National Aquatic Animal Health Laboratory System Report on the Development and Validation of a qPCR Assay for Detection of *Mikrocytos mackini*

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NATIONAL AQUATIC ANIMAL HEALTH LABORATORY SYSTEM REPORT ON THE DEVELOPMENT AND VALIDATION OF A qPCR ASSAY FOR DETECTION OF *MIKROCYTOS MACKINI*

by

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ABSTRACT

Abbott, C., Polinski, M., Lowe, G., Gilmore, S., Meyer, G., Kim, E., and Flores, A.-M. 2026. National Aquatic Animal Health Laboratory System Report on the Development and Validation of a qPCR Assay for Detection of *Mikrocytos mackini*. Can. Tech. Rep. Fish. Aquat. Sci. 3753: ix + 22 p. https://doi.org/10.60825/yab1-h547

Mikrocytos mackini is a protistan parasite that causes Denman Island Disease, or 'mikrocytosis', in Pacific oysters (*Crassostrea gigas*), which is a federally regulated disease in Canada. High sensitivity detection of *M. mackini* has several applications, including disease surveillance and import/export testing, hence we developed (Polinski et al. 2015) and diagnostically validated (Polinski et al. 2021) a specific TaqMan qPCR assay. Analytical specificity testing using 10 host species and 14 aquatic pathogens showed no cross-reactivity, and analytical sensitivity was 2 – 5 target copies. Diagnostic accuracy of the qPCR assay was evaluated using a latent class analysis approach. Diagnostic sensitivity was 0.994 in populations with high infection intensity and 0.865 in populations with low infection intensity. Diagnostic specificity ranged from 0.963 in populations with high infection intensity and 0.990 in populations with low infection intensity. The assay demonstrated high repeatability, with intra-assay and inter-assay coefficients of variation ranging between 0.44 –1.83% and 0.74 – 2.36%, respectively, and inter-laboratory reproducibility was 98.6%. This *M. mackini* qPCR assay is used routinely for regulatory diagnostic testing in Canada; this report provides available assay validation data up to and including 2025.

RÉSUMÉ

Abbott, C., Polinski, M., Lowe, G., Gilmore, S., Meyer, G., Kim, E., and Flores, A.-M. 2026. National Aquatic Animal Health Laboratory System Report on the Development and Validation of a qPCR Assay for Detection of *Mikrocytos mackini*. Can. Tech. Rep. Fish. Aquat. Sci. 3753: ix + 22 p. https://doi.org/10.60825/yab1-h547

Mikrocytos mackini est un protiste parasite qui cause la maladie de l'île Denman, ou mikrocytose, chez l'huître creuse du Pacifique (*Crassostrea gigas*). Il s'agit d'une maladie réglementée par le gouvernement fédéral au Canada. La détection à haute sensibilité de *M. mackini* a plusieurs applications, dont la surveillance des maladies et les tests d'importation/d'exportation. C'est pourquoi nous avons mis au point (Polinski *et al*. 2015) et validé sur le plan diagnostique (Polinski *et al*. 2021) une épreuve de PCR quantitative TaqMan spécifique. Les tests de spécificité analytique, réalisés avec 10 espèces hôtes et 14 agents pathogènes aquatiques, n'ont montré aucune réactivité croisée, et la sensibilité analytique était de 2 à 5 copies cibles. La précision diagnostique de l'épreuve de PCR quantitative a été évaluée à l'aide d'une méthode d'analyse de structure latente. La sensibilité diagnostique était de 0,994 dans les populations présentant une forte intensité d'infection et de 0,865 dans les populations à faible intensité d'infection. La spécificité diagnostique variait de 0,963 dans les populations à forte intensité d'infection à 0,990 dans les populations à faible intensité d'infection. La méthode a démontré une grande répétabilité, avec des coefficients de variation dans les épreuves et entre les épreuves de 0,44 à 1,83 % à 0,74 à 2,36 %, respectivement, et une reproductibilité interlaboratoire de 98,6 %. Cette épreuve de PCR quantitative ciblant *M. mackini* est utilisée régulièrement pour les analyses réglementaires de détection des maladies au Canada; le présent rapport fournit les données de validation de l'épreuve disponibles jusqu'en 2025 inclusivement.

1. INTRODUCTION

Several protistan parasites are known to induce mortality in commercially important oyster species and contribute to substantial economic losses (Audemard et al. 2002). Among these is a small group of intracellular parasites called 'microcells' (Carnegie et al. 2003), typified by their extraordinarily small cell size. Microcell parasites belong to the genera *Bonamia* (order Haplosporida) and genus *Mikrocytos* (order Mikrocytida; Hartikainen et al. 2014) and are considered major threats to oyster populations globally (Carnegie et al. 2003). Of these, *Mikrocytos mackini* (Farley et al. 1988) is of particular concern to commercial oyster production interests in Canada (see review by Abbott and Meyer 2014). It's Canadian geographic range is restricted to the west coast around Vancouver Island (Bower et al. 1994) and its primary host is the Pacific oyster (*Crassostrea gigas*), which is the most economically important commercial shellfish species in British Columbia (DFO 2022).

M. mackini causes mikrocytosis, or 'Denman Island disease,' which is characterized by focal green lesions within the body wall or on the surface of the labial palps or mantle of infected Pacific oysters (Bower et al. 1994). Pathogenicity of *M. mackini* is temperature dependent (Bower et al. 1997) and varies seasonally; clinical signs of the disease usually occur in British Columbia between March and June (Carnegie and Cochennec 2004). Mortality rates induced among Pacific oysters by mikrocytosis have been recorded as high as 53% (Hervio et al. 1996), and although *M. mackini* is not infectious to humans, non-lethal cases of the disease still render oysters unmarketable (Bower et al. 2005). Long term annual survey data collected from Denman Island, British Columbia, between 1960 and 1994 found that the prevalence of *M. mackini* among Pacific oysters varied from 11% to 48% (Hervio et al. 1996).

In Canada, *M. mackini* is a federally reportable disease and is regulated by the National Aquatic Animal Health Program (NAAHP). Efficient and accurate pathogen detection is essential for minimizing the impact of diseases in commercial aquaculture. Three methods have been developed for detection of *M. mackini* in oyster tissue: visualization of the parasite through histopathology (Cochennec-Laureau et al. 2003; Meyer et al. 2005), conventional polymerase chain reaction (PCR) (Carnegie et al. 2003), and real-time quantitative PCR (qPCR) (Polinski et al. 2015). Histopathology often lacks sensitivity (Abbott and Meyer 2014), resulting in missed subclinical infections, and the conventional PCR designed for *M. mackini* detection (Carnegie et al. 2003) cross-reacts with other *Mikrocytos* species (Abbott and Meyer 2014). Detection through qPCR is currently recommended due to its greater diagnostic sensitivity and specificity for *M. mackini* (Polinski et al. 2021). The use of qPCR as a diagnostic tool allows high throughput, quick turnaround, and increased specificity over other methods (Kralik and Ricchi 2017).

This report describes the development and validation of the MQGL assay, a TaqMan qPCR assay for specific detection of *M. mackini* used for routine regulatory testing by DFO's National Aquatic Animal Health Laboratory System (NAAHLS). Formal assay validation is essential to confirm accurate and reliable results under defined laboratory

conditions, and to ensure the assay has been properly developed and optimized for its intended purpose (WOAH 2025). The validation pathway used here follows guidelines established by the World Organisation for Animal Health (WOAH) and includes the evaluation of both analytical and diagnostic characteristics, in addition to reproducibility across laboratories (WOAH 2025). We describe the design, intended purpose, and full validation of the MQGL assay, as well as information on how the assay is currently used within the NAAHP. This assay can be used for a variety of purposes, including (but not limited to) health certification of oyster imports and exports prior to transfer, disease outbreak investigations, and surveillance.

Substantial portions of this report draw from material published in *Molecular and Biochemical Parasitology* (Polinski et al. 2015) and *Diseases of Aquatic Organisms* (Polinski et al. 2021); we refer to these publications throughout the report.

2. TEST DESCRIPTION AND REQUIREMENTS

2.1. Intended purpose of the test

The MQGL assay for qPCR detection of *M. mackini* was developed to support the NAAHP (co-delivered by the Canadian Food Inspection Agency (CFIA) and DFO). It's intended purpose includes and is not limited to applications related to domestic movement, export and import testing, surveillance, and disease response.

2.2. Disease / analyte target

The MQGL assay is TaqMan-based and targets a region of ITS-2 and 5.8S rDNA that has high sequence divergence among *Mikrocytos* species but is 100% conserved within *M. mackini* (Abbott et al. 2011). The forward primer (MQGLf) and probe (MQGLp) targets the highly variable ITS-2 rDNA region, while the reverse primer (MQGLr) targets the conserved 5.8S rDNA region (Table 1; Polinski et al. 2015). The assay amplifies a 60 bp sequence.

Table 1. The MQGL qPCR assay developed by the NAAHLS for the detection of *Mikrocytos mackini* in oyster tissues. The forward (MQGLf) and reverse (MQGLr) primers target ITS-2 and 5.8S rDNA regions, respectively; the internal probe (MQGLp) binds to a highly conserved region within ITS-2.

Primers/Probe	Primer Sequence (5' - 3')
Forward (MQGLf)	GCCTATGACAGCACGAAGCA
Reverse (MQGLr)	TGGCCGAATGACGTAGTTG
Probe (MQGLp)	6FAM-CTGCACGCCGAAGC-MGB
Amplified sequence (60 bp)	TGGCCGAATGACGTAGTTGCCGCTTCGGCGTGCAGTCTCGTGCTTCGTGCTGTCATAGGC

2.3. Sampling Procedures including Preservation

The necropsy of Pacific oysters (*Crassostrea gigas*) and tissue specimen collection procedures were previously described by Polinski et al. (2015). DNA tissues samples

were approximately 25 mg total tissue and consisted of mantle, gonad, digestive gland and gills (Figure 1).

The following section was taken from the supplemental material of Polinski et al. (2015) and describes the procedures for necropsy and tissue sampling.

Necropsy and tissue sampling

All tools and surfaces should be disinfected thoroughly prior to necropsy and between handling individual specimens. A standardized method for sample collection and storage is as follows:

i. Open the oysters by inserting an oyster knife between the valves along the dorsal side adjacent to the adductor muscle and use a twisting motion to separate valves.
ii. Using a sterile scalpel sever the adductor muscle as close to the right valve as possible and pry off the right valve without damaging soft tissues.
iii. Loosen the oyster from the shell by severing the adductor muscle as close to the left valve as possible.
iv. Using forceps lift the oyster by the posterior end of both mantle margins, allow the mantle fluid to drain and transfer to a clean and sterilized area for dissection.
v. Examine the oyster for the presence of macroscopic lesions (particularly on the mantle, labial palps and adductor muscle). If present, use scissors and forceps to excise up to 3 representative lesions and preserve for DNA and histology. Large lesions (> 2mm) should be bisected, with half of each preserved for DNA and histology, while smaller lesions (< 2mm) that are too small to bisect can be fixed whole.
vi. Using a scalpel cut a single transverse tissue section approximately 2-3 mm in thickness through the middle region of the visceral mass (see Figure 1). Tissue representation should include mantle, gonad, digestive gland and gills. A second parallel cut can be made at this time and used for histological examination, if desired (Abbott and Meyer 2014).

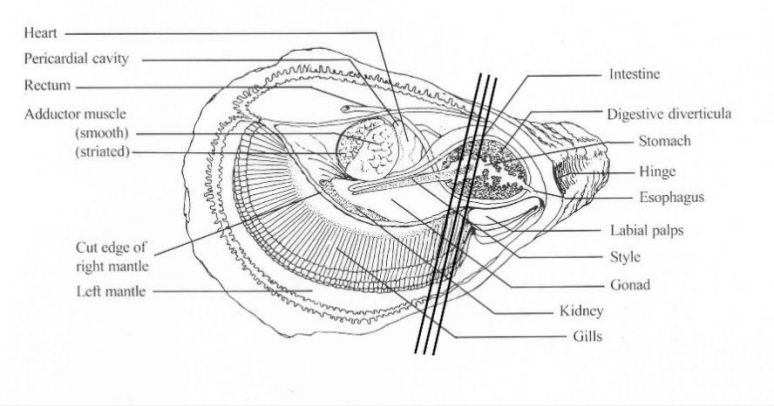

Figure 1. Lateral sketch of an oyster with anatomical notation and orientation of the mid-body cross-sectional slice indicated by bold lines (from Roberts et al. 2013).

vii. Dissect an approximate 3 mm x 8 mm area (~20-25 mg total) from the mid-body cross section where the gills attach to the visceral mass (Figure 2). Tissue representation should include digestive gland, connective tissue, gonad and gill. It is recommended that a replicate parallel section be excised and stored separately for potential future diagnostic use.

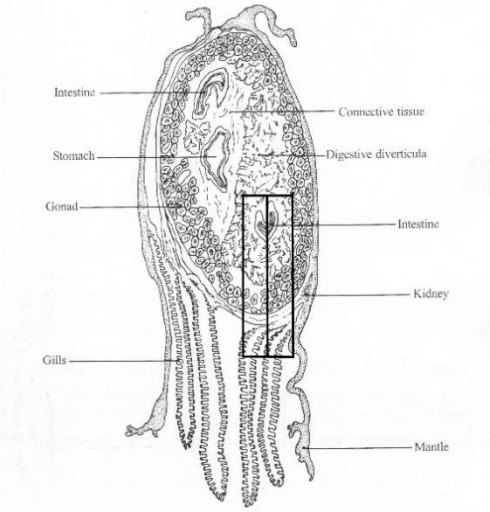

Figure 2. Frontal sketch of the mid-body cross-sectional oyster tissue slice with anatomical notation and location for obtaining representative replicate samples (from Roberts et al. 2013).

viii. Preserve the above samples and any lesions, if present, in 95% ethanol (ideally using 2 ml screw capped tubes). Microcentrifuge tubes are convenient for creating replicate samples from the same specimen; however, it is important the ratio of fixative to tissue is at least 10:1.

From Polinski et al. (2015) in *Molecular and Biochemical Parasitology*

Tissue specimen should be in good condition with sample containers intact and labelled. For fresh (i.e. not frozen) specimens, the interval between time of collection (if known) and necropsy / freezing / preservation of tissue samples must not exceed 72 hours. This restriction does not apply to preserved or frozen specimens. Specimen determined to be of insufficient quality for testing are deemed unfit and not tested.

2.4. Controls included in the MQGL assay

2.4.1. Positive controls

The positive control used for the MQGL assay is a synthetic DNA construct ('gBlock'; Integrated DNA Technologies) containing the target primer and probe binding sites and an artificial positive control (APC) probe binding site (Table 2).

Table 2. Sequence of the 491 bp gBlock sequence used here as a positive control for the MQGL assay. The primer binding sites are in bold, probe binding site is underlined, and the APC binding site is italicized.

M. mackini gBlock Sequence (5' - 3')
ATTTAGGTGACACTATAGAACACCAACTGTGAGGAGACTACCTACACAGA
GTGACGGGAGATTCAGTGTGGTGGCCCAGCAGTCCCGTCAAGAGGCCACT
CATACTTTCCACATGGCAACTGTCTCATGGTTCACACCAGCAGAGGGGG
GCCTATGACAGCACGAAGCACAGAC*ACCGTCTAGCATCCAGT*CCCCCTGCA
AATGAGCTGCACGCCGAAGCC**AACTACGTCATTCGGCCA**CCCAGCCTTCT
CCACCACGCGCCCCACTTGATTTTGGTCGGGATCTCGCTCCTGTGGTTGA
GGTCAATGAAGGGGTCCAAATCCGTTGACTCGGTGGATGTGGCTCGGTAA
ACAACACCTGGAACGGCTGGCGACGCAAAAGAAGATGCGGCTGAGGGCTG
CGGGCTCAATTTATAGAAACCGGGATGATATTCGGGGACAAATTGGGCGC
GGCGGCAGGCGGTGACTCGGACCCCGCCTCCCGCCGATATC

This *M. mackini* gBlock is used to create DNA extraction positive controls and qPCR positive controls. All positive controls are made ahead of time and their performance characterized and acceptance criteria generated before use in regulatory testing.

The DNA extraction positive control is prepared by adding between 1×10^6 and 1×10^2 copies/µl of *M. mackini* gBlock to the lysis buffer. qPCR positive controls are prepared by using the *M. mackini* gBlock instead of genomic DNA in the qPCR; typically one high positive control (containing a high concentration of gBlock) and one low positive control (containing a low concentration of gBlock) are used.

2.4.2. Negative controls

Two negative controls are included when using the MQGL assay: one DNA extraction negative control and one qPCR no template control. The DNA extraction negative control is either 200 µl of molecular grade water or a 'blank' (i.e. nothing adding to replace sample) or an approximately 25 mg slice of uninfected host tissue containing mantle, gonad, digestive gland and gills (as described in Section 2.3). The qPCR no template control is molecular grade water.

2.5. DNA extraction

DNA is extracted from oyster tissues using the Qiagen DNeasy Blood and Tissue Kit or the automated Kingfisher Flex Purification System paired with the MagMAX™ CORE Nucleic Acid Purification Kit.

A brief summary of the Qiagen kit DNA extraction is provided in the supplementary material of Polinski et al. (2015). For this method: (1) DNA is extracted following the manufacturer's quick-start protocol with the following exceptions: Buffer AE is heated to ~70°C before being added to the spin column, which is then incubated for 3 min at 56–70°C prior to elution; and (2) extracted DNA is quantified spectrophotometrically and normalized to a maximum concentration of 250 ng/µl.

When DNA is extracted using the automated Kingfisher Flex Purification System with the MagMAX™ CORE Nucleic Acid Purification Kit, the simple method for organ/tissue on the KingFisher™ Flex Purification System is used with the following modifications: (1) 180 µL of Qiagen™ ATL Buffer and 20 µL of Qiagen™ Proteinase K is added to each sample and incubated at 56°C for 3 h to overnight; (2) following digestion, samples are briefly vortexed and centrifuged for 5 min at 2250 x g to clarify homogenates; and (3) 200 µL of clarified lysate is transferred to the deep well plate. No normalization of extracted DNA is necessary when using the Kingfisher Flex for DNA extraction.

2.6. MQGL qPCR Assay

The master mix for the MQGL assay uses reagent volumes described in Table 3. See Table 1 for primer and probe sequences.

Table 3. Master Mix for the MQGL assay using ABI TaqMan Universal Master Mix (Life Technologies).

Reagent (Stock Concentration)	Final Concentration	Volume (µl)
Molecular grade water	-	x to 25 µl
F primer (10 µM)	0.6 µM	1.5
R primer (10 µM)	0.6 µM	1.5
TaqMan probe (10 µM)	0.2 µM	0.5
APC probe (Optional) (10 µM)	0.08 µM	0.2
ABI TaqMan Universal Master Mix (2 X)	1 X	12.5
DNA Template (maximum conc. of 250 ng/µl)	-	2
	Total Volume	25 µl

Thermocycling conditions are:

- 50°C for 2 min
- 95°C for 10 min
- 40 cycles x [95°C for 30 sec; 60°C for 60 sec*]
  * Fluorescence data is collected at this stage after every cycle

2.7. Interpretation of Ct Values

2.7.1. Assessing control results

i. Before use in regulatory cases, each batch of positive controls is characterized in several runs to determine the acceptable Ct range.

ii. Negative controls require a ‘No Ct’ result in all replicates to be considered acceptable.
iii. All controls are analyzed prior to sample analysis; if reported Ct values for all controls are within the acceptable range, controls pass and sample results are analyzed.

2.7.2. Assessing sample results

i. If the APC probe is used, confirm no APC amplification in sample wells; if there is, this indicates there was contamination with the APC and affected samples are to be repeated.
ii. If ‘No Ct’ value is observed in all technical replicates, the sample result is **Not Detected**.
iii. If a Ct value <40 is observed in all technical replicates, the sample result is **Detected**.
iv. If a Ct value <40 is observed in one but not all technical replicates, the sample result is **Inconclusive** and follow-up testing is required (e.g. repeat the qPCR or re-extract the sample from back-up tissue and re-do qPCR testing). If the sample remains inconclusive after follow-up testing, it is reported as such.

3. DEVELOPMENT AND VALIDATION OF THE MQGL ASSAY

3.1. Analytical characteristics

Analytical sensitivity and specificity of the MQGL assay were assessed by Polinski et al. (2015). Figure 3 and Tables 5, 6, and 7 are reproduced from this source; please refer to the original publication for details. The following section summarizes key results from the validation of the MQGL assay.

3.1.1. Repeatability

Methods: Repeatability refers to the ability of a diagnostic test to produce consistent results among runs using identical test samples (Bustin et al. 2009). Intra-assay repeatability refers to consistency of measurements of the same sample within a single test run, while inter-assay variation measures the variability among runs of the same test method within a single laboratory (Bustin et al. 2009). To evaluate both, a five-step serial dilution of a *M. mackini* positive Pacific oyster sample (#8275-440) was used. Three qPCR replicates of each dilution were used in each run to determine intra-assay repeatability; for inter-assay repeatability, the same serial dilution series was used. Intra-assay repeatability was assessed by calculating the coefficient of variation (CV) using the standard deviation of Ct values of technical replicates within a run, divided by the mean Ct value for those replicates and multiplied by 100. Inter-assay repeatability was assessed by calculating the CV using the standard deviation of Ct values across runs, divided by the mean Ct for those runs and multiplied by 100.

Results: Intra- and inter-assay repeatability results are in Table 4, including average Ct, standard deviation (SD), and intra- and inter-assay CV. From a 10^{-4} dilution of the positive oyster sample to undiluted concentration, intra-assay CV ranged from 1.83% to 0.44% and inter-assay CV ranged from 2.36% to 0.74%.

Table 4. Estimates of intra- and inter-assay variation for the MQGL assay.

Template	# of runs	Ct±SD	Intra-assay CV (%)	Inter-assay CV (%)
8275-440	14	26.16 ± 0.12	0.44	0.82
8275-440x10^{-1}	14	29.65 ± 0.13	0.45	0.74
8275-440x10^{-2}	14	33.15 ± 0.32	0.98	2.16
8275-440x10^{-3}	14	35.98 ± 0.55	1.52	1.66
8275-440x10^{-4}	14	39.40 ± 0.71	1.83	2.36

3.1.2. Analytical specificity

Methods: Analytical specificity refers to the qPCR assay's ability to detect the intended target species (species specificity) rather than non-target species (e.g., other infecting pathogens or host species) (Bustin et al. 2009; Polinski et al. 2015). To confirm specificity *in vitro,* Polinski et al. (2015) tested the MQGL assay with DNA from 10 shellfish hosts and 14 aquatic pathogens (Table 5). In each case, a mixture of host and pathogen DNA was used as the template in triplicate qPCR reactions, which were run for 40 amplification cycles as described in Section 2.6.

Results: No samples included in analytical specificity returned a Ct, indicating no cross-reactivity with any of the shellfish and pathogens tested and confirming assay specificity to *M. mackini*.

Table 5. Analytical specificity results for the MQGL assay (see Polinski et al. 2015 and references therein).

Template DNA / Host species	Infecting pathogen / condition	qPCR amplification
Edible crab (*Cancer pagurus*)	*Paramikrocytos canceri*	No
Pacific oyster (*Crassostrea gigas*)	*Mikrocytos mackini*	Yes
	Marteilioides chungmuensis	No
	Nocardia crassostreae	No
	Oyster Herpesvirus-1	No
Eastern oyster (*Crassostrea virginica*)	*Haplosporidium costale*	No
	Haplosporidium nelsoni	No
	Perkinsus sp.	No
Northern abalone (*Haliotis kamtschatkana*)	Coccidia	No
Blue mussel (*Mytilus edulis*) - experiencing disseminated neoplasia		No
European flat oyster (*Ostrea edulis*)	*Bonamia ostreae*	No
	Marteilia refringens	No
Olympia oyster (*Ostrea lurida*)	*Mikrocytos boweri*	No
Geoduck (*Panopea generosa*)		No

Yesso scallop (*Patinopecten yessoensis*)	*Perkinsus quguadi*	No
Manila clam (*Ruditapes philippinarum*)	*Perkinsus olseni*	No

3.1.3. Analytical sensitivity

Methods: Analytical sensitivity is synonymous with 'Limit of Detection' and refers to the minimum number of target copies in a sample that can be detected by the assay (Bustin et al. 2009). The sensitivity for targeted DNA detection of the MQGL assay in the absence of host gDNA was determined by Polinski et al. (2015) using a serial dilution of linearized recombinant plasmid in molecular grade water. This was analyzed in ten PCR replicates alongside no-template controls in a dynamic range of $1.0 \times 10^0 – 1.0 \times 10^7$ genome equivalents (copies) of 5.8S–ITS-2 rDNA per reaction. To determine the impact of host gDNA on the sensitivity of the MQGL assay*, C. gigas* gDNA was spiked into each of the three PCR replicates to a final concentration of 20, 40, or 80 ng/µL (0.5, 1 and 2 µg total DNA, respectively). Each of these was tested by qPCR after adding serially diluted linearised recombinant *M. mackini* plasmid within a dynamic range of 1.00×10^2 to 1.00×10^6 copies. Relative copy number estimation was compared to no-host controls in each instance following $\log_{10}$ transformation by a one-way analysis of variance and a Dunnett's multiple comparison post-test using GraphPad Prism software (Dunnett 1964).

Results: Analytical sensitivity for detecting *M. mackini* approached the theoretical limit for single copy detection. A limit of detection, as defined by a greater than 95% chance of target detection (Bustin et al. 2009), was between two and five copies with a theoretical limit following adjustment for dilution probability (Rodrigo et al. 1997) between two and four copies. The limit of quantification, as defined by less than a 20% CV in copy number estimation among identical replicates (N = 10), was between 10 and 50 copies, indicating high quantitative reproducibility for all but the lowest concentrations of target DNA. Further, the presence of host genomic DNA did not significantly reduce the sensitivity or quantitative ability for detection of *M. mackini*; copy number estimation was comparable to non-genomic standards in reactions containing up to 40 ng/µL host gDNA. However, under conditions of high genomic loading of host DNA (80 ng/µL), detection sensitivity was reduced by approximately 20% (Figure 3).

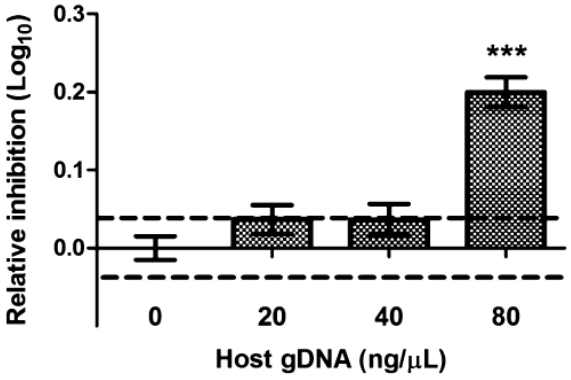

Figure 3. Inhibition of *M. mackini* qPCR detection by host genomic DNA from Polinski et al. (2015). Mean (±SE) $\log_{10}$ reduction in *M. mackini* DNA copy number estimation by qPCR in the presence of different amounts of Pacific oyster DNA is shown. Significant reduction relative to no-host controls (0 ng/µL) at $p<0.001$ is indicated by (***). Dotted lines represent upper and lower 95% confidence interval for variation within no-host controls (N = 15).

3.1.4. Standards of comparison

Methods: A standard of comparison is a reference or benchmark method used to determine whether a new assay or diagnostic test performs correctly and accurately detects its intended target. When performing the standard methods of comparison, the reference standard should be run in parallel on a small, carefully selected group of highly characterized test samples representing the linear operating range of the new method. The MQGL assay for the detection of *M. mackini* was compared to non-reference diagnostic tests, including macroscopic lesion observation, histopathology, and conventional PCR, as described by Polinski et al. (2015). For the methodology, please refer to the original publication.

Results: The MQGL qPCR assay has an increased ability to detect *M. mackini* in oyster tissue in comparison to the other diagnostic methods (Table 6, Table 7; Polinski et al. 2015). The MQGL assay detected 47% more infections than conventional PCR and 87% more than histopathology, with a false-negative rate of less than 1.2% across 197 samples.

Table 6. Comparative sensitivity of diagnostic tests for *M. mackini* detection from Polinski et al. (2015). The relative proportion (%) of infected individuals identified among 197 Pacific oysters is reported for each diagnostic test, with 127 individuals identified as infected. Relative detection within the subpopulation of infected individuals (individuals identified by at least one diagnostic test as showing signs of *M. mackini* infection) is also provided.

Diagnostic test	**Detection of *M. mackini***	
	Total population	Infected population
qPCR (MQGL assay)	57%	88%
Conventional PCR	37%	57%
Histopathology/microscopy	20%	31%
Gross lesion visualization	31%	48%

Table 7. Comparison between results of the MQGL qPCR assay and alternative, non-reference detection methods for *M. mackini* in 197 Pacific oysters from Polinski et al. (2015). Presence (+) or absense (-) of *M. mackini* DNA was identified by qPCR and compared separately to conventional PCR, histopathology, and gross lesion observation. Positive percent agreement (PPA), negative percent agreement (NPA), and overal percent agreement (OPA) are provided.

Agreement	**qPCR versus:**		
	PCR	Histopathology	Gross lesion
+/+	73	39	47
+/-	39	73	66
-/+	1	1	13

-/-	84	84	71
OPA	80	62	60
PPA	65	35	37
NPA	68	53	37

3.1.5. Accuracy of analytical methods

Analytical methods used to characterize or identify pathogens (e.g. PCR followed by DNA sequencing) are not considered diagnostic tests and are applied only after the presence of the pathogen has been detected. The accuracy of these methods needs to be verified before use.

If a detected (see section 2.7.2) qPCR result is obtained from the MQGL assay and analytical characterization of the pathogen is needed, a conventional PCR followed by DNA sequencing is used. Reference DNA sequences for multiple rDNA gene regions from *M. mackini* sampled throughout its endemic range were generated by Abbott et al. (2011) and available in GenBank. The 2020 bp reference sequence (GenBank Accession no. HM563060) contains almost the entire rDNA cistron of *M. mackini* from 18S to 28S, and is recommended to use as a reference sequence for species identification.

3.2. Diagnostic characteristics

Diagnostic sensitivity and specificity of the MQGL assay for *M. mackini* detection was assessed by Polinski et al. (2021) and is summarized here. All figures and tables are reproduced from Polinski et al. (2021); please refer to the original publication for details.

3.2.1. Study Design

Diagnostic sensitivity (DSe) is the ability of the assay to correctly identify the presence of the target (*M. mackini*) in samples; diagnostic specificity (DSp) is the ability of the assay to correctly identify the absence of the target in negative samples (Bustin et al. 2009). Several approaches can be used to determine diagnostic sensitivity and specificity. Given the absence of reference test methods, a latent class analysis (LCA) approach was used by Polinski et al. (2021) to assess the diagnostic accuracy of PCR, qPCR, and histology to detect *M. mackini* in Pacific oysters. This was done by collecting 802 Pacific oysters from seven geographically distinct locations. Because Denman Island disease (DID) is seasonal, one site was sampled twice and another was sampled three times to increase the probability of including all phases of the seasonality of the disease. As DID typically affects oysters over two years of age, older oyster populations were specifically targeted.

3.2.2. Negative / Not-detected reference samples

Negative or not-detected reference samples are samples that lack exposure to or infection with *M. mackini*. A presumed *M. mackini*-free population of Pacific oysters was

sampled in December 2012 (Polinski et al. 2021). The population sampled had no prior history of DID and sampling was conducted at a time of year when DID was not observed on Vancouver Island.

3.2.3. Positive / Detected reference samples

Positive or detected reference samples are samples with known exposure to or infection with *M. mackini*. Table 8 and Figure 5 present information on the Pacific oyster populations sampled by Polinski et al. (2021).

Table 8. Collection sites and sampling dates for Pacific oysters used in the diagnostic validation study of the MQGL assay by Polinski et al. (2021). Sites of known historical occurrence of *Mikrocytos mackini* infection were sampled during the infection season. One site with no historical occurrence of the parasite was sampled out of season (Sample Event 12). DID – Denman Island disease. Sampling locations – A: Nutchatlitz Island; B: Lemmens Inlet; C: Henry Bay; D: Deep Bay; E: Pipers Lagoon; F: Ladysmith Harbor; G: Coffin Point.

Sample event	Date (MM/DD/YYYY)	Location	n	Stock	Age (Year)	Substrate	Comment
1	2/13/2011	B1	28	cultured	3+	rope	Historical detection of *M. mackini*
2	3/4/2011	D1	73	cultured	2	rope	Historical detection of *M. mackini* and DID
3	3/12/2011	D2	38	wild	3+	intertidal	Historical detection of *M. mackini* and DID
4	3/28/2011	A	58	cultured	5+	intertidal	Historical detection of *M. mackini*
5	4/6/2011	C	45	cultured	5+	intertidal	Historical detection of *M. mackini* and DID
6	4/11/2011	D1	93	cultured	2	rope	5-10% recent DID-associated mortality
7	4/13/2011	A	69	cultured	5+	intertidal	Historical detection of *M. mackini*
8	4/20/2011	G	65	cultured	5+	intertidal	Historical detection of *M. mackini*
9	5/3/2011	F	24	wild	3+	intertidal	Historical detection of *M. mackini*
10	5/5/2011	B2	149	cultured	5+	intertidal	Historical detection of *M. mackini*
11	5/24/2011	D1	60	cultured	2	rope	5-10% recent DID-associated mortality
12	12/11/2012	E	100	wild	3+	intertidal	No historic detection of *M. mackini* or DID

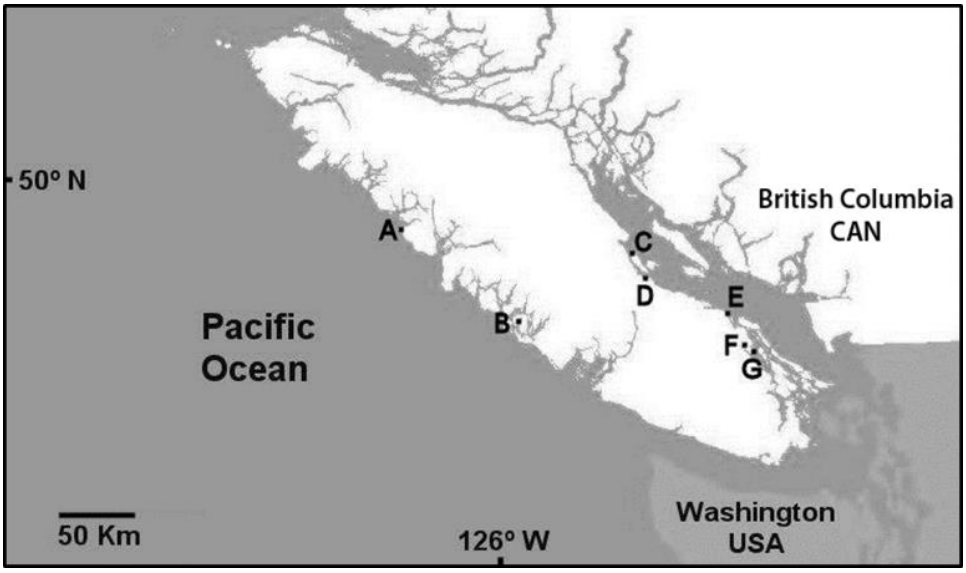

Figure 4. Locations of Pacific oyster samples collected for the diagnostic validation study of the MQGL assay by Polinski et al. (2021). Sampling locations – A: Nutchatlitz Island; B: Lemmens Inlet; C: Henry Bay; D: Deep Bay; E: Pipers Lagoon; F: Ladysmith Harbor; G: Coffin Point.

3.2.4. Diagnostic sensitivity and specificity

Methods: All 12 sampling events were considered in the determination of DSe and DSp of the MQGL assay; however, it was anticipated that the basic LCA assumptions of constant DSp and/or DSe estimates for all populations and conditional independence of each test were unlikely met. To mitigate potential violations of these assumptions, inform model assessments, and improve model fit, we: (1) used test-derived knowledge of *M. mackini* prevalence and severity within the sampled populations to separately assess DSp and DSe within groups of populations with similar prevalence/severity; (2) incorporated an informative prior to estimating the likelihood of *M. mackini* presence in the presumed negative population (sampling event 12); and (3) added co-variance terms for PCR and qPCR detections.

Results: Median posterior estimates for prevalence, DSe, and DSp are presented in Table 9. Diagnostic specificity did not vary much among tests or between infection intensities. Diagnostic sensitivity varied highly among tests and between infection intensities, ranging from 51% to 99% in high intensity infections and 21% to 87% in low intensity infections. The MQGL assay had a far higher analytical sensitivity than both PCR and histopathology; PCR and histopathology generally failed at detecting *M. mackini* when qPCR quantity estimates were below 300 copies per reaction, a level that was commonly detected by qPCR from infected populations (Polinski et al. 2021). The higher DSe of qPCR indicates fewer false negative results than PCR and histopathology; false positive results are expected to be similar across all three test methods as the DSp was similar.

Table 9. Posterior estimates [median and 95% probability interval (PI)] of prevalence, diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for histopathology (Histo), conventional PCR (PCR), and

real-time quantitative PCR (qPCR) from Polinski et al. (2021). The presumed negative population (N: Sample Event 12) was used in each model to inform DSp estimations.

Parameter	Models			
	High-intensity pops & N		**Low-intensity pops & N**	
	Median	95% PI	Median	95% PI
Prevalence				
High	0.723	0.660 - 0.765	-	-
Low	-	-	0.136	0.064 - 0.233
N	0.003	0.000 - 0.015	0.003	0.001 - 0.015
DSe				
Histo	0.510	0.460 - 0.565	0.205	0.050 - 0.496
PCR	0.827	0.781 - 0.887	0.630	0.332 - 0.949
qPCR	0.994	0.977 - 1.000	0.865	0.559 - 0.993
DSp				
Histo	0.977	0.953 - 0.992	0.996	0.980 - 1.000
PCR	0.989	0.967 - 0.999	0.994	0.973 - 1.000
qPCR	0.963	0.965 - 0.999	0.990	0.956 - 1.000

3.2.5. Comparison of performance between tests

Methods: Polinski et al. (2021) evaluated the agreement of MQGL assay results compared to PCR and histopathology. Refer to the original publication for methodology.

Results: The likelihood of test agreement was highly load dependent (Figure 5); there was no detection of *M. mackini* by any of the three tests in the presumed negative population. See Table 9 for estimates of prevalence, DSe and DSp for each test method in high and low intensity populations.

A

Agreement	**qPCR versus:**	
	Histopathology	PCR
+/+	232	376
+/-	270	124
-/+	3	2
-/-	297	300
OPA	66	84
PPA	46	75
NPA	52	70

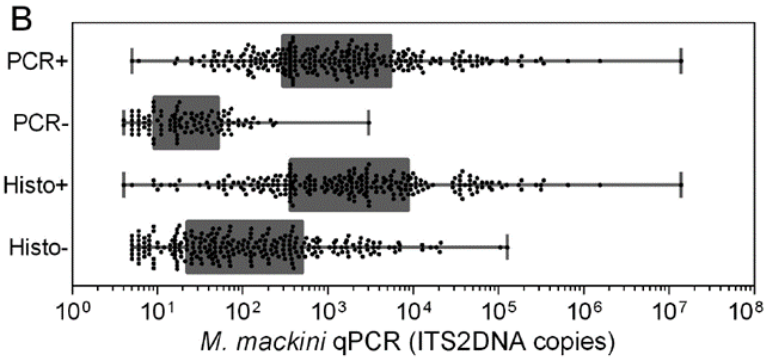

Figure 5. (A) Comparison between qPCR, histopathology (Histo) and conventional PCR (PCR) for the detection of *M. mackini* in 802 Pacific oysters tested by Polinski et al. (2021). Presence (+) or absence (−) of *M. mackini* and positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA) are provided. (B) Quantitative estimates of *M. mackini* DNA as determined by qPCR presented in relation to the presence (+) or absence (-) of *M. mackini* detected by conventional

PCR and histopathology. Bars spanning minimum and maximum values, as well as the 1st through 3rd quartile interval (shaded), are indicated.

3.3. Reproducibility

3.3.1. Analysis of reproducibility

Assay reproducibility evaluates variation in results among runs or different laboratories (WOAH 2025; Bustin et al. 2009). Reproducibility for *M. mackini* detection by qPCR was assessed: (1) between two thermocycler instruments; (2) between an experienced and inexperienced operator; and (3) among three diagnostic laboratories within the NAAHLS.

Plasmid preparation and DNA extraction procedures were completed as described in Section 2.5. Copy estimations were performed using using RStudio IDE (v3.1.2) implementing the cm3 mathematical model within the qPCR package (v1.4; Spiess et al. 2008). This model accounts for factors influencing quantitative PCR analysis, including baseline adjustment errors, reaction efficiency, template abundance, and signal loss per cycle (Carr and Moore 2012).

3.3.1.1. Thermocycler instrument reproducibility

Methods: A Stratagene Mx3000P real-time PCR system (Agilent Technologies) was compared with a Step-One Plus real-time PCR system (Applied Biosystems) to evaluate: (1) plasmid insert copy number estimation; and (2) diagnostic detection agreement using either plasmid or DNA extracted from host tissues. A single DNA extraction or plasmid dilution was used as template and run on both instruments (three replicates per instrument) in a six-step dilution series (A – F) in decreasing concentrations for all comparisons. Mean copy number within replicates was reported for each platform per dilution, and the mean, SD, and CV were calculated.

To assess the ability of the MQGL assay to consistently detect infected oysters, DNA was separately extracted from the mid-body section of six Pacific oysters: one exhibiting overt disease signs (Sample 8275-440) and five with no visible pathology (Samples 8275-104, 105, 107, 111, and 112). Each DNA sample was analyzed in triplicate at one, two, or three days post-extraction using two real-time detection platforms (see next section).

Results: When plasmid template was used, the MQGL assay demonstrated reproducible copy number estimates across a wide dynamic range (Table 10). At high template concentrations (Replicate A), the variability between platforms was low (CV = 0.04), indicating high reproducibility. At lower template concentrations (Replicates E and F), the assay still detected target DNA, although variability between platforms was higher (CV = 0.10 and 0.11, respectively). Overall, the MQGL assay reliably detected *M. mackini* across several orders of magnitude. When using *M. mackini*-specific DNA as a template in a serial dilution, the MQGL assay detected all replicate dilutions (A – F) in

triplicate on both qPCR platforms (Table 11). There was 100% agreement between instruments within a dynamic range of $10^{1.5}$ – $10^{6.8}$ target copies of plasmid construct (N = 36 total replicates). The MQGL assay also successfully determined which tissue sample was free of infection and which samples were infected (Table 12). There was 100% agreement between instruments for detecting *M. mackini* DNA within host tissues (N = 108 total replicates).

Table 10. Mean copy number estimation within replicates (N = 3) for each thermocycling instrument using a plasmid positive control as template in a series of six decreasing concentrations (A – F). The mean, standard deviation (SD) and coefficient of variance (CV) for copy number estimation between instruments is provided.

Replicate Set	Mean copy Estimation		Mean	SD	CV
	Stratagene	Step-One Plus			
A	6075642	6394244	6234943	225285	0.04
B	520000	400519	460260	84486	0.18
C	29568	48166	38867	13151	0.34
D	3787	3739	3763	34	0.01
E	223	258	241	25	0.1
F	33	38	36	4	0.11

Table 11. Presence (+) of *M. mackini* specific DNA relative to the total number of replicates tested (N) for each dilution set (A – F) as assessed by either a Stratagene Mx3000P or Step-One Plus thermocycling instruments. Detection agreement (%) between the two instruments is also provided.

Replicate Set	***M. mackini* detection (+/N)**		Instrument detection agreement
	Stratagene	Step-One Plus	
A	3/3	3/3	100%
B	3/3	3/3	100%
C	3/3	3/3	100%
D	3/3	3/3	100%
E	3/3	3/3	100%
F	3/3	3/3	100%

Table 12. Presence (+) of *M. mackini* specific DNA relative to the total number of replicates (N) for DNA extracted from each of six Pacific oysters of unknown infection status as assessed by either one of two Stratagene Mx3000P thermocycling instruments. Detection agreement (%) between the two instruments for each oyster is also provided.

Sample	***M. mackini* detection (+/N)**		Instrument detection agreement
	Stratagene-1	Stratagene-2	
8275-440	9/9	9/9	100%
8275-104	0/9	0/9	100%
8275-105	0/9	0/9	100%
8275-107	0/9	0/9	100%
8275-111	0/9	0/9	100%
8275-112	0/9	0/9	100%

3.3.1.2. Between operator reproducibility

Methods:

Comparisons of detection ability between operators and template storage duration utilized the undiluted elution of DNA from the individual oyster used in quantitative comparisons (Sample 8275-440), alongside DNA extracted from five additional oysters which did not show gross signs of disease pathology (Samples 8275-104,-105,-107,-111, and -112). A single aliquot was used to assess quantitative DNA loads of *M. mackini* in a four-step, 10-fold dilution series of the sample. Estimates were made within one week (6 – 9 days) of DNA extraction by an experienced operator in three runs on each of two real-time PCR systems, and following 3 months (91 – 93 days) of -20°C storage by an inexperienced operator.

Results: The MQGL assay reliably detected *M. mackini* across a four-step serial dilution using DNA from a single diseased Pacific oyster on two instruments, with samples analysed after one week and three months of storage (Table 13). The dynamic range of genomic DNA was between $10^{1.7}$ – $10^{4.7}$ target copies. Both experienced and inexperienced operators detected the targeted DNA in all dilutions on both instruments, which verifies the assay's robustness across users and platforms. Precision was higher for the experienced operator, with lower standard deviation (SD) and coefficient of variation (CV) values. Variability increased at lower target DNA concentrations, which indicated reduced reproducibility near the assay's lower detection limit, as expected. The overall mean CV was 34.5% across operators, instruments, and storage times. Re-analysis of the samples after three months indicated that the DNA was stable over time and the assay was robust to template storage conditions. The MQGL assay, for both experienced and inexperienced operators, was able to distinguish tissue samples that were free of infection and to confirm those that visually showed overt signs of infection (Table 14). There was 98.8% agreement between operators and instruments for detecting *M. mackini* DNA within host tissues (N = 216 total replicates).

Table 13. Mean copy number estimation within replicates (N = 3) as assessed by either one of two Stratagene Mx3000P (Strat) thermocycling instruments. DNA was extracted from a single *M. mackini* infected Pacific oyster and tested in a four-step 10-fold dilution series. DNA was analysed between 6 - 9 days post extraction by an experienced operator (EO1) and then by an inexperienced operator (IO1) after it had been stored for 91 - 93 days at -20°C. The mean, standard deviation (SD) and coefficient of variance (CV) for copy number estimation among runs for each operator (N = 6) as well as for both operators combined (N = 12) is also provided.

Operator(s)	Run	Date	Machine	concentration			
				Neat	x 10^{-1}	x 10^{-2}	x 10^{-3}
EO1	1	10/18/2011	Strat-1	55495	4376	373	62
	2	10/19/2011	Strat-2	47448	3200	253	36
	3	10/19/2011	Strat-1	56773	3900	439	61
	4	10/20/2011	Strat-2	39164	2594	265	37
	5	10/20/2011	Strat-1	52841	3183	351	52
	6	10/21/2011	Strat-2	51557	4395	452	62
			EO1 SD	6461	731	84	12

			EO1 CV	0.128	0.203	0.237	0.237
IO1	7	1/11/2012	Strat-2	37030	NA	295	28
	8	1/12/2012	Strat-1	62060	5324	385	48
	9	1/12/2012	Strat-2	31924	2763	253	26
	10	1/13/2012	Strat-1	25948	1890	201	35
	11	1/13/2012	Strat-2	20324	2751	204	21
	12	1/13/2012	Strat-1	28280	2290	207	7
			IO1 SD	14731	1347	72	14
			IO1 CV	0.430	0.448	0.282	0.495
			Overall SD	13782	1045	91	18
			Overall CV	0.325	0.314	0.296	0.446

Table 14. Presence of *M. mackini* specific DNA relative to the total number of replicates for DNA extracted from each of six Pacific oysters, as assessed by an experienced (EO1) or inexperienced (IO1) operator. Three replicates were tested in each of three runs using either one of two Stratagene Mx3000P thermocycling instruments. Detection agreement (%) between the two operators for each oyster is also provided.

Sample	EO1		IO1		Agreement (%)
	Stratagene-1	Stratagene-2	Stratagene-1	Stratagene-2	
8275-440	9/9	9/9	9/9	9/9	100
8275-104	0/9	1/9	2/9	0/9	95.3
8275-105	0/9	0/9	0/9	0/9	100
8275-107	0/9	0/9	0/9	0/9	100
8275-111	0/9	0/9	1/9	0/9	97.2
8275-112	0/9	0/9	0/9	0/9	100

3.3.1.3. Inter-laboratory reproducibility

Methods: Reproducibility of the MQGL assay was assessed by distributing replicate DNA tissue samples with unknown infection status to three National Aquatic Animal Health Laboratory System (NAAHLS) laboratories: PBS (Nanaimo, British Columbia), the Freshwater Institute (FWI; Winnipeg, Manitoba), and the Gulf Fisheries Centre (GFC; Moncton, New Brunswick). These laboratories conduct regulatory diagnostic testing for aquatic animal diseases and are accredited to ISO 17025 for various methods. Forty Pacific oysters were collected near Vancouver Island, Canada, and transported to PBS, where triplicate cross-body tissue sections were collected and preserved in ethanol (as described in Section 2.3). One sample replicate was retained at PBS and the other replicates were sent to FWI and GFC. Each lab independently extracted the DNA from the cross-body tissue sections and performed the qPCR assays. Extracted DNA was analysed in triplicate at all laboratories.

Results: All three Canadian diagnostic laboratories detected the presence of *M. mackini*-specific DNA using qPCR on the DNA extracted from replicate tissue samples of 40 Pacific oysters with unknown infection status (Table 15). Across all laboratories, the detection agreement was 98.6% (N = 360 total replicates).

Table 15. Results of assay reproducibility as assessed at three Canadian federal laboratories: FWI, GFC, and PBS. Presence (+) of *M. mackini* specific DNA relative to the total number of replicates (N) for DNA extracted from each of 40 Pacific oysters of unknown infection status is shown. Detection agreement (%) among laboratories for each oyster is also provided.

Sample	Laboratory (+/N)			Agreement (%)
	FWI	GFC	PBS	
8275-103	0/3	1/3	0/3	88.9
8275-104	0/3	0/3	0/3	100
8275-105	0/3	0/3	0/3	100
8275-107	0/3	0/3	0/3	100
8275-111	0/3	0/3	0/3	100
8275-112	0/3	0/3	0/3	100
8275-169	3/3	3/3	3/3	100
8275-180	3/3	3/3	3/3	100
8275-182	3/3	3/3	3/3	100
8275-187	3/3	3/3	3/3	100
8275-195	3/3	3/3	3/3	100
8275-198	3/3	3/3	3/3	100
8275-226	3/3	3/3	3/3	100
8275-234	3/3	3/3	3/3	100
8275-256	3/3	3/3	3/3	100
8275-287	3/3	3/3	3/3	100
8275-290	3/3	3/3	3/3	100
8275-292	3/3	3/3	3/3	100
8275-295	3/3	3/3	3/3	100
8275-316	3/3	3/3	3/3	100
8275-322	3/3	3/3	3/3	100
8275-330	3/3	3/3	3/3	100
8275-338	3/3	3/3	3/3	100
8275-368	3/3	3/3	3/3	100
8275-409	1/3	0/3	0/3	88.9
8275-411	1/3	0/3	1/3	88.9
8275-412	0/3	1/3	0/3	88.9
8275-414	0/3	0/3	0/3	100
8275-440	3/3	3/3	3/3	100
8275-555	3/3	3/3	3/3	100
8317-001	0/3	0/3	0/3	100
8317-002	0/3	0/3	0/3	100
8317-003	0/3	0/3	1/3	88.9
8317-004	0/3	0/3	0/3	100
8317-005	0/3	0/3	0/3	100
8317-006	0/3	0/3	0/3	100
8317-007	0/3	0/3	0/3	100
8317-008	0/3	0/3	0/3	100

8317-009	0/3	0/3	0/3	100
8317-010	0/3	0/3	0/3	100

4. CONCLUSIONS

The MQGL assay described here, developed and validated by the DFO NAAHLS, demonstrated greater analytical sensitivity, specificity, and repeatability than other diagnostic methods. Reproducibility assessments involving multiple operators, thermocycling instruments, and diagnostic laboratories showed consistent results, establishing assay reliability. Given its high diagnostic sensitivity and specificity, this assay is the preferred diagnostic method for regulatory testing for the presence of *M. mackini*, and its broad adoption within diagnostic laboratories is recommended.

5. ACKNOWLEDGEMENTS

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