Format	NQC-1	NQC-2
					10^4 copies/µL	10^2 copies/µL
23	NHP	OIE NHP 16S rRNA qPCR	pOIE_NHP_16SrRNA_qPCR	qPCR	Yes	Yes
24	SDDV	SDDV de Groof qPCR	pSDDV_degroof_qP	qPCR	Yes	Yes
25	CMNV	CMNV Pooljun RT-qPCR	pCMNV_POOLJUN_RT-qPCR	RT-qPCR	Yes	Yes
26	TiLV	TiLV Hong Liu RT-qPCR	pTiLV_HongLiu_RT-q	RT-qPCR	Yes	Yes
27	EHP	EHP Liu qPCR	pEHP_Liu_qPCR	qPCR	Yes	Yes
28	DIV1 (SHIV)	SHIV Qui qPCR	pSHIV_Qui_qPCR	qPCR	Yes	Yes
29	SAV	OIE SAV RT-qPCR	pOIE_SAV_RT-qPCR	RT-qPCR	Yes	Yes
30	*X. californiensis*	*Xenohaliotis californiensis* Friedman qPCR	pXcal_Friedman_qPCR	qPCR	Yes	Yes

Appendix 4. List of pathogens and molecular assays and associated positive control plasmids for conventional PCR assays.

Plasmid	Pathogen	Assay	Plasmid Name	Format	NQC-1	NQC-2
31	EHNV	OIE EHNV PCR	pEHNV_OIE_PCR	PCR	Yes	No
32	WSSV	OIE WSSV nPCR	pWSSV_OIE_nPCR	nPCR	Yes	No
33	POMV	AFDL POMV RT-nPCR	pPOMV_AFDL_RT-nPCR	RT-nPCR	Yes	No
34	YHV1	AFDL YHV1 RT-nPCR	pYHV1_AFDL_RT-nPCR	RT-nPCR	Yes	No
35	ISAV	OIE ISAV Seg2 RT-PCR	pISAV_OIE_Seg2_RT-PCR	RT-PCR	Yes	No
36	TSV	OIE TSV RT-PCR	pTSV_OIE_RT-PCR	RT-PCR	Yes	No
37	NNV	ANZSDP NNV RT-nPCR	pNNV_ANZSDP_RT-nPCR	RT-nPCR	Yes	No
38	TABV	CSIRO TABV RT-PCR	pTABV_CSIRO_RT-PCR	RT-PCR	Yes	No
39	VHSV	OIE VHSV 2009 RT-PCR	pVHSV_OIE_2009_RT-PCR	RT-PCR	Yes	No
40	AbHV	OIE AbHV 1617 PCR	pAbHV 1617 PCR	PCR	Yes	No
41	CMNV	Covert Mortality Nodavirus RT-nPCR	pCMNV_ZHANG_RT-nPCR	RT-nPCR	Yes	No
42	DIV1 (SHIV)	SHIV Qui nPCR	pSHIV_QIU_nPCR	nPCR	Yes	No

Appendix 5: Details provided with the plasmid positive controls.

CSIRO AUSTRALIAN CENTRE FOR DISEASE PREPAREDNESS
www.csiro.au
5 Portarlington Road, East Geelong VIC 3219
Private Bag 24, Geelong VIC 3220, Australia
T (03) 5227 5000 • ABN 41 687 119 230

15 February 2021

Recipient Name
Recipient Laboratory
Address 1
Address 2

Phone:
Email:

Re: Positive control plasmids – DIV1

Dear .....,

Please find enclosed positive control plasmids for the DIV1 ATPase (Qui et al, 2018) qPCR assay.

Table 1: Positive control plasmid details

Name	Assay	Volume	Expected C_T
DIV1 ATPase qPCR Positive Control Plasmid NQC-1 (200µl) Batch: 1812-07-1002	DIV1 ATPase qPCR	2 × 200µL	25.58 (2µL per assay)
DIV1 ATPase qPCR Positive Control Plasmid NQC-1 (200µl) Batch: 1812-07-1004	DIV1 ATPase qPCR	2 × 200µL	32.38 (2µL per assay)

Plasmids are stabilised in TE Buffer (10mM Tris, 1mM EDTA, pH 8.0) containing 50ng/µL yeast tRNA.

The C_T values correspond to the addition of 2µL per assay and were obtained using a threshold of 0.1.

Yours sincerely,

Nick Moody BSc (Hons), PhD
Research Group Leader – ACDP Fish Diseases Laboratory
Australian Centre for Disease Preparedness | CSIRO
nick.moody@csiro.au | +61 3 5227 5749 | 0436 694 252
5 Portarlington Road, East Geelong, VIC 3219, Australia
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T +61 (03) 5227 5000 • ABN 41 687 119 230

Commercial Invoice

15 February 2021

Re: Import of reagents into ……..

AWB: XXX XXX XXX

Consignee:

Recipient Name
Recipient Laboratory
Address 1
Address 2

Phone:
Email:

This is to state the following in regard to the import of material from the Australian Centre for Disease Preparedness to the ……

Items to be brought into …… (contents of package):

Description of Contents	Batch Number	Animal Source	Country of Manufacture	Number of Items	Total Value
Scientific Research Specimens for molecular testing – purified plasmid (DNA)	1812-07-1002 Decapod iridescent virus 1 control material	n/a	Australia	2x tubes (200 µL/tube)	$5.00
Scientific Research Specimens for molecular testing – purified plasmid (DNA)	1812-07-1004 Decapod iridescent virus 1 control material	n/a	Australia	2x tubes (200 µL/tube)	$5.00
			Total Value of Package (AUD)		**$10.00**

The material will be received at the ……..

The materials are non-infectious, non-hazardous, are for scientific research purposes only and have a commercial value of AUD$10 for export purposes.

Yours sincerely,

Nick Moody BSc (Hons), PhD
Research Group Leader – ACDP Fish Diseases Laboratory
Australian Centre for Disease Preparedness | CSIRO
nick.moody@csiro.au | +61 3 5227 5749 | 0436 694 252
5 Portarlington Road, East Geelong, VIC 3219, Australia
Private Bag 24, Geelong VIC, 3220, Australia

REAL-TIME PCR FOR DECAPOD IRIDESCENT VIRUS 1 (DIV1 Qiu qPCR)

SAN: _____

DIV1 Qiu qPCR (188 bp)

Operator: _____ Date prepared: _____

Reagent	Volume for 1 rxn	Volume for ____ rxns
Water Batch: _____	6.75 µl	
TaqMan Universal PCR Master Mix Batch: _____ Control Date: _____	12.5 µl	
Primer name: **DIV1-Qiu-F** (18 µM) Batch: _____	1.25 µl	
Primer Name: **DIV1-Qiu-R** (18 µM) Batch: _____	1.25 µl	
TaqMan probe: **DIV1-Qiu-Probe** (5 µM) Batch: _____	1.25 µl	
Total volume	23 µl	

Thermal Cycler Program

Cycles	Conditions
1	50°C for 2 minutes
1	95°C for 10 minutes
45	95°C for 15 seconds 60°C for 60 seconds

Run Details

Date run: _____

Thermal cycler: _____

Operator: _____

Primer sequences:

Primer	Sequence
DIV1-Qiu-F	5'- AGG AGA GGG AAA TAA CGG GAA AAC -3'
DIV1-Qiu-R	5'- CGT CAG CAT TTG GTT CAT CCA TG -3'
Probe	
DIV1-Qiu-Probe	5'-**FAM**- CTG CCC ATC TAA CAC CAT CTC CCG CCC -**TAMRA**-3'

Reference:

Qiu L, Chen M-M, Wan X-Y, Zhang Q-L, Li C, Dong X, Yang B, Huang J (2018) Detection and quantification of shrimp hemocyte iridescent virus by TaqMan probe based real-time PCR. Journal of Invertebrate Pathology 154, 95-101.

END.

Appendix 6. List of Researchers and project staff

Name	Position	Organisation
Dr Nick Moody	Group Research Leader	CSIRO ACDP Fish Diseases Laboratory
Dr Peter Mohr	Team Research Leader	CSIRO ACDP Fish Diseases Laboratory
Dr Mark Crane	Senior Principal Research Scientist	CSIRO ACDP Fish Diseases Laboratory
Dr David Cummins	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Lynette Williams	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Mr John Hoad	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Stacey Valdeter	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Joanne Slater	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Mr Reuben Klein	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Dr Mike Snow	Senior Supervising Scientist	Fisheries – Western Australia
Dr Cecile Dang	Principal Research Scientist	Dept Primary Industries & Regional Development, Western Australia
Dr Terry Miller	Research Scientist	Dept Primary Industries & Regional Development, Western Australia

FRDC FINAL REPORT CHECKLIST

Project Title:	Aquatic Animal Health and Biosecurity Subprogram: Development of stable positive control material and development of internal controls for molecular tests for detection of important endemic and exotic pathogens		
Principal Investigators:	Dr Nick Moody		
Project Number:	2014/002		
Description:	Thirty-two positive control plasmids (22 for real-time assays and 10 for conventional assays) have been prepared and are in routine use in over 20 laboratories nationally and internationally. A further 10 plasmid positive controls (8 for real-time assays and 2 for conventional assays) are undergoing final quality checks. Therefore, a total of 42 plasmid positive controls for 25 different pathogens have been generated as a result of this project. In addition, T4 and QBeta bacteriophages (phages) have been evaluated as heterologous internal positive controls for DNA and RNA targets, respectively, for use in establishing that generic aspects of PCR testing (e.g. nucleic acid extraction and absence of PCR inhibitors) are performing as expected. Implementation of the use of the T4 and QBeta phages as internal positive controls has improved the quality of molecular testing, through more sensitive assessment of the effect of PCR inhibitors and confidence in results generated when testing atypical samples (i.e. plankton, dirt, feed).		
Published Date:	01/03/2021	**Year:**	2021
ISBN:	978-1-925994-19-3	**ISSN:**	Not applicable
Key Words:	Molecular diagnostic testing, non-infectious positive controls, heterologous internal controls, diagnostic capability, PCR		

Please use this checklist to self-assess your report before submitting to FRDC. Checklist should accompany the report.

	Is it included (Y/N)	**Comments**
Foreword (optional)	N	Optional
Acknowledgments		
Abbreviations	Y	Not relevant. Project staff listed in Appendix 6
Executive Summary		
- **What the report is about**	Y	
- **Background – why project was undertaken**	Y	
- **Aims/objectives – what you wanted to achieve at the beginning**	Y	
- **Methodology – outline how you did the project**	Y	
- **Results/key findings – this should outline what you found or key results**	Y	
- **Implications for relevant stakeholders**	Y	

– **Recommendations**	N	In the body of the Final Report
Introduction	Y	
Objectives	Y	
Methodology	Y	
Results	Y	
Discussion	Y	
Conclusion	Y	
Implications	Y	
Recommendations	Y	
Further development	Y	
Extension and Adoption	Y	
Project coverage	Y	
Glossary	N	List of Abbreviations is provided
Project materials developed	Y	
Appendices	Y	