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Design of a TaqMan MGB quantitative PCR assay for the invasive crab, *Hemigrapsus sanguineus* (DeHaan, 1853)

Wiltshire, K.H., Giblot-Ducray, D. and Deveney, M.R.

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Report to the Department of Agriculture, Water and the Environment

Government of South Australia
Department of Primary Industries and Regions

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Department of Agriculture, Water and the Environment

SARDI
SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

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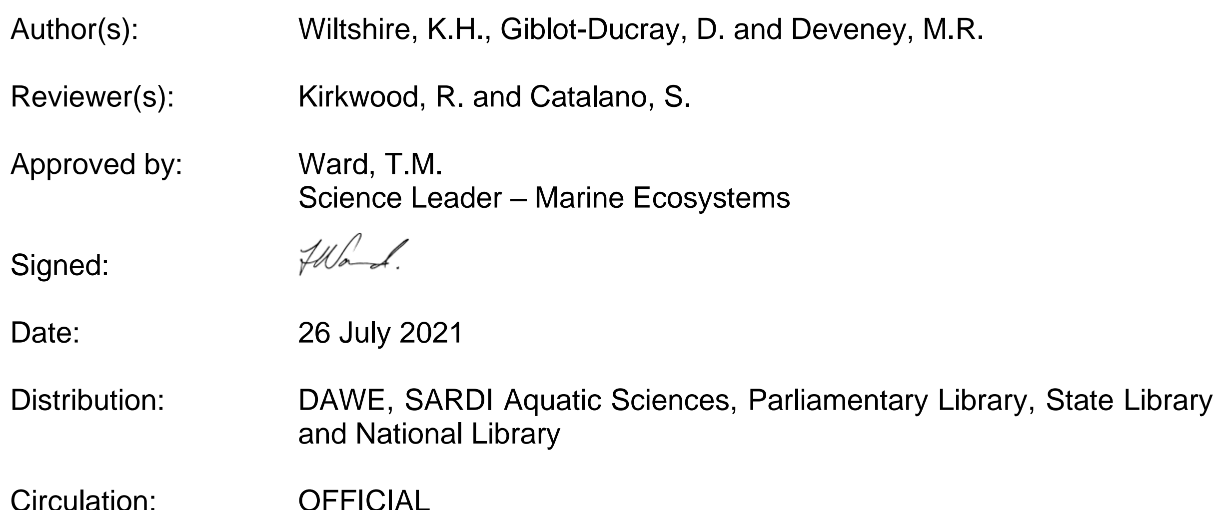

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS VI
EXECUTIVE SUMMARY 1
1. INTRODUCTION 2
1.1. Background 2
1.2. Objectives 4
2. METHODS 5
2.1. Assay design 5
2.2. Laboratory validation 5
3. RESULTS 7
3.1. Primer and probe details 7
3.2. Assay efficiency and limit of detection 7
4. DISCUSSION 9
5. CONCLUSIONS 11
6. REFERENCES 12
7. APPENDIX 1 TARGET SEQUENCE AND AMPLICON 15

LIST OF FIGURES

Figure 1. Regression of C_T value against copies (log scale) for determination of assay efficiency. 8

LIST OF TABLES

Table 1. TaqMan® MGB qPCR assay for detecting *Hemigrapsus sanguineus* showing target gene region, sequences for Forward and Reverse primers and probe, sequence length (base pairs, bp), melt temperature (Tm), and percentage guanine and cytosine (GC %). 7

Table 2. Results of standard curve testing 7

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Cover photo: *Hemigrapsus sanguineus* at Dunkirk. LeBourgmestre, CC BY-SA 4.0, via Wikimedia Commons.

EXECUTIVE SUMMARY

The Asian shore crab, *Hemigrapsus sanguineus*, is an intertidal, omnivorous varunid, native to northern Asia, that has established invasive populations in North America and Europe. In 2020 a population of *H. sanguineus* was detected in Port Phillip Bay, Victoria, Australia. Surveillance for exotic pests such as *H. sanguineus* is important to achieve early detection and effectively manage incursions. SARDI has developed a molecular surveillance system using plankton samples tested with qPCR assays for pests of concern. This molecular method provides greater survey confidence than traditional survey methods (dive visual, traps, trawl sampling) for detection of target pests, while requiring less than half the staff time and equipment costs for sample collection and processing. Many pest species have specific qPCR assays designed for them. A qPCR assay for *H. sanguineus* was developed in Denmark, but used Black Hole Quencher™ (BHQ) probe chemistry, while most assays applied by SARDI's Molecular Diagnostics Laboratory use TaqMan® Minor Groove Binder (MGB) probe chemistry, which typically provides improved diagnostic performance. This project therefore adapted and laboratory validated an MGB qPCR assay for *H. sanguineus*. The assay has high efficiency and analytical sensitivity and displays good *in silico* specificity. Further testing and field validation are now required, which will confirm performance of the assay or indicate if refinement is needed.

Keywords: Marine pests, *Hemigrapsus sanguineus,* qPCR, assay validation.

1. INTRODUCTION

1.1. Background

The risk of aquatic species introductions by shipping, via propagules in ballast water or hull-fouling, is increasing with accelerating global trade (Minchin *et al.* 2009; Hewitt and Campbell 2010; Banks *et al.* 2015; Sardain *et al.* 2019). Although not all introduced species become pests, those that do have wide ranging impacts on ecosystems, marine industries, infrastructure and amenity (Hayes *et al.* 2005; Schaffelke and Hewitt 2007; Molnar *et al.* 2008; Katsanevakis *et al.* 2014). Aquatic species are rarely able to be eradicated once established, and costs of management or reparation are ongoing (Arthur *et al.* 2015b; Beric and MacIsaac 2015). Australia's marine pests strategic plan (Department of Agriculture and Water Resources 2018) aims to minimise the risk of pest introduction, prevent further spread of pests already present in Australia, and strengthen surveillance to support these aims and to assist with early detection of new pest incursions.

The Asian shore crab *Hemigrapsus sanguineus* is an intertidal varunid crab native to parts of China, Japan, Korea and Russia, which has formed invasive populations in North America and Europe (Dauvin *et al.* 2009; Epifanio 2013). In its invaded range, this crab has demonstrated impacts on other species, particularly mussels and crustaceans, through competition and predation (Dauvin *et al.* 2009; Epifanio 2013; Blakeslee *et al.* 2017; Baillie and Grabowski 2018a; Baillie and Grabowski 2018b). *Hemigrapsus sanguineus* was detected in Port Phillip Bay, Victoria, in October 2020 with subsequent surveillance confirming an established population in the area (DAWE 2021a). Shipping is a likely vector for *H. sanguineus* introductions globally (Epifanio 2013; Blakeslee *et al.* 2017). The presence of this species in Port Phillip Bay and in source ports for shipping to Australia, and its environmental impacts where established means that this species is considered high risk for introduction to other regions of Australia.

The importance of surveillance for introduced marine species is recognised in Australia's marine pest plan (Department of Agriculture and Water Resources 2018). Port areas are at high risk of shipping-mediated introductions and provide typically suitable conditions for establishment of marine pests, and may also act as nodes for further spread of pest species (Glasby *et al.* 2007; Ojaveer *et al.* 2014; Lehtiniemi *et al.* 2015; Couton *et al.* 2019). Surveillance of ports is consequently important for early detection and prevention of spread of marine pest species (Bott *et al.* 2010b; Lehtiniemi *et al.* 2015).Traditional surveillance, e.g. using diver visual surveys,

trawls, or trapping, is expensive, however, which is an impediment to performing regular routine surveillance (Arthur *et al.* 2015a).

Molecular techniques for marine pest surveillance offer cost and time savings over traditional techniques (Bott *et al.* 2010b; Department of Agriculture and Water Resources 2018; Wiltshire *et al.* 2019a). The South Australian Research and Development Institute (SARDI) has developed qPCR assays for detection of 10 marine pest species (Ophel-Keller *et al.* 2007; Bott *et al.* 2010a; Bott and Giblot-Ducray 2011a, b, 2012; Bott *et al.* 2012), and has developed and refined plankton sampling and preservation methods and quality controls for molecular marine pest surveillance (Giblot-Ducray and Bott 2013; Deveney *et al.* 2017). The plankton sampling molecular method has been field validated using parallel molecular and traditional surveys and is fit-for-purpose for marine pest surveillance (Wiltshire *et al.* 2019a). The molecular method provides higher survey confidence than traditional techniques, while incurring less than half the field costs and requiring less than half the person-hours for collection and post-collection processing.

Several molecular technologies have been applied for surveillance, but PCR-based approaches, such as those applied in the SARDI molecular surveillance system, perform best of the currently available molecular technologies for detection of specific target species (Darling and Blum 2007; Zaiko *et al.* 2018; Wiltshire *et al.* 2019b). In addition to the assays developed by SARDI, qPCR assays have been developed for a number of other priority marine pests (e.g. Simpson *et al.* 2018) and implemented in the SARDI testing system, expanding the range of species that can be targeted with the plankton sampling molecular surveillance method. A molecular test for *H. sanguineus* was developed in Denmark (Knudsen and Møller 2020), but used a probe based on Black Hole Quencher™ (BHQ) fluorophore chemistry and had thermodynamic properties (primer and probe melt temperatures) that were unsuitable for implementation in the SARDI system. All qPCR assays designed by SARDI, and the majority of other assays implemented in the SARDI Molecular Diagnostics laboratory, use TaqMan® Minor Grove Binder (MGB) probes. The chemistry of these probes provides high performance (sensitivity and specificity), including in complex environmental samples (Yao *et al.* 2006; Alonso *et al.* 2007; Gasparic *et al.* 2010). A new assay was therefore designed for *H. sanguineus* that has suitable primer and probe melting temperatures for compatibility with the SARDI testing system and which uses TaqMan® MGB probe chemistry. Analytical sensitivity of the new assay was also assessed.

1.2. Objectives

This project aimed to design a TaqMan® MGB qPCR assay for the Asian shore crab, *Hemigrapsus sanguineus*, and assess analytical sensitivity of the new assay.

2. METHODS

2.1. Assay design

Knudsen and Møller (2020) sequenced specimens of *H. sanguineus* and identified a diagnostic region of the cytochrome oxidase 1 (CO1) gene to target for assay design. The nucleotide sequence for this gene target was used to design a TaqMan® MGB assay.

CO1 sequences from *H. sanguineus* and related decapods were downloaded from GenBank, grouped by family, and aligned in BioEdit (Hall 1999) using the Clustal W alignment to delineate heterologous species. *Hemigrapsus sanguineus* specific primers and TaqMan® MGB probe sequences with suitable thermodynamic properties were generated using Primer3 software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and checked in Primer Express v3.0.1 (ThermoFisher), an application that designs primers and TaqMan® MGB probes with suitable thermodynamic properties and nucleotide content for efficient amplification.

2.2. Laboratory validation

Laboratory validation involved preparation of a standard curve to determine the analytical limit of detection and assay efficiency.

A double-stranded 120 bp gBlock fragment was designed and synthesized (Integrated DNA Technologies) for use as a qPCR positive control to avoid laboratory contamination with a target-derived positive control. The gBlock fragment contained the *H. sanguineus* specific primer and probe sequences but otherwise comprised random nucleotides corresponding to no known sequences, making it differentiable from the target CO1 amplicon by melt curve analysis or sequencing. An initial qPCR run using 4 µl gBlock per reaction indicated that a standard curve should be developed using the gBlock (starting concentration: 2.0 E^{10} gene copies μl^{-1}) serially diluted 10-fold from $1E^{-7}$ to $1E^{-12}$. The C_T values from ten replicates of each dilution in ultrapure water of *H. sanguineus* gBlock were used to develop a standard curve. The lowest concentration of gBlock producing a C_T value in at least five of ten replicates was considered the limit of detection (LoD), based on the definition of LoD as the concentration providing ≥ 50% chance of detection (OIE 2019).

Efficiency of a qPCR assay is defined as the proportion of target DNA that is successfully amplified in each cycle, and is an important aspect of assay performance (Rebrikov and Trofimov 2006; Kralik and Ricchi 2017). Efficiency can be determined from the slope of a regression of C_T values

against log concentration (Burns and Valdivia 2008; Caraguel *et al.* 2011). Linear regression was therefore performed, using R statistical software (R Core Team 2021), with the natural logarithm of copy number as a covariate and C_T value as the response. Efficiency was calculated from the coefficient (β) for log copy number as: Efficiency = $\exp(-1/\beta) - 1$.

2.2.1. qPCR methods

Initial testing of a range of primer and probe concentrations determined the optimal combination to be: 12.5 µl QIAGEN® QuantitTect® Probe master mix, 0.5 µM forward and reverse primer, 0.1 µM TaqMan® MGB probe, 2 µl template DNA, and 7.75 µl of nuclease-free water, in a 25 µl total reaction volume. These concentrations were used for all subsequent testing. For development of the standard curve, the template DNA was 2 µl gBlock fragment.

qPCR was carried out in 96 well plates for analysis using a StepOnePlus (Applied Biosystems). PCR cycling parameters were 15 min at 95°C (activation) followed by 45 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing). Each plate included a gBlock positive control and no template (nuclease free water) negative control.

3. RESULTS

3.1. Primer and probe details

Details of the primers and probe design for the TaqMan® MGB *H. sanguineus* assay and thermodynamic properties are given in Table 1.

Table 1. TaqMan® MGB qPCR assay for detecting *Hemigrapsus sanguineus* showing target gene region, sequences for Forward and Reverse primers and probe, sequence length (base pairs, bp), melt temperature (Tm), and percentage guanine and cytosine (GC %).

Target	Type	Sequence (5´ to 3´)	Length (bp)	Tm (°C)	GC %
CO1	Forward	CCTGGGCCGGTATAGTAGGT	20	57	60%
	Probe	6FAM-ATCATTACCAATTAAGCTTC-MGB	20	68	30%
	Reverse	CGAAAGCATGAGCTGTAACAA	21	56	43%

3.2. Assay efficiency and limit of detection

The assay demonstrated high analytical sensitivity, with LoD ≤ 0.2 copies μL^{-1} (Table 2). The assay response was linear over the range assessed (Figure 1), with the regression of C_T on copy number having $R^2 = 0.984$. Assay efficiency calculated from this regression was 95.2%.

Table 2. Results of standard curve testing

copies μL^{-1}	detections	C_T (mean ± s.e.)
2000	10/10	22.9 ± 0.1
200	10/10	26.7 ± 0.3
20	10/10	30.1 ± 0
2	10/10	33.1 ± 0.1
0.2	10/10	36.5 ± 0.3
0.02	3/10	41.2 ± 0.7

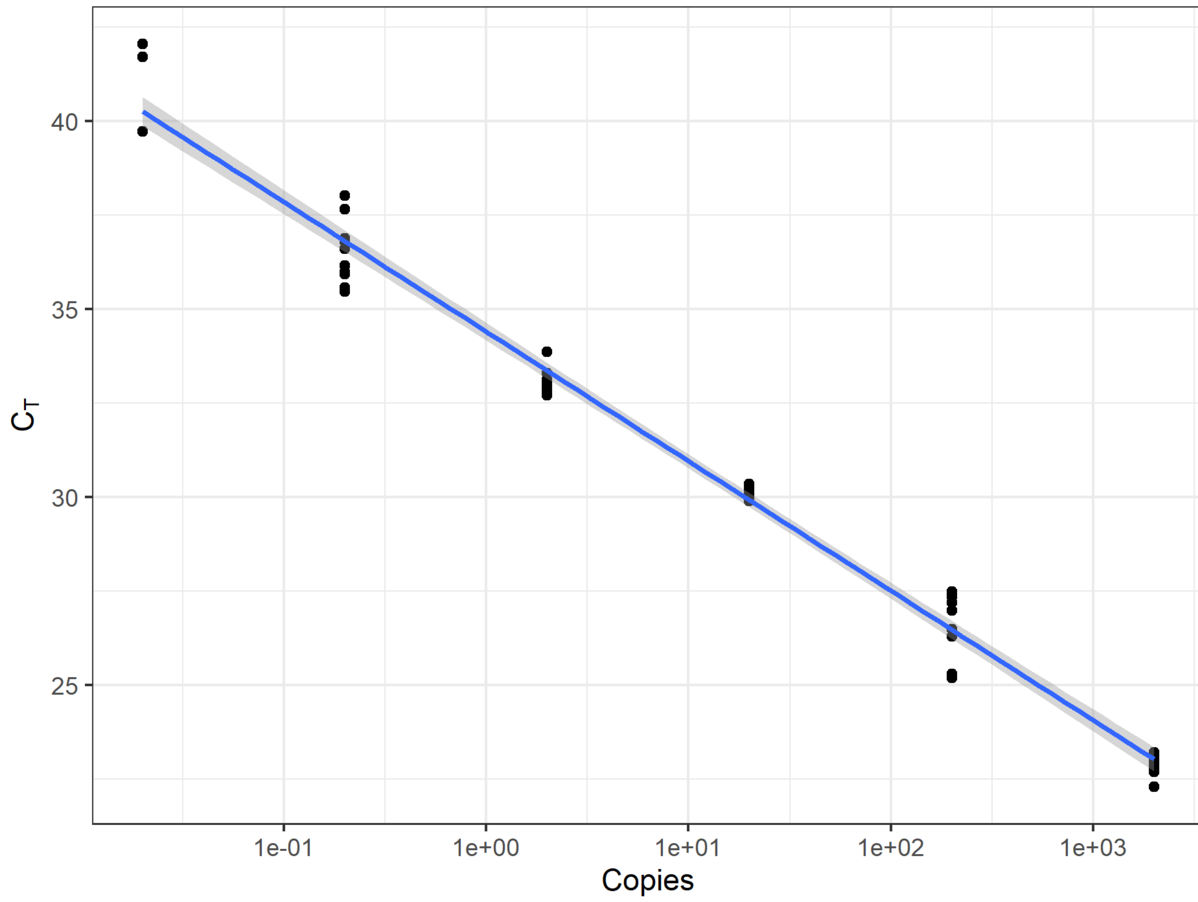

Figure 1. Regression of C_T value against copies (log scale) for determination of assay efficiency.

4. DISCUSSION

A *H. sanguineus* qPCR assay developed by Knudsen and Møller (2020), which used BHQ probe chemistry was adapted to TaqMan® MGB probe chemistry for implementation in the SARDI diagnostics laboratory testing system. MGB probes have better performance than probes using alternative chemistries because they are dual-labelled, shorter, have more favourable thermodynamic properties and are more sensitive to mismatches, with this combination of characteristics providing both better sensitivity and specificity (Yao *et al.* 2006; Gasparic *et al.* 2010). The Knudsen and Møller (2020) assay produces a 136 bp amplicon of CO1; in comparison, the redesigned assay has a shorter amplicon of 120 bp, primers that have been modified to have suitable melt temperatures for use in the SARDI testing system and to maximise mismatches with non-target species for the sequence alignment, and MGB rather than BHQ probe chemistry.

Knudsen and Møller (2020) assessed their assay for specificity using gDNA from decapods that occur in Europe: *Cancer pagrus*, *Callinectes sapidus*, *Hyas araneus*, *Rhithropanopeus harrisii*, *Eriocheir sinensis, Nephrops norvegicus* and *Hemigrapsus takanoi*. The assay designed by this project lies within the Knudsen and Møller (2020) assay and should have similar or better specificity, with the MGB chemistry increasing the binding affinity of the probe to the target sequence (Yao *et al.* 2006). *In silico* testing showed that sequence mismatches in the assay design will provide high specificity against Australian decapods and other marine organisms for which sequences are available. The most similar sequences found in GenBank belong to other *Hemigrapsus* spp., specifically *H. takanoi* (Asakura and Watanabe, 2005) and *H. penicillatus* (De Haan, 1835). Based on the available GenBank data, the redesigned assay provides at least six mismatches from *H. takanoi* and two mismatches from *H. penicillatus* CO1 sequences, with the testing by Knudsen and Møller (2020) also demonstrating that their assay did not detect *H. takanoi.* No *H. penicillatus* material was available for assessment, but the mismatches provided by the redesigned assay in combination with the use of MGB probe chemistry should provide specificity for the target sequence. Testing of the assay against *H. penicillatus* gDNA (or synthesised DNA if material cannot be obtained), however, should be prioritised to confirm this.

Two *Hemigrapsus* species: *H sanguineus* and *H. takanoi*, are invasive (Dauvin *et al.* 2009; Epifanio 2013; Markert *et al.* 2014). When a *Hemigrapsus* sp. was first recorded in Europe in 1994, *H. takanoi* was not described and the invading species was identified as *H. penicillatus* (see Noél *et al.* 1997; Dauvin *et al.* 2009). Asakura and Watanabe (2005) separated *H. takanoi* from *H. penicillatus* and determined that the initial invasion in Europe was *H. takanoi*, as confirmed by Dauvin *et al.* (2009) and Markert *et al.* (2014). *Hemigrapsus sanguineus* was recorded in Europe

in 1999 and also established invasive populations (Breton *et al.* 2002; Dauvin *et al.* 2009). It should be noted that although *H. penicillatus* is not recorded as invasive, it is potentially invasive because it has similar biology to its invasive cogeners, and it is not native to, or recorded in, Australia. If the redesigned *H. sanguineus* assay also detects *H. penicillatus*, detections by this assay may need to include follow-up by sequencing or traditional surveillance and taxonomy to accurately identify which of these non-native crabs has been detected. The ability to confidently correlate positive qPCR results with known introductions or discovery of specimens of pest species is important because it builds confidence in assay utility and may identify problems with specificity from DNA of species the assays have not been tested against, and which may have not been sequenced. A separate qPCR assay would need to be applied for molecular detection of *H. takanoi*, which is not known to occur in Australia but is considered to pose a risk of introduction (DAWE 2021b).

Further specificity testing was not possible within the timeframe and scope of this project, but, when possible, this assay will be tested against DNA of Australian species, particularly decapods, and DNA extracted from plankton at a range of Australian sites to provide further data on specificity. Relatively few marine species have been sequenced, therefore, it is necessary to assess specificity using DNA from a wide a range of related species and environmental samples from target survey areas to provide confidence in field specificity.

5. CONCLUSIONS

The TaqMan® MGB qPCR assay developed in this study is laboratory validated and will be implemented at SARDI's Molecular Diagnostics laboratory for use in molecular surveillance for marine pests. Laboratory validation demonstrated high assay efficiency, detection of ≤ 0.2 copies μL^{-1}, and *in silico* specificity against relevant species. These characteristics show that this assay will provide good sensitivity for detection while having high specificity. Further investigations are needed, however, to accurately determine the assay's diagnostic specificity and sensitivity when applied to field samples from Australia. Understanding diagnostic performance allows surveillance to be designed such that negative surveillance results can be interpreted to provide a specified confidence of absence of the target and provides assurance that detections are of target species DNA rather than a cross-reaction with DNA of species that the assay has not been tested against and which may not have been sequenced. In Australia, *H. sanguineus* has been recorded only in Port Phillip Bay, Victoria, and surveillance using specific, sensitive tools such as the assay developed here can contribute to management steps that will aid in preventing further spread.

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7. APPENDIX 1 TARGET SEQUENCE AND AMPLICON

Hemigrapsus sanguineus CO1 target sequence with primer regions highlighted in red and probe region highlighted in yellow.

```
5’aaccttatat tttatctttg gtgcctgggc cggtatagta ggtacatcac taagattaat
  tattcgagca gaattaagac aaccaggaag cttaattggt aatgatcaaa tctataatgt
  agttgttaca gctcatgctt tcgtaataat tttctttata gttataccaa ttataattgg 3’
```

120 bp qPCR amplicon generated by the assay developed in this project.

```
cctgggccggtatagtaggtacatcactaagattaattattcgagcagaattaagacaaccaggaagcttaattggt
aatgatcaaatctataatgtagttgttacagctcatgctttcg
```