World Organisation for Animal Health
Founded as OIE

Template for the validation report form for tests recommended in the WOAH *Aquatic Manual*

Section 1. Guide for applicants/contributors to the *Aquatic Manual*

1.1. Information to fill out in this form

The purpose of this template is to provide pre-publication validation data so that the Aquatic Animal Health Standards Commission (the Aquatic Animals Commission) can consider inclusion of proposed tests in the WOAH *Manual of Diagnostic Tests for Aquatic Animals* (*Aquatic Manual*). Only tests that have been validated to level 2 will be considered for inclusion in the *Aquatic Manual*. If the proposed assay is accepted for inclusion in the *Aquatic Manual*, the completed template will be made available on the WOAH website.

Before filling in this form and submitting an application, applicants should consult Chapter 1.1.2 of the WOAH *Aquatic Manual*

WOAH *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Terrestrial Manual*) and supporting chapters 2.2.1, 2.2.2, and 2.2.3 which provide information for validation of fundamentally different assays such as for the detection of antibodies, antigens and nucleic acid.

As shown in Figure 1, from the WOAH *Aquatic Manual* chapter 1.1.2, the following parameters have to be addressed: intended purpose(s), optimisation and standardisation, analytical sensitivity (ASe) and analytical specificity (ASp), repeatability, cut-off, diagnostic sensitivity (DSe) and diagnostic specificity (DSp), reproducibility, and conclusion about fitness for purpose. It is important that validation information supports the specific purpose, e.g. a screening test would need to show high DSe and a confirmatory test high DSp to conclude fitness for purpose.

Section 2. General information

2.1. Information about the applicant

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2.2. Name and purpose of the test

2.2.1. Type of method

Indirect or competitive ELISA, conventional or real-time PCR, etc.

Real-time reverse transcription PCR.

Through-out this report the YHV1 real-time reverse transcription PCR will be referred to as the YHV1 RT-qPCR.

2.2.2. Commercial name (if applicable)

Not Applicable

2.2.3. Intended purpose(s) of the test

Please select the specific purpose(s) of the test from the list of intended purposes provided below. Suitable data need to be provided to substantiate fitness for each selected purpose in the application.

Double click on a check box to indicate the purpose of the test. Select 'Checked' ☒ *to indicate Yes. Select 'Not Checked'* ☐ *to indicate No.*

1	Surveillance of apparently healthy animals	☒
2	Presumptive diagnosis of clinically affected animals	☒
3	Confirmatory diagnosis of a suspect result from surveillance or presumptive diagnosis	☐

2.3. Test description

2.3.1. Protocol of the test

Include your detailed working protocol here to allow users to set up the test in their laboratory and interpret the results. Please include targets in analytical terms, the species and specimens that can be examined and the positive and negative controls included. Include definitions of test positivity cut-offs or results categories.

4.4. Nucleic acid amplification

PCR assays should always be run with the controls specified in Section 5.5 Use of molecular and antibody-based techniques for confirmatory testing and diagnosis of Chapter 2.2.0 General information (diseases of crustaceans). Each sample should be tested in duplicate.

Extraction of nucleic acids

Different kits and procedures can be used for nucleic acid extraction. The quality and concentration of the extracted nucleic acid is important and can be checked using a suitable method as appropriate to the circumstances.

4.4.1. Real-time PCR

The real-time PCR methods was developed by the reference laboratory.

Pathogen /Target	Primer/probe (5'-3')	Concentration	Cycling parameters
YHV1 /ORF1	YHV1-12-qF: AGT-CTA-CAG-TGC-TCT-GAT-CT YHV1-12-qR: GAT-TCT-TGA-AGC-GCA-TGA-GT YHV1-12-qPr: FAM-TCT-CAT-GTG/ZEN/TCA-TGA-TAT-TCT-CAA-GCG-AGT-IABkFQ	900 nM of each primer 250 nM of probe	Reverse transcription at 48°C/30 min 1 cycle 95°C/10 min 45 cycles of 95°C/15 sec and 60°C/60 sec

The protocol in place at the ACDP Fish Diseases Laboratory is as follows:

Tissue samples can be homogenised by manual pestle-grinding or by bead-beating. Depending on the number of samples to be tested, nucleic acids are extracted with either the QIAamp Viral RNA Mini Kit (Qiagen) or MagMAX-96 Viral RNA Isolation Kit (Applied Biosystems) according to the manufacturer's instructions. A negative extraction control, consisting of extraction reagents only, should be included when test samples are extracted.

The YHV1 RT-qPCR is a YHV1-specific real-time PCR screening test run as; template (2 µl) is added to 23 µl reaction mixture containing 12.5 µl 2× AgPath-ID One-step RT-PCR Buffer, 1 µl 25× RT-PCR Enzyme Mix (ThermoFisher International), 900 nM of each YHV1-12-qF and YHV1-12-qR primer, 250 nM of YHV1-12-qPr probe and molecular grade water. After 1 cycle of 48°C for 30 minutes and 95°C for 10 minutes, PCR amplification consists of 45 cycles of 95°C for 15 seconds, 60°C for 60 seconds.

2.3.2. Disease target/analyte target

State targets in analytical terms.

Yellow head virus genotype 1 nucleic acid, ORF1 gene.

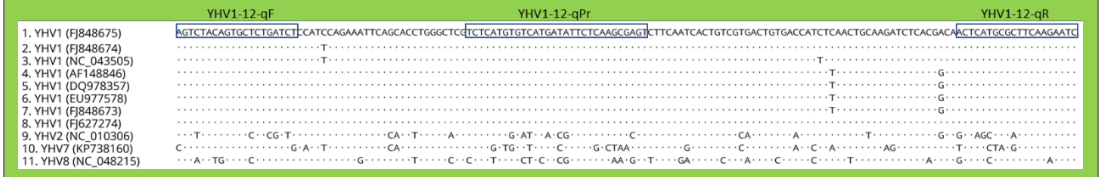
Section 3. Development and validation of the assay

3.1. Assay development pathway

3.1.1. Design, development, optimisation and standardisation of the assay for the intended purpose

For guidance, refer to Section Assay development – the experimental studies of Chapter 1.1.2 of the WOAH Aquatic Manual

The YHV1 RT-qPCR was designed to detect specifically YHV1, with primers and a Taqman probe identical in sequence with available YHV1 strain nucleotide sequences, that also incorporated mismatches with other available YHV genotype sequences.



The primer and probe concentrations, reagents and cycling parameters adopted were those routinely used for all real-time reverse-transcription PCR assays at the ACDP Fish Diseases Laboratory Laboratory for the detection of aquatic pathogens with RNA genomes. Initial analytical sensitivity, analytical specificity, repeatability estimates and testing of known YHV1 positive and negative samples, indicated that the YHV1 RT-qPCR assay performed as designed and therefore a more detailed evaluation of the validation pathway was undertaken as detailed below.

3.2. Validation pathway stage 1 – analytical characteristics

3.2.1. Stage 1. Repeatability data

Repeatability is the level of agreement between replicates of a sample both within (intra-assay) and between (inter-assay) runs of the same test method in a single laboratory. Repeatability is estimated by evaluating variation in results of replicates. The number of replicates should preferably be determined in consultation with a statistician with a suggested minimum of three different samples representing analyte activity within the operating range of the assay. Within or intra-assay variation can be assessed using three or more replicates of each sample in one run (one operator). Intra-assay and inter-assay variation can be assessed by testing the panel of samples over several days, using two or more operators, e.g. for a total of 10–20 runs.

The data/detail provided must be clear, including:

i) the number of different isolates use, ideally minimum of three covering analytical range of test (strong/moderate/weak)

ii) the number of replicates per sample for intra-assay and inter-assay analysis

iii) the number of different operators used at a single site

Include statistical data where applicable, e.g. coefficients of variation or upper and lower ranges.

Repeatability of the YHV1 RT-qPCR has been estimated when testing two concentrations of plasmid DNA positive controls (synthetic DNA plasmid containing the 150 bp YHV1 target sequence prepared in TE Buffer + 50 ng/µL tRNA) on each PCR plate tested. The two positive controls were tested in duplicate on each plate, by two operators, on a total of 52 plates. The testing of both positive controls by both operators on each plate fell within the laboratory acceptance criterion. The ACDP Fish Diseases Laboratory acceptance criterion for positive control C_T is set at the mean ± 2 C_T of the mean, that is determined by testing all aliquots of the prepared control. Coefficient of variation (CV <5%) indicated that there was no significant difference in repeatability between plates tested by each operator or all operators. The 95% predictive interval (95% PI) for each operator or all operators was within the laboratory acceptance criterion.

Operator	No. plates	Plasmid Positive Control 1			Plasmid Positive Control 2		
		C_T Mean ± SD	CV (%)	95% PI	C_T Mean ± SD	CV (%)	95% PI
1	28	25.54±0.42	1.65	25.43-25.65	32.57±0.56	1.71	32.43-32.72
2	24	25.43±0.48	1.88	25.29-25.56	32.25±0.58	1.79	32.08-32.41
All Operators	52	25.49±0.45	1.76	25.40-25.57	32.42±0.59	1.81	32.31-32.54

SD = Standard Deviation, CV – Co-efficient of variation, PI = Prediction interval

The approach taken above is the same as that published for the performance characteristics of two WSSV real-time PCR assays for the detection of WSSV (Moody et al, 2022, Disease of Aquatic Organisms v150, p169-182). This publication has been used to update the WOAH *Aquatic Manual* chapter for infection with white spot syndrome virus, adopted in May 2023.

Repeatability of the YHV1 RT-qPCR was also estimated using genomic RNA extracted from a YHV1 positive control. The genomic YHV1 positive control was tested in duplicate on each plate, by nine operators, on a total of 81 plates. The testing of the positive control by the nine operators on each plate fell within the ACDP Fish Diseases Laboratory acceptance criterion. Coefficient of variation (CV <5%) indicated that there was no significant difference in repeatability between plates tested by each operator or all operators. The 95% predictive interval (95% PI) for each operator or all operators was within the laboratory acceptance criterion.

Operator	No. plates	Genomic YHV1 RNA		
		Ct Mean ± SD	CV (%)	95% PI
1	3	28.71±0.12	0.43	28.61-28.80
2	31	28.85±0.22	0.78	28.79-28.90
3	4	29.01±0.12	0.43	28.92-29.09
4	10	29.12±0.19	0.64	29.04-29.21
5	2	28.94±0.14	0.47	28.80-29.07
6	2	28.70±0.32	1.10	28.39-29.01
7	15	28.81±0.36	1.27	28.68-28.95
8	9	28.92±0.27	0.95	28.79-29.05
9	5	29.04±0.25	0.86	28.88-29.19
All Operators	81	28.90±0.04	0.95	28.85-28.94

SD = Standard Deviation, CV – Co-efficient of variation, PI = Prediction interval

3.2.2. Stage 1. Analytical specificity data (as appropriate for the test type and disease)

Analytical specificity is the degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false positives. The assessment of analytical specificity is qualitative, and the choice and sources of sample types, organisms and sequences for the assessment should reflect test purpose and assay type. Analytical specificity is further characterised by determining:

a) selectivity, which is the extent to which a method can accurately quantify the targeted analyte in the presence of interferents, for example, a) of matrix components such as inhibitors of enzymes in the reaction mix, b) degradants (toxic factors), c) nonspecific binding of reactants to a solid phase, e.g. conjugate of an ELISA absorbed to well of microtiter plate, and d) antibodies to vaccination that may be confused with antibodies to active infection,

b) exclusivity, which is the capacity of the assay to detect an analyte or genomic sequence that is unique to a targeted organism, and excludes all other known organisms that are potentially cross-reactive. This would also define a confirmatory assay, and

c) *inclusivity, which is the capacity of an assay to detect several strains or serovars of a species, several species of a genus, or a similar grouping of closely related organisms or antibodies thereto. It characterises the scope of action for a screening assay.*

The selectivity and inclusivity of the YHV1 RT-qPCR was demonstrated by testing all known YHV1 nucleic acid extracts available at the ACDP Fish Diseases Laboratory. All YHV1 known positive material was test positive. All nucleic acid was extracted from infected prawns/shrimp.

Nucleic acid extract	YHV1 RT-qPCR
YHV1 1998	Positive
YHV1 2001	Positive
YHV1 2002	Positive
YHV1 2003	Positive
YHV1 2008	Positive
YHV1 2012	Positive
YHV1 2017	Positive

The exclusivity of the YHV1 RT-qPCR was demonstrated by testing a wide range of nucleic acid extracts containing various YHV genotypes, prawn pathogens or from various prawn species. All non-YHV1 material was test negative.

Nucleic acid extract	YHV1 RT-qPCR
Yellow head virus genotype 2	Negative
Yellow head virus genotype 3	Negative
Yellow head virus genotype 4	Negative
Yellow head virus genotype 5	Negative
Yellow head virus genotype 6	Negative
Yellow head virus genotype 7	Negative
Yellow head virus genotype 9	Negative
Acute hepatopancreatic necrosis disease	Negative
Infectious myonecrosis virus	Negative
Infectious hypodermal and haematopoietic necrosis virus	Negative
Hepatobacter penaei	Negative
Taura syndrome virus	Negative
White spot syndrome virus	Negative
Penaeus monodon	Negative
Penaeus esculentus	Negative
Penaeus merguiensis	Negative

3.2.3. Stage 1. Analytical sensitivity data

Analytical sensitivity is synonymous with 'Limit of Detection', the smallest detectable amount of analyte in a specified matrix that would produce a positive result with a defined certainty. An analyte may include antibodies, antigens, nucleic acids, or live organisms. The WOAH Terrestrial Manual *Chapter 2.2.1 suggests each dilution in the series should be tested in 10 replicates, however, three to five replicates are acceptable. The dilution series must extend to at least one dilution past end-point (negative/not detectable). Criteria for end-point dilution must be established, e.g. the end-point is the last dilution for which all replicates are positive. A precise estimate of ASe is often not available for assays for infectious diseases, except for PCR where it is possible to calculate the threshold number of copies of a target nucleic acid sequence that can be detected by the assay. Alternatively, it is possible to compare the limit of detection between the candidate test and reference test to obtain a relative estimate for ASe.*

Include statistical data where applicable, e.g. coefficients of variation or upper and lower ranges.

To estimate the analytical sensitivity of the YHV1 RT-qPCR with plasmid DNA, a 10-fold dilution series of a synthetic DNA plasmid (containing the 150 bp YHV1 target sequence) was prepared in TE Buffer + tRNA (50 ng/µL). The dilution series was prepared independently on three separate occasions from the same plasmid stock. Plasmid copy number was calculated from the DNA concentration of the plasmid stock, as determined by Qubit 2.0 fluorometer and a plasmid size of 3089 bp. Each dilution series was tested in triplicate PCR reactions. The limit of detection (LOD) was defined as the final dilution where all test replicates tested positive in all dilution series. All positive and negative controls performed as expected (data not shown). The YHV1 RT-qPCR LOD (highlighted in blue) when testing plasmid DNA was estimated to be 20 copies per reaction.

Plasmid copies per reaction	Dilution Series 1			Dilution Series 2			Dilution Series 3			Total reactions (positive /tested)
	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	
200000	21.47	21.82	21.64	19.69	20.11	20.34	19.75	20.42	20.32	9/9
20000	25.21	25.35	24.91	23.31	23.65	23.91	23.57	23.77	23.70	9/9
2000	28.60	28.70	27.96	26.64	27.42	27.53	27.36	27.43	27.25	9/9
200	32.54	32.31	32.19	29.95	30.72	30.79	30.22	30.45	30.19	9/9
20	35.21	35.60	35.13	33.69	33.49	34.85	32.95	32.98	34.62	9/9
2	-	-	40.37	36.60	44.33	-	36.73	40.59	34.74	6/9
0.2	-	-	-	40.69	-	-	-	-	-	1/9
0.02	-	-	-	-	-	-	-	-	-	0/9

To estimate the analytical sensitivity of the YHV1 RT-qPCR on genomic YHV1 RNA, 10-fold dilutions of nucleic acid extracted from YHV1 positive *P. monodon* haemolymph were prepared in TE Buffer + tRNA (50 ng/µL). The dilution series was prepared independently on three separate occasions from the same haemolymph stock. The YHV1 RNA copy number of each dilution was calculated from a standard curve generated by simultaneously testing 5 DNA plasmid standards (200,000 to 20 copies/reaction) in triplicate on each genomic RNA test plate. Each dilution series was tested in triplicate PCR reactions. The limit of detection (LOD) was defined as the final dilution where all test replicates tested positive in all dilution series. All positive and negative controls performed as expected. The YHV1 RT-qPCR LOD (highlighted in blue) when testing genomic YHV1 RNA was estimated to be approximately 6 copies per reaction in the 10^{-4} dilution.

YHV1 RNA dilution (copies per reaction)	Dilution Series 1			Dilution Series 2			Dilution Series 3			Total reactions (positive /tested)
	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	Rep 1 C_T	Rep 2 C_T	Rep 3 C_T	
10^{-1} (4659)	23.44	23.53	23.41	23.58	23.62	23.55	23.60	23.62	23.49	9/9
10^{-2} (470)	26.96	26.98	26.89	26.80	26.92	26.79	26.98	26.91	27.10	9/9
10^{-3} (47)	30.28	30.34	29.96	30.58	30.50	30.13	30.47	30.47	30.34	9/9
10^{-4} (6)	33.21	33.34	33.52	32.65	34.62	32.94	32.65	33.88	34.59	9/9
10^{-5} (0.8*)	-	-	-	-	-	-	36.51	-	-	1/9
10^{-6} (0.7*)	-	-	-	-	36.38	-	-	-	36.47	2/9
10^{-7} (n/a)	-	-	-	-	-	-	-	-	-	0/9
10^{-8} (n/a)	-	-	-	-	-	-	-	-	-	0/9

* Copies per reaction calculated from less than three replicates.

3.2.4. Stage 1. Standard of comparison

For a preliminary evaluation, the standard method(s) of comparison (reference standard) should be run in parallel on a small but select group of highly characterised test samples representing the linear operating range of the new method(s). Identify and cite the reference method(s) and protocol(s) used in the study.

WOAH *Aquatic Manual* chapter 2.2.9 Infection with yellow head virus genotype 1 (version adopted in May 2019), section 4.3.1.2.3 Molecular Techniques, describes three reverse-transcription polymerase chain reactions (RT-PCR). Protocol 1 is a 1-step RT-PCR (Wongteerasupaya et al. 1997) that can detect YHV1 in affected shrimp. Protocol 2 is a multiplex nested RT-PCR (Cowley et al. 2004) that can differentiate YHV1 from GAV in diseased shrimp or for screening healthy carriers. Protocol 3 is a multiplex nested RT-PCR (Wijegoonawardne et al. 2008) that can detect 7 YHV genotypes but will not discriminate between genotypes. As the chapter lacks a real-time PCR the analytical sensitivity of the YHV1 RT-qPCR was compared to Protocol 1 and Protocol 2 RT-PCR assays.

To estimate the analytical sensitivity of the YHV1 RT-qPCR, YHV1 Protocol 1 RT-PCR and YHV1 Protocol 2 nested RT-PCR, 10-fold dilutions of nucleic acid extracted from YHV1 positive *P. monodon* haemolymph were prepared in TE Buffer + tRNA (50 ng/µL). The dilution series was prepared independently on three separate occasions from the same haemolymph stock. The YHV1 RNA copy number of each dilution was calculated from a standard curve generated by simultaneously testing 5 DNA plasmid standards (200,000 to 20 copies/reaction) in triplicate on each YHV1 RT-qPCR test plate. Each dilution series was tested in triplicate PCR reactions. The limit of detection (LOD) was defined as the final dilution where all test replicates tested positive in all dilution series. All positive and negative controls performed as expected (data not shown). The YHV1 RT-qPCR LOD (highlighted in blue), when testing genomic YHV1 RNA, was estimated to be approximately 6 copies per reaction in the 10^{-4} dilution (as per section 3.2.3 results). The YHV1 RT-qPCR was ten-fold more sensitivity than the YHV1 Protocol 1 RT-PCR that had a LOD of approximately 47 copies per reaction in the 10^{-3} dilution. The YHV1 RT-qPCR had comparable sensitivity with the YHV1 Protocol 2 nested RT-PCR that also had a LOD of approximately 6 copies per reaction in the 10^{-4} dilution.

YHV1 RNA dilution (copies per reaction)	YHV1 RT-qPCR			YHV1 Protocol 1 RT-PCR			YHV1 Protocol 2 nested RT-PCR		
	Series 1	Series 2	Series 3	Series 1	Series 2	Series 3	Series 1	Series 2	Series 3
10^{-1} (4659)	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^{-2} (470)	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^{-3} (47)	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^{-4} (6)	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3
10^{-5} (0.8*)	0/3	0/3	0/3	0/3	0/3	0/3	1/3	2/3	1/3
10^{-6} (0.7*)	0/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3	0/3

* Copies per reaction calculated from less than three replicates.

3.3. Stage 2 – Diagnostic performance of the assay

3.3.1. Study design(s)

Ideally, study design(s) should be done with the assistance of a statistician to ensure that the sample size and the experimental approach are valid. Give an overview of the chosen approach used for determination of diagnostic specificity and sensitivity estimates. Include rationale for statistical design, choice of populations, animals or animal models, numbers of animals used to generate confidence intervals for sensitivity and specificity etc. Field samples are preferable to samples from experimental infection studies which may not be representative of natural infection and often result in overestimation of Dse and DSp. Host variables in the target population must be represented and recorded. The true status (positive/negative, etc.) of the reference animal populations should be independently verified by a different technique. Bayesian latent class model (LCM) Chapter 1.1.6 can be used to account for imperfect reference tests and test accuracy data from animals of unknown status. LCMs do not assume that the reference test is a perfect "gold standard" but estimate the accuracy of the reference test and the candidate test *with the joint test results.*

YHV1 is exotic to Australia and it has not been possible to source international field samples of apparently healthy or clinically diseased prawns infected with YHV1. Therefore clinically diseased *P. monodon* and *P. merguiensis* infected with YHV1 were created by experimental infection at the ACDP Fish Diseases Laboratory to fulfil section 3.3.4 'Stage 2. Experimentally infected or vaccinated reference animals'.

Farmed *P. monodon* (~7-9 cm in length) and farmed *P. merguiensis* (~6-8 cm in length) were placed in round (infection by feeding) or square (infection by co-habitation) polypropylene tanks containing 80 L of 100-µm-filtered seawater, a canister filter and air stones. Prawns were distributed across 3 rooms:

a) *P. monodon* and *P. merguiensis* negative controls

b) *P. monodon* exposed to YHV1 by feeding and co-habitation

c) *P. merguiensis* exposed to YHV1 by feeding and co-habitation

Each tank held 22 to 25 prawns with water temperature maintained at 29°C using immersion heaters. Animals were monitored and fed commercial prawn pellets daily (unless being fed YHV1 positive tissue) and 30% water exchanges were undertaken every 2 days. Prior to infection, 25 animals of each species were sacrificed, 10 of which were fixed in 80% (v/v) ethanol for PCR testing and the other 15 fixed in Davidson's Fixative for histological analysis. The farmed prawns were pre-screened by PCR for YHV2 and YHV7 (known to be enzootic in Australia), with only *P. monodon* testing positive for YHV2. Histological pre-screening observed that the lymphoid organ tubules of negative control *P. monodon* and *P. merguiensis* appeared normal. Two *P. monodon* contained low-level Type A lymphoid organ spheroids with no lymphoid organ spheroids observed in *P. merguiensis*.

YHV1 Feeding: Previous experimentally YHV1 infected prawns (*P. monodon* or *P. merguiensis* with low YHV1 RT-qPCR C_T values) stored at -80°C, were the source of infected material for the feeding tanks. For feeding trials only YHV1 infected *P. merguiensis* were feed to *P. merguiensis* and YHV1 infected *P. monodon* were feed to *P. monodon*. Pools of 3 or 4 frozen infected prawns were taken into the feeding rooms and sliced in half longitudinally then cut into pieces approximately 5 mm in length. The prawn segments were sprinkled on the surface of the water for each feeding tank and the tank observed to ensure each prawn was consuming prawn tissue (this generally occurred within about 30 seconds). The prawns were fed once daily with the infected prawns, for five days, and were then fed commercial prawn pellets. After exposure to YHV1 by feeding, in both *P. monodon* and *P. merguiensis*, 100% mortality was observed at 5-6 days and 4-5 days post-inoculation in duplicate tanks, respectively. Pleopods were sampled from each YHV1 fed mortality and fixed in 80% (v/v) ethanol for PCR testing. One tank of negative control prawns for each species was fed frozen negative control prawns as for exposed prawns. Pleopods were sampled from each negative control prawn at the termination of the experiment and fixed in 80% (v/v) ethanol for PCR testing.

YHV1 Co-habitation: Two square polypropylene tanks were joined by two pieces of piping and an immersible pump used to transfer water out of one tank and into the second tank to create a flow-through system. Mesh was tied over the ends of the piping to stop movement of prawns from one tank to the other. One tank contained 25 prawns injected with YHV1 (infected prawns) and the other tank contained prawns that had no treatment applied to them (co-habitant prawns). YHV1 inoculum was prepared from archived YHV1 haemolymph, that had been stored in liquid nitrogen, that was thawed and diluted in PBSA (pH 7.4). The YHV1 inoculum was pre-screened and found to be negative by PCR for YHV1, WSSV, TSV, IMNV, IHHNV and AHPND. After injection with YHV1, in both *P. monodon* and *P. merguiensis*, 100% mortality was observed at 5-6 days post-inoculation, respectively. Mortality rates in both *P. monodon* and *P. merguiensis* co-habitants reached 100% at 9-10 days, respectively. Pleopods were sampled from only co-habitant mortalities and fixed in 80% (v/v) ethanol for PCR testing. One tank of negative control prawns for each species was mock injected. Pleopods were sampled from each negative control prawn at the termination of the experiment and fixed in 80% (v/v) ethanol for PCR testing.

Details above have been extracted from Moody, Mohr & Crane (2021). Aquatic Animal Health and Biosecurity Subprogram: Determining the susceptibility of Australian *Penaeus monodon* and *Penaeus merguiensis* to newly identified enzootic (YHV7) and exotic (YHV9 and YHV10) Yellow head virus (YHV). FRDC 2015-005 Final Report. CSIRO Centre for Disease Preparedness

3.3.2. Stage 2. Reference animal populations

It is imperative that estimates of DSe and DSp are as accurate as possible. Ideally, they are derived from testing a panel of samples from reference animals, of known history and infection status relative to the disease/infection in question and relevant to the country or region in which the test is to be used.

3.3.2.1. Negative reference animals/samples

True negative samples, from animals that have had no possible infection or exposure to the agent, may be difficult to locate. It is often possible to obtain these samples from countries or zones that have eradicated or have never had the disease in question. Provide details of the source of the samples and the tests used to define status of animals.

YHV1 is exotic to Australia and therefore farmed *P. monodon* and *P. merguiensis* are known to be truly negative animals. Farmed *P. monodon* and *P. merguiensis* sourced by the ACDP Fish Diseases Laboratory for use for pre-trial testing or negative control prawns sampled at the end of experimental trials have been tested with the YHV1 RT-qPCR. Ethanol-fixed pleopods or gills have tested negative for YHV1 from 56 farmed *P. monodon* and 20 *P. merguiensis*. No false positives have been detected from the truly negative animals.

3.3.2.2. Positive reference animals/samples

Positive refers to known exposure to, or infection with, the agent in question. Provide details of the tests used to define status of animals or prevalence within population. It is generally problematic to find sufficient numbers of true positive reference animals, as determined by isolation of the pathogen. It may be necessary to resort to samples from animals that have been identified by another test of sufficiently high accuracy, such as a validated nucleic acid detection assay. The test is applied to these reference samples and results (*positive and negative*) *are cross-classified in a 2 × 2 table. This has been called the "gold standard model" as it assumes the reference standard is perfect.*

Positive reference animals of apparently healthy or clinically diseased prawns infected with YHV1 do not exist due to the limited geographical distribution of YHV1 infected prawns. Therefore YHV1 positive reference samples (clinically diseased) were created by experimental infection via feeding or co-habitation of farmed *P. monodon* and *P. merguiensis* as described in section 3.3.1 to fulfil section 3.3.4.

3.3.3. Stage 2. Samples from animals of unknown status

A way to overcome the problem of an imperfect reference standard is to perform a latent class analysis of the joint results of two tests assuming neither test is perfect. LCMs estimate the accuracy of the candidate test and the reference standard with the joint test results. If Bayesian latent class analysis was used, please describe sampling from the target population(*s*), *the characteristics of other tests included in the analysis, the appropriate choice of model and the estimation methods based on peer-reviewed literature.*

Currently the ACDP Fish Disease Laboratory do not receive or have access to samples from animals of unknown health status to test for YHV1.

3.3.4. Stage 2. Experimentally infected or vaccinated reference animals

In cases when the near-impossibility of obtaining suitable reference samples from naturally exposed animals necessitates the use of samples from experimental animals for validation studies, the resulting DSe and DSp measures should be considered as less than ideal estimates of the true DSp and DSe. Multiple serially acquired pre- and post-exposure results from individual animals are not acceptable for establishing estimates of DSe and DSp because the statistical requirement of independent observations is violated. Single time-point sampling of individual experimental animals can be acceptable but the strain of organism, dose, and route of administration to experimental animals are examples of variables that may introduce error when extrapolating DSe and DSp estimates to the target population. Please provide complete description: age, sex, breed, etc. Immunological status. Type of exposure, inoculation, source, aerosol, contact, sampling plan and procedures, etc.

YHV1 positive reference samples (clinically diseased) were created by experimental infection via feeding or co-habitation of farmed *P. monodon* and *P. merguiensis* as described in section 3.3.1. The 130 samples from clinically diseased prawns infected with YHV1 were tested by YHV1 RT-qPCR as per section 2.3.1.

Species (YHV1 Exposure)	**n =**	**Positive (C_T range)**	**Negative**
P. monodon (Cohabitation)	23	23 (17.01 - 29.68)	0
P. monodon (Feeding)	43	43 (16.82 - 30.69)	0
P. merguiensis (Cohabitation)	22	22 (18.49 – 33.40)	0
P. merguiensis (Feeding)	42	42 (17.49 - 29.65)	0
Total	130	130 (16.82 – 33.40)	0

3.3.5. Stage 2. Cut-off (threshold) determination

The selection of the cut-off(s) should reflect the intended purpose of the assay and its application, and must support the required DSe and DSp of the assay. Options and descriptive methods for determining the best way to express DSe and DSp are available (*Branscum* et al., *2005; Georgiadis* et al., *2003; Greiner* et al., *1995; 2000; Jacobson, 1998; Zweig & Campbell, 1993; and Chapter 2.2.5 of the* Terrestrial Manual).

From Moody et al. (2022); To increase sensitivity and avoid false-negative results, no C_T cut-offs were used. Within AFDL no C_T cut-offs are used for real-time PCR assays as all real-time PCR tests generating a typical amplification curve are considered positive and further investigations are undertaken, which can include re-testing, testing by conventional PCR and amplicon sequence analysis and collection of additional samples for testing from the source population for increased monitoring of the population. This is considered a valid alternative to C_T cut-off values (Caraguel et al., 2011).

3.3.6. Stage 2. Diagnostic sensitivity and specificity estimates – with defined reference animals

Complete either 3.3.6 if defined reference animals were used, or 3.3.7 if a latent class model was used.

Diagnostic sensitivity is the proportion of known infected reference animals that test positive in the assay; infected animals that test negative are considered to have false-negative results. Diagnostic specificity is the proportion of known uninfected reference animals that test negative in the assay; uninfected reference animals that test positive are considered to have false-positive results. Please include a 2×2 table and confidence intervals for estimates of these parameters.

For quantitative diagnostic tests, a useful adjunct to estimates of diagnostic sensitivity and specificity is an estimate of the area under the receiver operating characteristic (ROC) curve. Please include this information where relevant.

Diagnostic sensitivity and diagnostic specificity estimates – with defined reference animals, YHV1 positive reference samples (clinically diseased) from section 3.3.4 and YHV1 negative reference samples from section 3.3.2.1.

Diagnostic Sensitivity 2x2 tables:

P. monodon experimentally infected with YHV1 by cohabitation

		Clinical Appearance	
		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	23
	Negative	0	0

DSe = true positives / true positives + false negatives = 23/23 + 0 = 1.0 = 100%

P. monodon experimentally infected with YHV1 by feeding

		Clinical Appearance	
		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	43
	Negative	0	0

DSe = 43/43 + 0 = 0.100 = 100%

P. merguiensis experimentally infected with YHV1 by cohabitation

		Clinical Appearance	
		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	22
	Negative	0	0

DSe = 22/22 + 0 = 1.0 = 100%

P. merguiensis experimentally infected with YHV1 by feeding

		Clinical Appearance	
		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	42
	Negative	0	0

DSe = 42/42 + 0 = 1.0 = 100%

Diagnostic Specificity 2x2 tables:

Farmed *P. monodon* known to be YHV1 negative

		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	0
	Negative	56	0

DSp = true negatives / true negatives + false positives = 56/56 + 0 = 1.0 = 100%

Farmed *P. merguiensis* known to be YHV1 negative

		Healthy	Diseased
YHV1 RT-qPCR	Positive	0	0
	Negative	20	0

DSp = 20/20 + 0 = 1.0 = 100%

3.3.7. Stage 2. Diagnostic sensitivity and specificity estimates – without defined reference animals

Complete either 3.3.6 if defined reference animals were used, or 3.3.7 if a latent class model was used.

Complete description of LCM used (*Bayesian or maximum likelihood*). *Describe rationale for use of this approach, and sources of priors (e.g. experts and published papers) for Bayesian models providing relevant, supporting data. Population selection criteria should be presented, including prevalence estimates. Other test methods evaluated should also include the standard method of comparison. The source data tables with cross-classified test results should be presented for each test population. Using best available priors, choose test populations with appropriate prevalences and select animals in sufficient numbers to generate estimates of sensitivity and specificity with an allowable error of ± 5% at a level of 95% confidence. If multiple laboratories are involved in the study design, data on reproducibility should be presented in Section 3.4.3.*

Not applicable.

3.3.8. Stage 2. Comparison of performance between tests

For standard method(s) of comparison (reference methods) used in full field studies, indicate diagnostic sensitivity and specificity estimates as determined in either Section 3.3.6 or 3.3.7. The reference method could also be used to calculate relative DSe and DSp of the candidate test. Provide statistical measures of agreement between the reference method(s) and the new test being validated and suggest explanations for results not in agreement.

Not applicable. For practical reasons, experimental samples were only tested using the YHV1 RT-qPCR.

3.4. Stage 3 – Reproducibility

Reproducibility is the ability of a test method to provide consistent results when applied to aliquots of the same sample tested by the same method in different laboratories. Where possible, the reproducibility assessment should include data from tests conducted at an WOAH Reference Laboratory or national laboratory. The panel should contain at least 20 samples and at least three laboratories should participate in the reproducibility testing. Further information is available in the WOAH *Terrestrial Manual*.

3.4.1. Stage 3. Laboratory identification

State the number of laboratories included (minimum of three), which should also include WOAH Reference Laboratories or Collaborating Centres, or national laboratories where they exist.

Ten laboratories participated in a YHV1 interlaboratory comparison (ILC) activity, including the ACDP Fish Diseases Laboratory.

3.4.2. Stage 3. Evaluation panel

Description of test panel used for independent reproducibility study (interlaboratory comparisons), nature and number of samples and assessment of homogeneity and stability.

Each laboratory received 30 uniquely labelled samples that consisted of 20 YHV1 positive samples that ranged from strong to weak, YHV1 negative samples and samples containing YHV genotype 2, YHV genotype 7 and YHV genotype 9 (see Table below). The samples consisted of frozen haemolymph or tissue homogenate supernatants that had been inactivated by treatment with 50 kGy gamma irradiation. The samples were stabilised with the addition of 20% foetal bovine serum prior to gamma irradiation. The YHV1 ILC program was conducted under the principles of ISO 17043.

For YHV1 positive samples with expected C_T values <30 (i.e. YHV1 strong, moderate and weak-1), homogeneity was assessed from 6 aliquots of each preparation. For YHV1 positive samples with expected C_T values >30 (i.e. YHV1 weak-2, weak-3, weak-4 and weak-5), homogeneity was assessed from 10 aliquots of each preparation. The coefficient of variation was <5% for each preparation and therefore they were deemed to be acceptable samples for the program. The homogeneity of 10 aliquots of YHV genotype 2, YHV genotype 7 and YHV genotype 9 was also assessed with the same criteria and the samples were also deemed to be acceptable for the program.

Pathogen	Expected result YHV1 assay	Number of samples per panel
YHV1 Strong	Positive	2
YHV1 Moderate	Positive	2
YHV1 Weak-1	Positive	2
YHV1 Weak-2	Positive	2
YHV1 Weak-3	Positive	4
YHV1 Weak-4	Positive	4
YHV1 Weak-5	Positive	4

Negative diluent only	Negative	4
YHV2	Negative	2
YHV7	Negative	2
YHV9	Negative	2

The stability of the samples used in the activity was not assessed due to;

a) samples being provided to participants via shipment on dry ice
b) the stability of equivalent YHV1 positive samples had previously been demonstrated after assessment at 4 temperatures (-20°C, 4°C, 24°C and 37°C) over 4 time points (0, 1, 2 and 4 weeks).

3.4.3. Stage 3. Analysis of reproducibility

Interpretation and statistical analysis of results.

Instructions were provided regarding nucleic acid extraction from 50 µL for each sample with testing to be by usual diagnostic procedures for YHV1. Results are only shown in the table below for laboratories using the YHV1 RT-qPCR (i.e. results from laboratories using different assays, or where the assay used was unclear, have been excluded). Variables between testing laboratories included nucleic acid extractions, TaqMan probe labelling, concentration of primers and probe, PCR master mix, PCR cycling parameters, thresholds and real-time PCR instruments.

The ACDP Fish Diseases Laboratory (Lab 4) and four other laboratories (Lab 2, Lab 3, Lab 7 and Lab 9) reported all YHV1 positive and negative samples as expected with the YHV1 RT-qPCR. A sixth laboratory using the YHV1 RT-qPCR detected the negative samples as expected (Lab 10) and reported one false negative sample from the twenty YHV1 positive samples.

Sample	Lab 2	Lab 3	Lab 4	Lab 7	Lab 9	Lab 10
YHV1 strong	POS	POS	POS	POS	POS	POS
YHV1 strong	POS	POS	POS	POS	POS	POS
YHV1 moderate	POS	POS	POS	POS	POS	POS
YHV1 moderate	POS	POS	POS	POS	POS	POS
YHV1 weak-1	POS	POS	POS	POS	POS	POS
YHV1 weak-1	POS	POS	POS	POS	POS	POS
YHV1 weak-2	POS	POS	POS	POS	POS	POS
YHV1 weak-2	POS	POS	POS	POS	POS	POS
YHV1 weak-3	POS	POS	POS	POS	POS	POS
YHV1 weak-3	POS	POS	POS	POS	POS	POS
YHV1 weak-3	POS	POS	POS	POS	POS	POS
YHV1 weak-3	POS	POS	POS	POS	POS	POS
YHV1 weak-4	POS	POS	POS	POS	POS	POS
YHV1 weak-4	POS	POS	POS	POS	POS	POS
YHV1 weak-4	POS	POS	POS	POS	POS	Neg
YHV1 weak-4	POS	POS	POS	POS	POS	POS
YHV1 weak-5	POS	POS	POS	POS	POS	POS
YHV1 weak-5	POS	POS	POS	POS	POS	POS
YHV1 weak-5	POS	POS	POS	POS	POS	POS
YHV1 weak-5	POS	POS	POS	POS	POS	POS
Negative - diluent only	Neg	Neg	Neg	Neg	Neg	Neg
Negative - diluent only	Neg	Neg	Neg	Neg	Neg	Neg
Negative - diluent only	Neg	Neg	Neg	Neg	Neg	Neg
Negative - diluent only	Neg	Neg	Neg	Neg	Neg	Neg

Negative - YHV2	Neg	Neg	Neg	Neg	Neg	Neg
Negative - YHV2	Neg	Neg	Neg	Neg	Neg	Neg
Negative - YHV7	Neg	Neg	Neg	Neg	Neg	Neg
Negative - YHV7	Neg	Neg	Neg	Neg	Neg	Neg
Negative - YHV9	Neg	Neg	Neg	Neg	Neg	Neg
Negative - YHV9	Neg	Neg	Neg	Neg	Neg	Neg

POS = Positive, Neg = not detected

3.5. Stage 4 – Monitoring the performance

To retain the status of a validated assay it is necessary to assure that the assay as originally validated consistently maintains the performance characteristics as defined during validation of the assay.

Reproducibility is assessed through external quality control programmes such as at least annual, proficiency testing. This is an essential requirement of ISO 17025 accredited laboratories.

At minimum a plan for monitoring the performance is essential for the initial acceptance as an WOAH validated test and implementation of that plan will be required to maintain its status as an WOAH validated test.

3.5.1. Stage 4. Routine performance

Description of procedures in place or planned, to monitor the assay's daily performance within the applicant's laboratory. This monitoring differs from assessment of repeatability. Repeatability determined as part of the assay validation pathway, is the level of agreement between results of replicates of a sample, both within and between runs of the same method in one laboratory. Daily monitoring concerns the inclusion of quality control samples for example a strong and weak positive control, in the assay to confirm that the results fall within established limits which may be defined by the repeatability studies. Please include details of the controls included in the assay to determine whether it is performing as expected and the measurement of uncertainty.

A YHV1 genomic RNA positive control with a C_T value of approximately 29 has been tested in duplicate within each YHV1 RT-qPCR plate tested at the ACDP Fish Diseases Laboratory since 2015. Four independent batches of positive control have been prepared and tested on 163 PCR plates with the YHV1 RT-qPCR assay and all have passed. The acceptance criteria for the positive control is based on a C_T mean calculated from the testing of all aliquots within a batch ± 2 C_T. The coefficient of variation within the testing for each batch has been ≤1.08%.

Positive Control Batch	Testing Date Range	Batch Mean C_T	No. of Tests	Coefficient of Variation %
1	05/06/2015 to 28/06/2016	28.52	10	0.76%
2	30/06/2016 to 27/04/2017	28.45	16	0.84%
3	27/04/2017 to 05/06/2018	29.92	26	0.58%
4	08/06/2018 to 23/10/2023	29.04	111	1.08%

3.5.2. Stage 4. Reproducibility

Description of external proficiency programme in place or planned, to monitor reproducibility. Provide details including frequency and source of proficiency panel.

Since 2020 the ACDP Fish Diseases Laboratory has participated in the EURL Crustacean Diseases inter-laboratory proficiency testing panel for WSSV, TSV and YHV1. The YHV1 samples in the 2020 panel were *P. vannamei* pleopods in RNAlater and from 2021 FTA cards infused with shrimp homogenate at a high and low concentration. Each year 100% correct results have been reported after testing with the YHV1 RT-qPCR assay.

EURL Crustacean Diseases interlaboratory proficiency testing panels

Year	Panel Score
2020	6/6
2021	6/6
2022	6/6
2023	8/8

Since 2015 the ACDP Fish Diseases Laboratory has participated in the Australian National Aquatic Proficiency Testing program. This program contains YHV1-positive samples and testing has been undertaken using the YHV1 RT-qPCR. From 2015 to 2019 the YHV1 samples were provided as ethanol fixed prawn tissue suspension in a YHV1 panel. From 2021 the YHV1 samples were provided as gamma irradiated, lyophilised material in a crustacean panel for WSSV and YHV1. Each year 100% correct results have been reported after testing with the YHV1 RT-qPCR assay.

Australian National Proficiency Testing program testing results and assessment

Year	Panel/Sample	Panel Score/Assessment
2015	YHV1 panel / ethanol fixed tissue suspension	6/6 Satisfactory
2017	YHV1 panel / ethanol fixed tissue suspension	6/6 Acceptable
2019	YHV1 panel / ethanol fixed tissue suspension	6/6 Acceptable
2021 Round 1	Crustacean panel / gamma irradiated, lyophilised	WSSV and YHV1 Acceptable
2021 Round 2	Crustacean panel / gamma irradiated, lyophilised	WSSV and YHV1 Acceptable
2022	Crustacean panel / gamma irradiated, lyophilised	WSSV and YHV1 Acceptable
2023	Crustacean panel / gamma irradiated, lyophilised	WSSV and YHV1 Acceptable

3.5.3. Summary

Please include a summary of relevant parameters and a statement about whether the assay is fit for all purposes, its scope and limitations. Ideally this summary should include a succinct table inclusive of source population information as it relates to fitness for purpose, and also inclusive of numbers of animals in each relevant category (infected/uninfected). Examples (from the *Aquatic Manual*):

Table 4.1. WOAH recommended diagnostic methods and their level of validation for surveillance of apparently healthy animals and investigation of clinically affected animals

Method [amend or shade in as relevant]	Surveillance of apparently healthy animals				Presumptive diagnosis of clinically affected animals				Confirmatory diagnosis1 of a suspect result from surveillance or presumptive diagnosis			
	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV	Early life stages[2]	Juveniles[2]	Adults	LV
YHV1 RT-qPCR	++	+++	+++	4	++	+++	+++	4				

Table 4.4.1.1. Primers and probes (sequences) and cycling conditions for YHV1 RT-qPCR real-time PCR

Target gene	Primer/probe (5'–3')	Concentration	Cycling parameters[a]
Method 1: Reference; GenBank Accession No.: xxx			
ATPase	F: AGG-AGA-GGG-AAA-TAA-CGG-GAA-AAC R: CGT-CAG-CAT-TTG-GTT-CAT-CCA-TG: FAM-CTG-CCC-ATC-TAA-CAC-CAT-CTC-CCG-CCC-TAMRA	500 nM 200 nM	40 cycles of 95°C/100 sec and 60°C/30 sec

For presumptive diagnosis of clinically affected animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation
YHV1 RT-qPCR	Diagnosis	Infected by co-habitation or feeding	Pleopods	*Penaeus monodon, P. merguiensis*	100% (n=130)	100% (n=130)	Real-time PCR	This document

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.

For surveillance of apparently healthy animals

Test type	Test purpose	Source populations	Tissue or sample types	Species	DSe (*n*)	DSp (*n*)	Reference test	Citation

DSe = diagnostic sensitivity, DSp = diagnostic specificity, *n* = number of samples used in the study, PCR: = polymerase chain reaction.