MBIE CAWX1904
Marine Biosecurity Tool Kit
RA1.3 Detect

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Exploration of LAMP focused on the COX 1 DNA barcode to detect invasive species of significance to New Zealand marine environments.

LAMP Primer Table

Species	Primer Types	Sequence 5'->3'	Analytical LoD molecules/reaction
Charybdis japonica	F3 B3 FIP BIP	• GAG CAT ATT TTA CAT CAG CTA CT • ATA TCA ATG GAA GAG TTT GCT AA • AGT GCC GTG AAG AGT TCT AAG TTA ATT ATT GCT GTG CCT ACT GG • ATA GAC CTT CTA TAT TAT GGG CCC TAC TCC TGT AAG ACC TCC TAC	22,800
Carcinus maenas	F3 B3 FIP BIP	• CCT TAC TTT TAA TAA GAG GGA TGG • AAG GTA TCT GGT CTA TTG TCA • CAA CTG AAG CAC CAG CAT GGA AAG AGG AGT TGG AAC AGG • TTC TCT TTA CAT TTA GCC GGG GTT TTG CCG AAA GAA CGC ATA T	30,000
Asterias amurensis	F3 B3 FIP BIP LF LB	• ACC GGA GTG GTT TTA GCT • CGC CTA TAA ACA TTA CCG CA • GCC CCC ATG GAT AAT ACG TAG TGT CTT CCA TTG ATA TAA TTC TTC ACG • CTT TGC AAT ATT TGC TGG CTT TAC CCT TTC TTC ATA GGG GGT GTA • TGG GCA ACA ACG TAG TAT GTG T • ACT GGT TTC CAC TAT TTT CTG GAG T	15,500
Undaria pinnatifida	F3 B3 FIP BIP	• GGTCTGTTTTAATTACAGCGTT • CCGAAAACTGGTTTTCTTGA • GCCCGCAGGATCAAAAAAAGTAGTATCATTGCCGGTTTTAGCA • ATTTTGGTTCTTTGGTCATCCTGAATATGACTAACAATACCGAATCC	22,800 - 2,400,000 (unresolved)

Executive Summary

Loop Mediated Isothermal Amplification (LAMP) assays were created for four invasive species of significance to New Zealand marine environments. These species were *Charybdis japonica, Carcinus maenas, Asterias amurensis* and *Undaria pinnatifida.* Assays were designed to the COX1 mitochondrial sequence, corresponding to qPCR assays also under investigation by the University of Otago team. COX1 references available from GenBank, NCBI, provided sequence information for LAMP primer design and the WarmStart LAMP reagent and primer design tool from New England BioLabs (NEB) used to create each assay.

Each assay successfully amplified both genomic DNA reference samples and specific synthetic gBLOCK DNA. None of the assays demonstrated sufficient sensitivity to form stand alone assays suitable for citizen scientist applications or typical eDNA samples. Therefore, these assays were not pursued. The assays, though not suitable for stand alone use, may prove helpful as seed amplification tools for CRISPR-based applications. LAMP has potential, as seen in the published literature, but alternative loci to COX1 should be explored for future assay design.

Introduction

Six invasive species formed the focus of molecular assay development for MBIE CAWX1904. These were *Sabella spallanzanii* (Mediteranian fanworm), *Undaria pinnatifida* (Wakame), *Carcinius maenus* (European Green Crab), Caulerpa sp. (Green Feather Kelp), *Charybdis japonica* (Asian Paddle Crab) and *Asterias amurensis* (Northern Pacific SeaStar). Some of these organisms have already been detected in New Zealand while others have yet to arrive. In this work four species were candidates for LAMP assay development: Undaria, Carcinus, Charybdis and Asterias. An RPA assay had already been developed for Sabella and insufficient genetic information was available for LAMP assay design for the invasive Caulerpa species. For these reasons Sabella and Caulerpa were not included in this LAMP experimental program.

LAMP is an isothermal DNA amplification technology (1,2). Basically, this technique, like PCR amplifies specific DNA sequences but, unlike PCR, LAMP uses a strand-displacement polymerase meaning temperature cycling is not required to produce single stranded DNA for primer annealing. LAMP assays target 4 to 6 primers to a specific diagnostic sequence used to identify the species of interest. Primers amplify the target sequence and the amplicons form dumbell shaped products. The LAMP primer set continues to anneal and amplify these amplicons in a chain reaction that produces large concatenated structures of the same target sequence. In addition, detection of target amplification is possible using a variety of methods. This can be a simple colour change using a pH indicator dye in the reaction mix, fluorescence when used with an intercallating dye or FRET systems or turbidity increase due to salt precipitation during the reaction. The simplicity of using single temperture amplification and the flexibility of detection mode has made LAMP attractive for citizen science and field based applications (3,4).

For the assays described here primer design was restricted to the cytochrome c oxidase subunit 1 gene (COX1) on the mitochondria, generally used as a species barcode, and the same region targeted by qPCR assays already designed or used as part of the MBIE CAWX1904 program. The NEB primer design tool was used to select compatible LAMP primers (5). It was noted that the COX1 sequence presented significant problems for primer selection with most assay options limited to sets of 4 LAMP primers only. Challenges ensuring species specificity of these assays were also encounted, further limiting choice. All assays used the 2x NEB WarmStart colourmetric reagents (Cat No. M1800) and were supplemented with 50x Fluorescence mix (Cat. No. B1700) where indicated.

The sections below summarise assay development and characterisation for each target species. Also note that the *Undaria pinnatifidia* assay was designed for the Marine Biosecurity Tool Kit by Dr Paul Czechowski using the NEB tool set.

The Assays

Charybdis japonica

Charybdis japonica (6) or the Asian Paddle Crab is a large swimming crab (Figure 1) native to South East Asia and has been detected in New Zealand waters (7). This subtidal zone inhabitant likely entered New Zealand in ballast water or as hull fouling sometime around 2000. The adults are able to swim large distances and are prolific breeders with the long life of the laerva potentially facilitating spread. These large crabs are highly versitile being able to live on a range of substrates from reefs to fine mud. They are currently found in Waitamata and Whangarei harbours and Waikare Inlet in Northland with their range potentially restricted to temperatures >20°C. The risk posed by *C.japonica* is their ability to outcompete native species for habitat and food and their consumption of culturally and economically important shellfish (7).

Five potential LAMP primer sets were suggested using the online design tool provided by NEB (5) with default settings and the COX1 mitochondrial reference MW446892.1 from GENBank. A 1000bp gBLOCK synthetic DNA was also selected from this reference that spanned the qPCR assay site from previous work. Figure 2 shows the gBLOCK sequence indicating the location of the qPCR Charybdis assay. Using the NEB tool with the GenBank reference sequence, potential LAMP primer sets were restricted to the last 500bp of this gBlock sequence and no LOOP primers (LF, LB: optional primers that can improve reaction speed and sensitivity) were possible, meaning all potential LAMP assays consisted of four primer types: F3, B3, FIP and BIP.

Charybdis LAMP primer sets were subject to Primer BLAST searches (8) prior to synthesis to explore potential primer specificity for *C. japonica*. Both the F3 and B3 primers for set P24 were non-specific leading to this primer set being excluded from further analysis. LAMP assays P49, P74, P58 and P83 were synthesised for testing despite all sets having one F3 or B3 primer showing some non-specificity by Primer BLAST.

Each Charybdis LAMP primer set was tested against a ten-fold serial dilution of the Charybdis gBLOCK synthetic DNA. Only LAMP primer set P83 (Table 1, Figure 2) successfully generated product from the gBLOCK with a suggested Limit of Detection (LoD) of 2280 DNA molecules/reaction (Figure 3). Conflicting LoD was apparent between the colour indicator (228,000 copies) and agarose gel analysis (2280 copies) requiring further investigation and suggesting sensitivity differences may exist between signal detection methods. Primer dimers were not detected.

Table 1: P83 primers sequences for LAMP

Primer	Sequence 5'->3'
F3	GAG CAT ATT TTA CAT CAG CTA CT
B3	ATA TCA ATG GAA GAG TTT GCT AA
FIP	AGT GCC GTG AAG AGT TCT AAG TTA ATT ATT GCT GTG CCT ACT GG
BIP	ATA GAC CTT CTA TAT TAT GGG CCC TAC TCC TGT AAG ACC TCC TAC
Hinge FIP	AGT GCC GTG AAG AGT TCT AAG TTT TTT AAT TAT TGC TGT GCC TAC TGG
Hinge BIP	ATA GAC CTT CTA TAT TAT GGG CCT TTT TCT ACT CCT GTA AGA CCT CCT AC

Figure 4 shows a direct comparison of the P83 LAMP assay with qPCR. This experiment was performed using a 10-fold serial dilution of the Charybdis gBLOCK and a 1/10 dilution of the laboratory's Charybdis reference gDNA sample (PTC). A fluorescence indicator was added to the LAMP reaction (f-LAMP) for fluorometric detection by the QuantStudio qPCR instrument and all reactions received the same input DNA: 2.5µl dilution of gBLOCK. The gBLOCK dilution series was made fresh and run with both assays on the same day. An agarose gel comparison was not performed for these experiments.

Comparison of the f-LAMP and qPCR results for the PTC showed each assay determined the same molecule count for the gDNA reference; approximately $1x10^7$ molecule input. Also, the gBLOCK signals over the detection range for f-LAMP were linear ($R^2$0.986, data not shown). Taken together this suggests the f-LAMP assay was accurate for samples within its range of detection. However, comparing the indicative LoD for the Charybdis LAMP and qPCR assays suggests a minimum of two orders of magnitude difference in sensitivity. qPCR potentially detected less that 228 molecules/reaction, while both f-LAMP and colourmetric LAMP stop detecting Charybdis sequence at 22,800 molecules/reaction.

One way to potentially increase reaction time and sensitivity of LAMP is to modify the FIP and BIP primers by addition of a short stretch of T bases between the forward and reverse complimentary protions of these primers (1). Modified P83 primers were synthesised and a matrix assay performed to determine whether these generated product. As shown in Figure 5, all combinations of hinged and non-hinged primers amplified the gBlock sequence, however, efficiency of amplification may have been compromised.

The hinged matrix primer experiment was repeated, incorporating a fluorescent indicator. This was to test whether reaction speed increased, something not able to be measured easily by end-point detection methods. Fresh primer mixes and LAMP reagent were used for this experiment. Results are given in Figure 6. Positive detection of Charybdis gBLOCKs did not exceed 22,800 molecules per reaction for any of the LAMP primer combinations indicating no increase in sensitivity. Comparison of the Cq across the PTC sample showed that Mix 1 and Mix 3 performed equally well (20.843 and 20.828 respectively) followed by Mix 2 (26.727) and Mix 4 (29.625) indicating that hinged primers did not confer a speed advantage over non-hinged primers. Colourmetric indicators were difficult to differentiate from negative results in some cases. This work also indicated that older primer mixes should be discarded as their performance appears to degrade over time. Based on these results, hinged primers had no impact on reaction speed or sensitivity.

[Image: Chris Woods, NIWA]

Figure 1: Two forms of the Asian Paddle Crab (*Charybdis japonica*). (https://www.marinebiosecurity.org.nz/charybdis-japonica/)

tattactgccattcttctactcctctcgcttcctgtgttagctggagctattactatattattaacagaccgaaacttaa
atacttcattcttcgaccctgctgggggtggggatcctgttctttatcaacacttgttttgattctttggacatcctgaa
gtttatattttaatcttacctgccttcggaataatttcacatattgtcagacaagaatctggtaaaaaggaatcatttgg
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gaatagatgtagatactcgagcatattttacatcagctactaataattattgctgtgcctactggaattaaaatttttaga
tgacttagaactcttcacggcactcaaattaactatagaccttctatattatgggccctaggatttatttcttattcac
agtaggaggtcttacaggagtagttttagcaaactcttccattgatattattcttcacgacacatactatgtggtagccc
actttcattacgttctttctataggagctgtatttggaattttcgccggcattgcccactgattccccttatttaccgga
ctttcattaaatcctaaatgaataaaaattcattttactattatatttattggtgtaaatattactttctttcctcaaca
ctttttaggactaaatggcatgcctcgtcgttattctgactaccccgatgcatacactacgtgaaatattgtctcttcta
taggatcaatagtatcacttattgctatactaatttttataattgtaatttgagaagcacttatctcaaatcgacctgtt
ctattttctcctttccttccttcttctattgaatgaaaccactcttacccacctgcagatcactcatacatagaaatccc
cctaatctctaacttctaaaatggcagatagatgtatagg

Figure 2: gBLOCK sequence for cox1 gene taken from MW446892.1. Sequence spans positions 561 to 1560 of reference. qPCR assay forward, reverse and probe sequences are indicated in red italics (Gert-Jan Jeunen, personal communication). LAMP primers are F3 in Pink; B3 in Green; FIP in Blue with reverse complement underlined; BIP in Orange with reverse complement underlined.

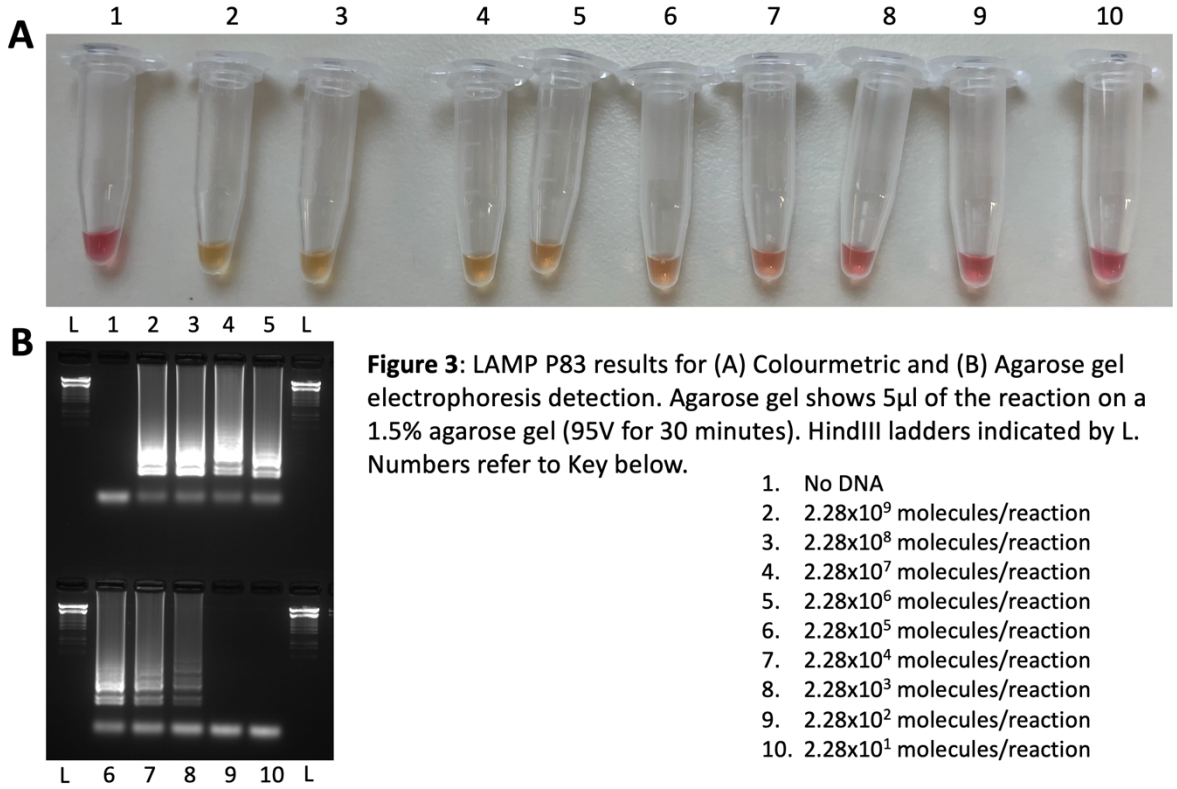
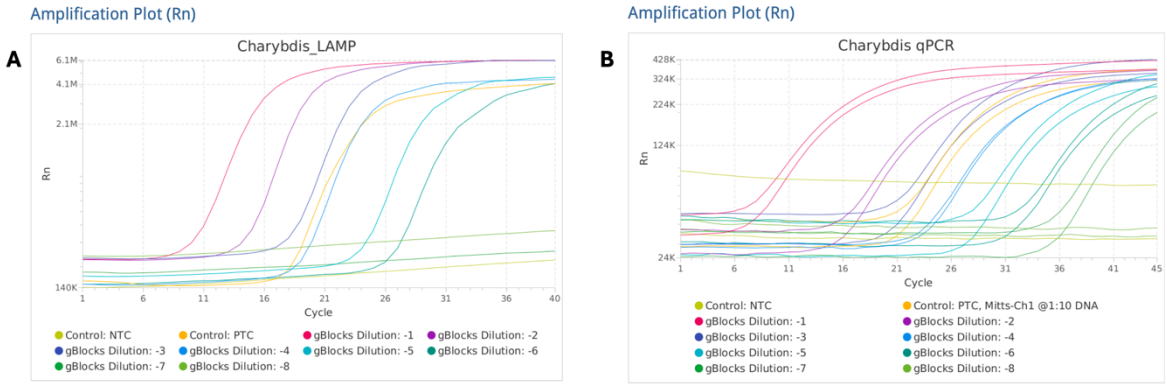


Figure 3: LAMP P83 results for (A) Colourmetric and (B) Agarose gel electrophoresis detection. Agarose gel shows 5µl of the reaction on a 1.5% agarose gel (95V for 30 minutes). HindIII ladders indicated by L. Numbers refer to Key below.

1. No DNA
2. 2.28x10^{9} molecules/reaction
3. 2.28x10^{8} molecules/reaction
4. 2.28x10^{7} molecules/reaction
5. 2.28x10^{6} molecules/reaction
6. 2.28x10^{5} molecules/reaction
7. 2.28x10^{4} molecules/reaction
8. 2.28x10^{3} molecules/reaction
9. 2.28x10^{2} molecules/reaction
10. 2.28x10^{1} molecules/reaction



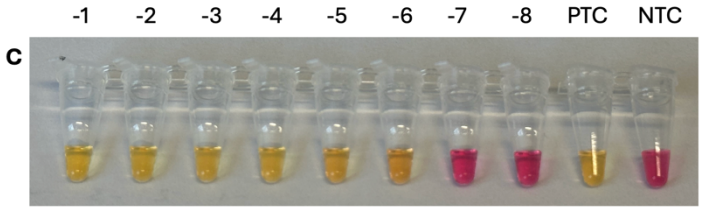


Figure 4: DNA input equal in all reactions. **A)** LAMP Fluoresence **B)** qPCR **C)** Colourmetric

Samples (molecules/reaction):

-1: 2.28x10^{9}
-2: 2.28x10^{8}
-3: 2.28x10^{7}
-4: 2.28x10^{6}
-5: 2.28x10^{5}
-6: 2.28x10^{4}
-7: 2.28x10^{3}
-8: 2.28x10^{2}
PTC: 1/10 Ref DNA
NTC: Water

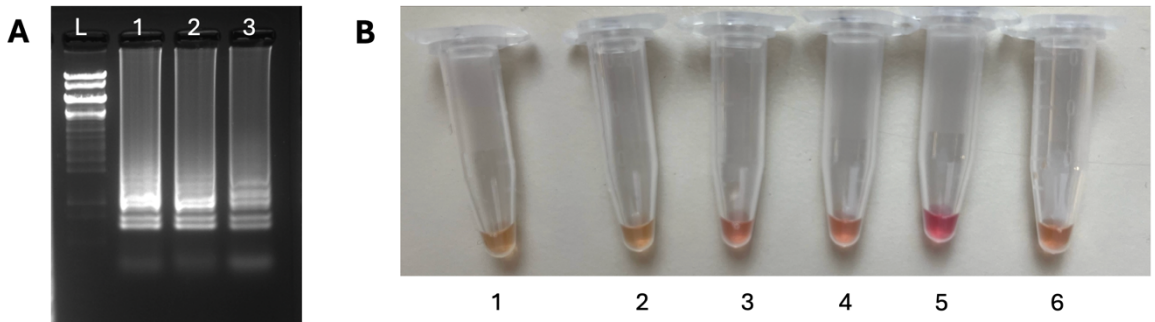


Figure 5: Hinge Matrix Experiment. 2.28x10^5 copies per reaction.
A: 1.5 % Agarose Gel with 5µl LAMP reaction (95V for 30 minutes)
B: Colourmetric colour change.

L: Ladder
1: Mix 1 New mix FIP+BIP
2: Mix 2 Hinge FIP + BIP
3: Mix 3 FIP + Hinge BIP
4: Mix 4 Hinge FIP + Hinge BIP
5: No template control
6: Original Mix FIP+BIP mix

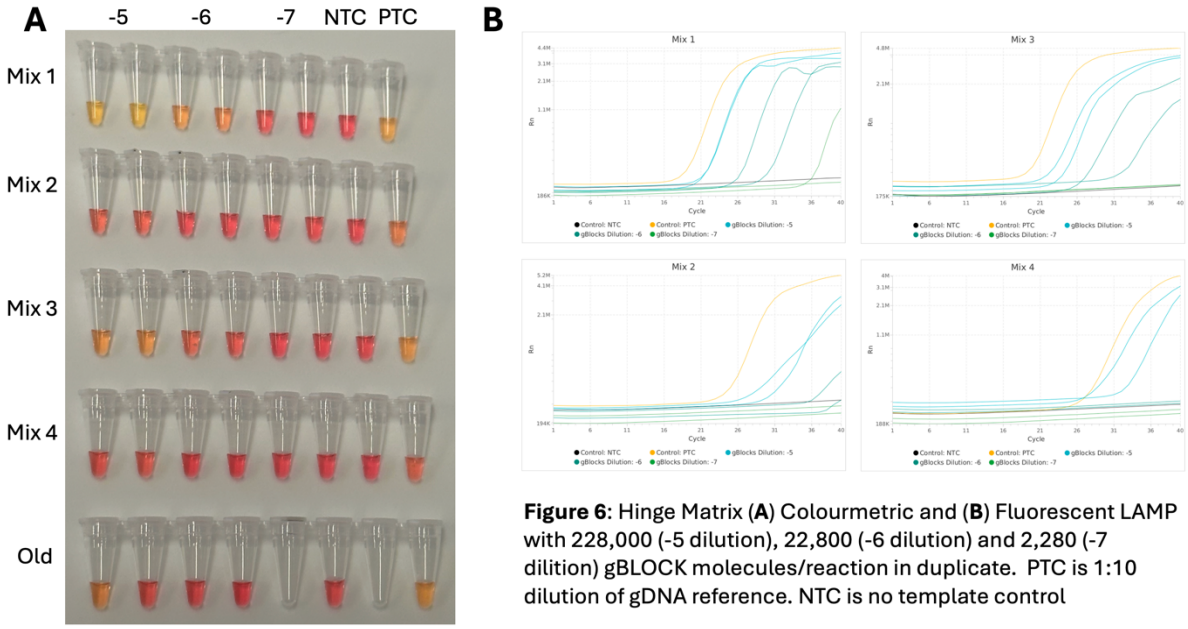


Figure 6: Hinge Matrix (**A**) Colourmetric and (**B**) Fluorescent LAMP with 228,000 (-5 dilution), 22,800 (-6 dilution) and 2,280 (-7 dilition) gBLOCK molecules/reaction in duplicate. PTC is 1:10 dilution of gDNA reference. NTC is no template control

Carcinus maenas

Carcinus maenas (7), the European Shore or Green Crab (Figure 7) , is native to the European Atlantic coast and north Africa. It is currently not found in New Zealand but has been spread to North America, Japan, South Africa and Australia (7). It poses a threat through predation and overwhelm of native environments. The Green Crab is a generalist feeder, including shellfish , and this impacts traditional food sources and the aquaculture industry. It tolerates wide ranges of salinity and temperature and can live in most marine habitates from the open ocean, intertidal zones and estuaries, including degraded environments with low oxygen and

pollution. Like Charybdis the larval stages of the Green Crab are long lived facilitating dispersal. They are prolific breeders (7).

The same approach to LAMP assay design was taken for Carcinus as for Charybdis. The NEB LAMP assay design tool suggested three primer sets for the Carcinus COX1 reference JQ306003.1 that spanned two published qPCR assays (9,10). Primer-BLAST (8) search of all suggested LAMP primers indicated two with potential species specificity: P35L1 and P20L1. Both consisted of 5 primers, B3, F3, BIP, FIP and LF (optional). Interestingly, the BIP primer was common to both P35L1 and P20L1. A synthetic gBLOCK was designed to span the COX1 region containing the LAMP and qPCR assays (Figure 8).

Two 10x primer mixes from each primer set were made to determine whether they could detect Carcinus DNA. One reaction mix included the LF primer (Mix 1) and the other omitted this primer (Mix 2, Table 2). This gave a total of four 10x primer mixes. These were tested against the Carcinus gDNA reference and results are given in Figure 9. Only primer set P35L1 without the LF primer gave amplified product. P35L1 without LF was selected for further analysis.

Table 2: P35L1 primers sequences for LAMP

Primer	Sequence 5'->3'
F3	CCT TAC TTT TAA TAA GAG GGA TGG
B3	AAG GTA TCT GGT CTA TTG TCA
FIP	CAA CTG AAG CAC CAG CAT GGA AAG AGG AGT TGG AAC AGG
BIP	TTC TCT TTA CAT TTA GCC GGG GTT TTG CCG AAA GAA CGC ATA T
LF	GCT AAA GGA GGA TAG ACT GTT CAT (Omitted from final assay)

The sensitivity of the P35L1 assay was probed by comparing an equal input of gBLOCK for LAMP and qPCR experiments. All comparisons were run on the same day and from the same gBLOCK dilution. Results are presented in Figure 10. LAMP detected Carcinus using both colourmetric assay and fluorescence. Examination of PTC signal shows both the qPCR and colourmetric LAMP (10^{-6} dilution) gave comparable results, indicating consistancy between the assays. Interestingly, though the colourmetric assay was strongly positive, the f-LAMP signal was low but detectable. The indicative LoD for colourmetric LAMP suggested a detection cutoff of $4x10^4$ molecules but was refined to $3x10^4$ molecules using the more granular f-LAMP measurement. The qPCR assay on the other hand appeared to detect <400 molecules. This suggests that the analytical LoD for the Carcinus assay is approx. 30,000 molecules, at least two orders of magnitude less sensitive than qPCR.

[Image: Michael Marmach]

Figure 7: Image of the European Shore Crab (7)

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1   gtagggactt ctttgagtct tattattcca gctgaattag ggcagccagg aactttaatc
61  ggtaacgacc aaatttataa cgttgttgta actgctcatg cttttgtaat aatttttttc
121 atagtaatac caattataat tggaggattt ggtaattgac ttgtacctct aatattagga
181 gggccagata tagcttttcc tcgaataaat aatataaggt tctgattact tcctccgtct
241 ttaacttac ttttaataag agggatggta gaaagaggag ttggaacagg atgaacagtc
301 tatcctcctt tagcaggggc tatcgccat gctggtgctt cagttgattt agggatttc
361 tctttacatt tagccggggt ttcttctatt ttaggagctg taaattttat aacaactatt
421 atcaatatgc gttctttcgg catgacaata gaccagatac ctttatttgt gtgagctgtt
481 tttattactg cgatcctttt attattatcg ttgccggttt tagcaggagc tattacaata
541 cttctaactg accgaaatct aaacacctca ttcttcgatc
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Figure 8: gBLOCK sequence for Carcinus COX1. LAMP P35L1 are highlighted in green. qPCR primers and probe described by Danzinger & Frederick (10) are in purple. qPCR primers and probe described by Roux et al (9) are in red

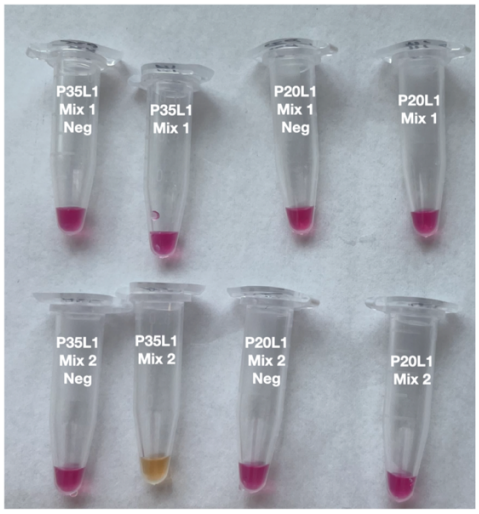

Figure 9: Comparison of LAMP colourmetric endpoint measurements from two primer sets with (Mix 1) and without (Mix 2) the LF primer option for Charybdis assays. A 1:10 dilution of the reference Charybdis gDNA was used as target.

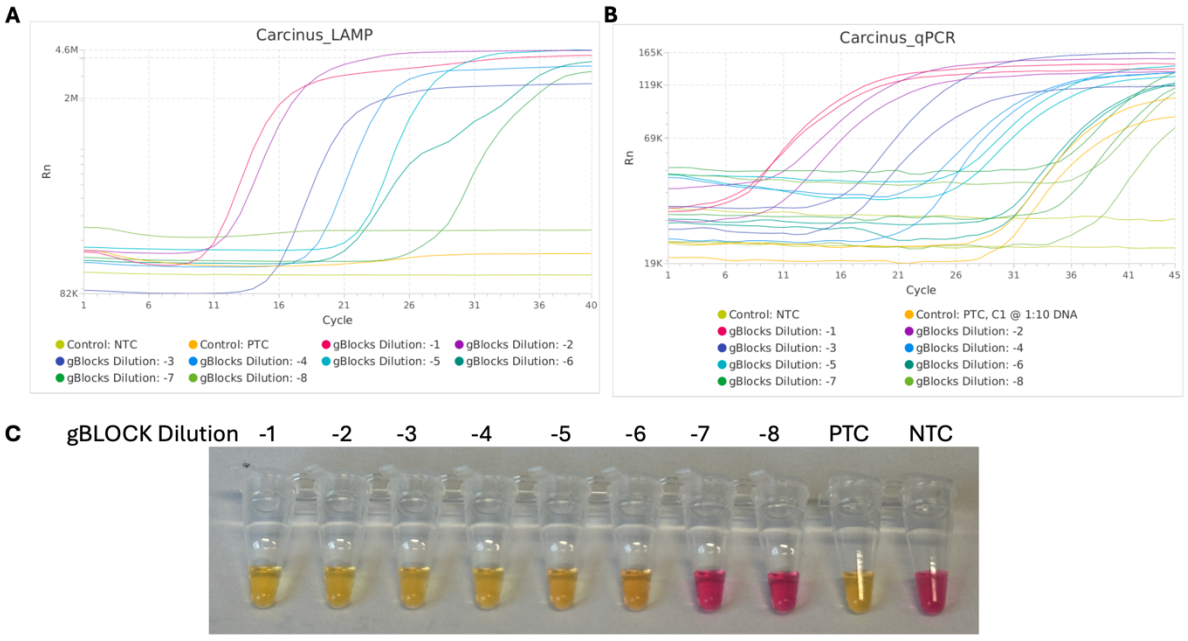

Figure 10: 2.5µl gBLOCK DNA input for (**A**) f-LAMP (**C**) Colourmetric and (**B**) qPCR assays. All experiments were performed on the same day with the same freshly made gBLOCK dilution series

Asterias amurensis

The North Pacific Sea Star (*Asterias amurensis* (7), Figure 11) is an unwanted organism currently not found in New Zealand. It is a coastal organism native to the Northwest Pacific that has invaded coastal estaries and bays in Tasmania and Victoria, Australia. It destroys native environments through heavy predation and will expand to large population numbers outside of it native habitat (7).

Using the COX1 Asterias reference AB183559.1 with the NEB LAMP design tool (5) two potential LAMP assays were selected. Manual Primer-BLAST searches (8) of all LAMP primers indicated that assay P225L1 was likely to be more species specific, where as the B3 and FIP primers of P139 were cross reactive with multiple species. Both assays were synthesised and tested against a gDNA reference for Asterias held by the Gemmell Lab. Only P225L1 was successful (Figure 12, agarose gel image not shown). LAMP primer sequences for P225L1 are given in Table 3.

A gBLOCK synthetic gene fragment was made for the COX1 gene containing both the LAMP assay and the qPCR assay for Asterias (figure 13). Figure 14 shows LAMP assay P225L1 used with a 10-fold dilution series of the gBLOCK and suggested higher sensitivity than other assays. The experiment was repeated with fresh gBLOCK dilutions and qPCR and LAMP performed on the same day in side-by-side assays with identical DNA input (2.5µl). Results are given in figure 15. The f-LAMP assay suggested an analytical LoD cut was 15,500 molecules/reaction. This was supported by the colourmetric results from the same LAMP samples, though the colour change for one of the duplicates was unconvincing. Comparison to the suggested analytical LoD for the Asterias qPCR assay of <150 molecules again indicates this LAMP assay was at least two orders of magnitude less sensitive than the qPCR COX1 assay.

Table 3: P225L1 LAMP Primer Sequences

Primer	Sequence 5'->3'
F3	ACC GGA GTG GTT TTA GCT
B3	CGC CTA TAA ACA TTA CCG CA
FIP	GCC CCC ATG GAT AAT ACG TAG TGT CTT CCA TTG ATA TAA TTC TTC ACG
BIP	CTT TGC AAT ATT TGC TGG CTT TAC CCT TTC TTC ATA GGG GGT GTA
LF	TGG GCA ACA ACG TAG TAT GTG T
LB	ACT GGT TTC CAC TAT TTT CTG GAG T

Figure 16 shows an initial application using hinged FIP and BIP primers for the Asterias LAMP assay. As previously, four thymine bases were inserted into the FIP and BIP primers at the junction between the forward and reverse portions of these primers. This creates a flexible spacer or hinge within the primer (1). Combinations of 10x primer mixes were made where the hinged primers were substituted for the FIP, BIP or both FIP and BIP primers. LAMP assays were performed on gBLOCK DNA at 27,400 molecules/reaction. All reactions except for the one containing both hinged FIP and hinged BIP, produced a colour change at the end of incubation (not shown). This is supported by the agarose gel results in figure 16. Figure 16 also suggested that LAMP amplification was less efficient when hinged FIP was substituted for FIP. This system was not explored further.

[Image: Serena Cox, NIWA].

Figure 11: *Asterias amurensis* or the North Pacific Sea Star

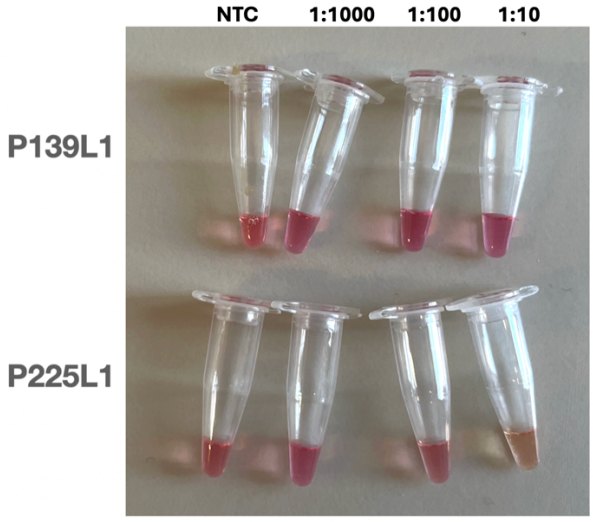

Figure 12: LAMP assays P139L1 and P225L1 tested against a dilution series of a gDNA reference for *Asterias amurensis*

attatatataataaattataaaacatgcaactaagacgctgactattttctactaaacataaggacattgggact
ctttatctaatatttggagcttgagctggtatgattggaactgctatgagagtaataattcgtactgagctcgca
caaccgggatctttacttcaagatgatcaaatttacaaagttatagtaactgctcatgctcttgtaatgatattt
tttatggtgatgcctattatgataggaggatttggtaaatgactaattcctcttatgataggtgccccagatatg
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gttgaaagaggagctggaactggctgaacgatttatcctcctttatctagaggactagctcatgcaggaggatcc
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cacgacacatactacgttgttgcccactttcactacgtattatccatgggggccgtctttgcaatatttgctggc
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tttttatttttaatttgggaagccttcttactaagtcacacggcctctccacccagagtctctac

Figure 13: Asterias COX1 gBLOCK. The qPCR primers and probe are marked in red (11). The P225L1primers are F3 = Blue; B3 = Green; FIP = Orange, reverse complement portion underlined; BIP = Purple, reverse complement underline

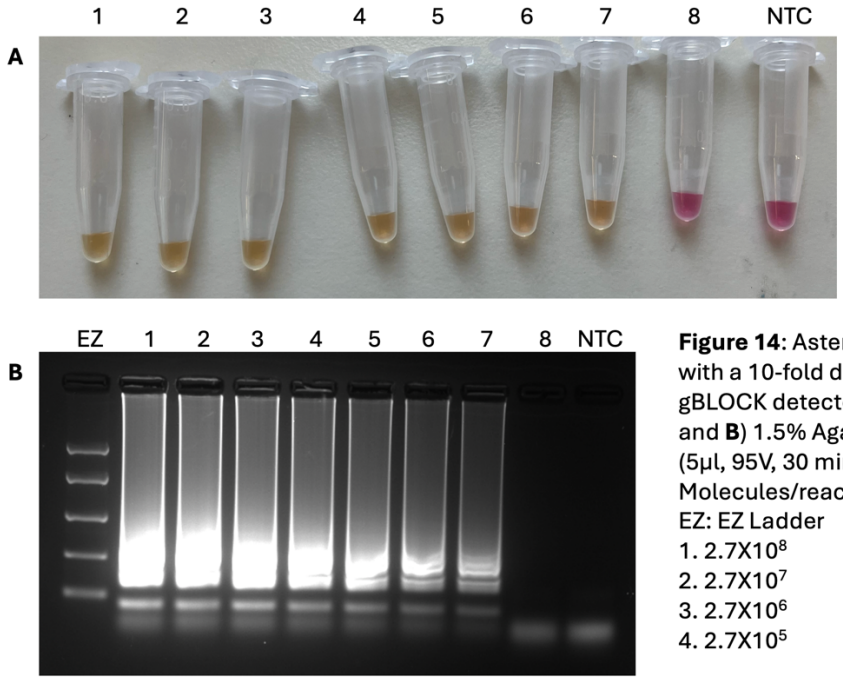

Figure 14: Asterias LAMP Assay P225L1 used with a 10-fold dilution series of COX1 gBLOCK detected by **A**) colourmetric assay and **B**) 1.5% Agarose Gel Electrophoresis (5µl, 95V, 30 minutes). Key units: Molecules/reaction)

EZ: EZ Ladder
1. $2.7X10^8$
2. $2.7X10^7$
3. $2.7X10^6$
4. $2.7X10^5$
5. $2.7X10^4$
6. $2.7X10^3$
7. $2.7X10^2$
8. $2.7X10^1$
NTC. No template

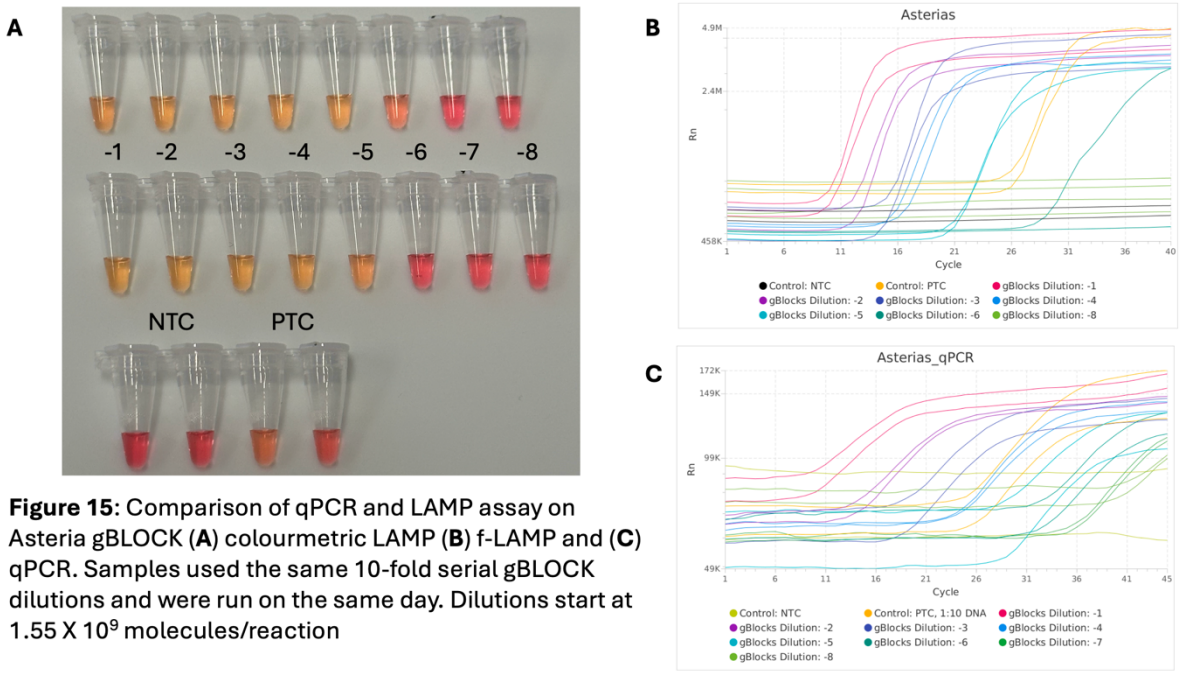

Figure 15: Comparison of qPCR and LAMP assay on Asteria gBLOCK (**A**) colourmetric LAMP (**B**) f-LAMP and (**C**) qPCR. Samples used the same 10-fold serial gBLOCK dilutions and were run on the same day. Dilutions start at 1.55×10^9 molecules/reaction

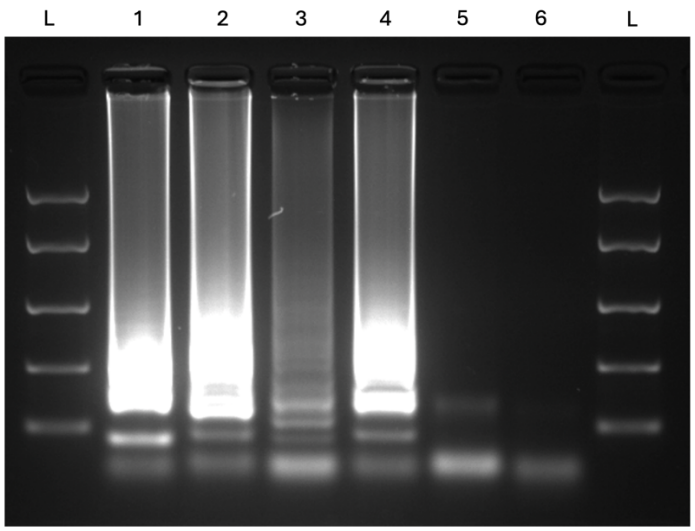

Figure 16: Hinge Matrix showing endpoint detection for the Asterias LAMP assay. 1.5% agarose gel electrophoresis with 5µl reaction mix, 95 V for 30 minutes. L = EZ Ladder; 1 = Original standard Asterias LAMP mix; 2 = Standard Asterias LAMP mix made with re-synthesized primers; 3 = Hinge FIP substituted for FIP; 4 = Hinge BIP substituted for BIP; 5 = Hinge FIP and Hinge BIP; 6 = No template control.

Undaria pinnatifida

Undaria pinnatifida or Wakame Asian kelp (Figure 17) was first detected in New Zealand in 1987 and it has since spread along the North and South Island east coasts. The mode of spread is as fouling on the hulls of vesels and it is currently under active management. The main threat it poses is through habitat exclusion for native species and it creates operational issues for the aquaculture industry (7).

A LAMP assay based on COX1 was designed by Dr Paul Czechowski using NEB LAMP design tools and a bioinformatics approach to check for species cross reactivity. This assay was also located near the COX1 qPCR assay (11) used for MBIE CAWX1904. LAMP primers for the Undaria assay are given in Table 4. A synthetic gBLOCK DNA based on Genbank Reference AB775223 was synthesised to span both the Undaria LAMP and qPCR priming sites for both assays (Figure 18).

Table 4: Undaria LAMP primer sequences

Primer	Sequence 5'->3'
F3	GGTCTGTTTTAATTACAGCGTT
B3	CCGAAAACTGGTTTTCTTGA
FIP	GCCCGCAGGATCAAAAAAAGTAGTATCATTGCCGGTTTTAGCA
BIP	ATTTTGGTTCTTTGGTCATCCTGAATATGACTAACAATACCGAATCC
LF	CTGTTAGTAACATTGTAACAGCACC (Omitted from final assay)

Testing the LAMP assay both with and without the LF primer using gDNA and gBLOCK reference samples showed that LF prevented amplification and was therefore omitted from the Undaria assay (data not shown). Figure 19 shows the performance of the Undaria LAMP

assay against a 10-fold serial dilution of the gBLOCK. Colourmetric detection of Undaria stopped at $2.28x10^6$ molecules/reaction but detection was appartent for 228,000 molecules/reaction with a faint suggestion of amplification at 22,800 molecules/reaction using agarose gel detection. This difference in sensitivity between methods was demonstrated repeatedly.

The experiment presented in figure 20 was performed using LAMP with two detection modes (fluorometric and colourmetric) and directly compared to qPCR. As previously, fresh gBLOCK dilutions were made and both qPCR and LAMP were performed on the same day using the same samples with 2.5µl DNA input. Neither f-LAMP or colourmetric LAMP was able to detect the Undaria positive control (PTC) however qPCR estimated that the PTC contained approximently 13,000,000 molecules/reaction. In comparison f-LAMP was able to detected 2,400,000 molecules/reaction of the gBLOCK dilution which should have been within the detection range for the PTC. The colourmentric LAMP detected less than 240,000,000 molecules/reaction putting this detecion method orders of magitude behind f-LAMP and qPCR, consistent with other Undaria experiments. qPCR could detect 2400 molecules/reaction in this comparison expermiment when using the same samples. These detection measurements are high for both detection technologies and, taken with earlier experiments may indicate degradation of the gBLOCK standard sample. Even so the performance of the Undaria LAMP assay is at least three orders of magnitude less sensitive than qPCR.

The use of hinged primers was explored as a potential method of improving Undaria LAMP assay sensitivity. In this work a modified FIP primer was trialed: Hinged FIP. A side-by-side performance comparison of the hinged and non-hinged FIP primer is given in figure 21. A colour change was apparent for both primer mixes indicating a detection cut-off of 22,800,000 molecules/reaction. Agarose gel electrophoresis revealed a difference between detection methods with both mixes indicating Undaria at below 2,280,000 molecules/reaction, an order of magnitude more sensitive. Interestingly, agarose gel detection using the hinged FIP primer hinted detection might be possible to 228,000 molecules/reaction. The non-hinged primer demonstrated clear detection of 228,000 molecules/reaction with potential detection at 22,800 molecules/reaction. Therefore the non-hinged FIP primer performed better than the hinged version, consistent with trials of other hinged LAMP assays in this study and showing sensitivity differences were only apparent when using agarose gel detection not the colourmetric method.

Before the Undaria LAMP assay had been fully evaluated an opportunity presented itself for screening eDNA samples in the laboratory. Table 5 presents test data for seven eDNA samples, four from a site known to be heavily infested with Undaria and three from a site thought to be Undaria free. Water samples were vacuum filtered and extracted using the QIAgen Blood and Tissue protocol. Results obtained in laboratory tests were in concordance with sample collection site descriptions and between detection method.

Table 5: eDNA test - QIAgen extracted samples supplied by Gert-Jan Jeunen.

Collection Site	**Sample number**	**Cp: Undaria qPCR**	**+/- Colorimetric LAMP**
Otago Harbour. Known Undaria site	1	33.45	**+**
	2	32.97	**+**
	3	33.65	**+**
	4	33.65	**+**
Deep Cove Fiordland. No know Undaria detected	0.1	-	**-**
	0.2	-	**-**
	0.3	-	**-**

Figure 17: Undaria pinnatifidia (12)

acattatatt taatttttgg gggtttttcg ggggttcttg gtacagcaat gtctgttttt
atccgattgc aattagctag ccctggtaat caatttttgg ggggaaatca tcagttgtat
aatgttattg taacagcaca tgctttctta atgatttttt ttatggttat gccaattctt
atcggtggat ttggtaactg gtttgtacct ttaatgattg gtgctcctga tatggctttt
cctcgtatga ataatattag tttttggtta ttacctccct ctttaattct tcttttagcc
tcttctttag tagagtctgg ggctggaaca ggttggacgg tataccctcc gcttagtggt
attcaagctc actcaggtcc ttcagttgat ttagctattt ttagtcttca cctttcagga
gctgcttcta ttttaggtgc tataaacttt attaccacaa tttttaatat gagagcacct
ggtatgacaa tggatagatt gcctcttttt gtgtggtctg ttttaattac agcgttttta
ttgttgttat cattgccggt tttagcaggt gctgttacaa tgttactaac agatcgtaat
tttaatacta ctttttttga tcctgcgggc ggtggtgatc cagtattata tcagcattta
ttttggttct ttggtcatcc tgaagtatat atattaattt taccaggatt cggtattgtt
agtcatatat tatctacttt atcaagaaaa ccagttttcg gttatttagg tatggtttat
gctatgcttt ctataggtat acttggtttt attgtatggg ctcatcacat gtttacagta
ggtttagata ttgatacaag agcttatttt acagcagcca ctatgattat tgcggtccct
acaggtatta aaatttttag ttgggtcgca actttgtggg gaggttctat tcggttaaaa

Figure 18: gBLOCK sequence for Undaria COX1 segment containing both the LAMP assay and qPCR primer and probe sites (11). qPCR primer and probe sites are indicated in green italics. LAMP primers are indicated in colours corresponding to primer type. Blue = F3; Teal = B3; Orange and Yellow forward and reverse complement of FIP; Light and Dark Pink = forward and reverse complement of BIP; Red bold = LF.

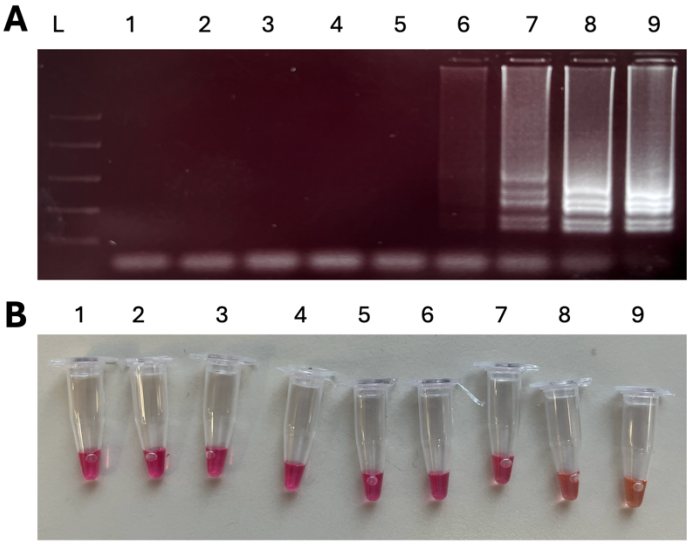

Figure 19: Detection of Undaria from a serial dilution of a gBLOCK standard using LAMP. Amplification was detected by (**A**) Agarose gel electrophoresis and (**B**) colourmetric methods. Electrophoresis of 5µl reaction at 95V for 30 minutes. Key: L = EZ Ladder, 1 = NTC; 2 = 2.3; 3= 23; 4= 228; 5 = 2280; 6 = 22,800; 7 = $2.28x10^5$; 8 = $2.28x10^6$; 9 =$2.28x\ 10^7$. Units are molecules/reaction

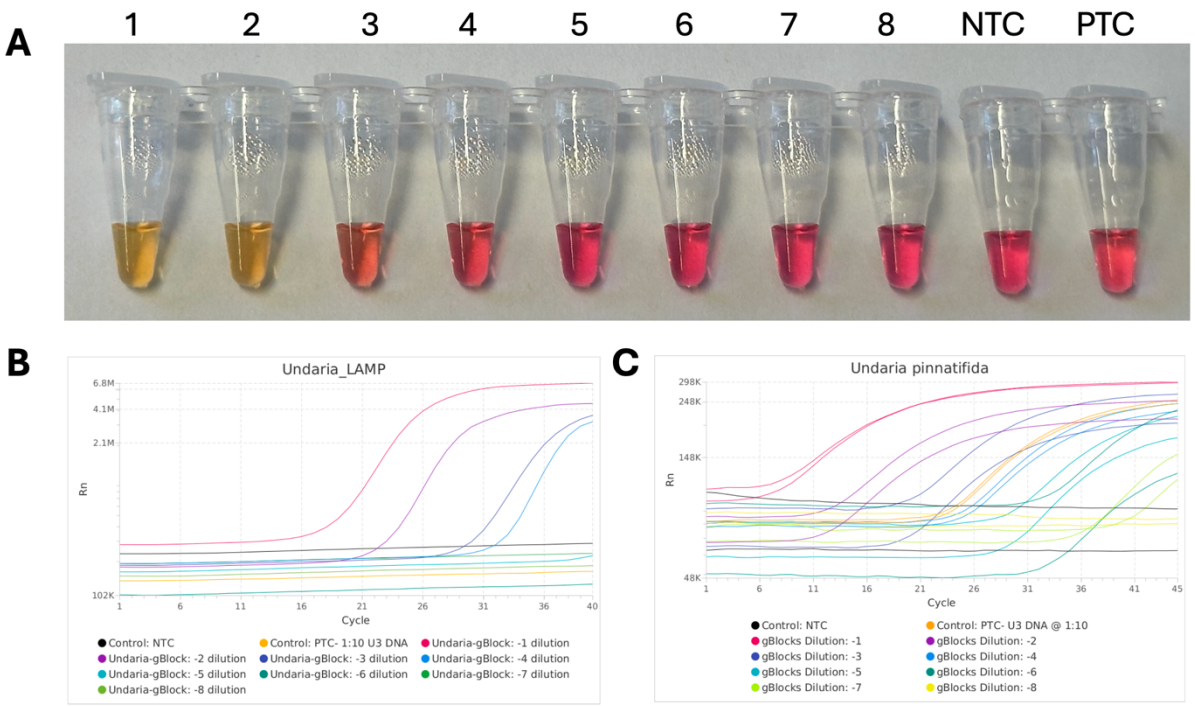

Figure 20: Comparison of qPCR and LAMP for Undaria detection from gBLOCK DNA serial dilution. 2.5µl gBLOCK DNA input for (**A**) Colourmetric and (**B**) f-LAMP and (**C**) qPCR assays. All experiments were performed on the same day with the same freshly made gBLOCK dilution series. Key: NTC = No template control; PTC = Positive template control; 1 and -1 dil = 2.4×10^9 molecules/reaction; 2 and -2 dil = 2.4×10^8 molecules/reaction; 3 and -3 dil = 2.4×10^7 molecules/reaction; 4 and -4 dil = 2.4×10^6 molecules/reaction; 5 and -5 dil = 2.4×10^5 molecules/reaction; 6 and -6 dil = 2.4×10^4 molecules/reaction; 7 and -7 dil = 2.4×10^3 molecules/reaction; 8 and -8 dil = 2.4×10^2 molecules/reaction

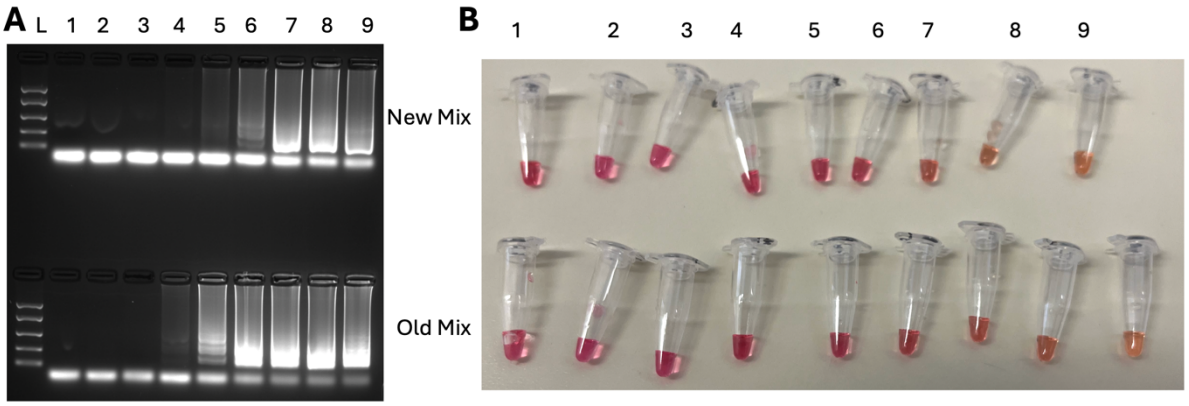

Figure 21: Side-by-side comparison of of two LAMP assay primer mixes by (**A**) Agarose gel electrophoresis and (**B**) Colourmetric detection of a serial dilution of Undaria gBLOCK DNA. Old Mix consisted of non-hinged primers. New Mix substituted a Hinged FIP primer for the standard FIP. Electrophoresis of 5µl reaction at 95V for 30 minutes. Key: L = EZ Ladder, 1 = NTC; 2 = 228; 3= 2280; 4= 22,800; 5 = 228,000; 6 = 2.28×10^6; 7 = 2.28×10^7; 8 = 2.28×10^8; 9 = 2.28×10^9. Units are molecules/reaction

Conclusion

This work describes considerations for future development of LAMP assays for biosecurity and citizen science applications. Though LAMP has already proven a useful tool for eDNA detection of specific species (e.g. 3,4,13) for the method to be useful it must be sensitive.

Keying LAMP design to qPCR assays for the COX1 gene impacted primer selection and was potentially detrimental to assay sensitivity. Using the NEB assay design tool for all four species restricted LAMP primer options with only a few sets of these potential assays proving successful for amplifying target from reference gDNA or gBLOCK standards. In addition, sensitivities for all of the assays were at least two orders of magitude or more less senisitive than qPCR. Ellis et al (2022)(14) found the LoD for COX1 qPCR for detection of Undaria and Asterias was 6.73 molecules/reaction and 3.6 molecules/reaction respectively, when adjusted for the system used in this work. None of the LAMP assays arising from this work reached these levels of sensitivity.

LAMP primers are complex and can form hairpin and primer dimer structures (Meagher et al 2018)(15). Using a dedicated design tool will avoid these issues. However, amplifying primer dimers were tested for by observing no template controls and checking for amplification on agarose gels. Hairpinning may explain the instability both in sensitivity and performance of the Undaria LAMP assay, however this was not investgated further. Adding a hinge to the FIP and BIP primers impacts performance, as shown here. In this approach the FIP and BIP primers are modified by including a linker between the forward and reverse complementary sequences. Traditionally this is a short stretch of thymine residues, however Lamas et al (2023) (16) suggest that the sequence of this linker can significantly impact LAMP performance. It would be interesting to investigate whether alternative linkers improve the sensitivity and speed of these assays.

The method used to detect LAMP amplification should be carefully considered. In the work presented here, colourmetic detection was less sensitive than agarose gel or fluorescent detection (e.g. figures 20 and 21). It is also possible for an acidic sample to trigger the colour change with no requirement for DNA amplification, giving a false positive result (J. Stanton, personal communication). In future work it is recommended colourmetric detection be avoided despite the attractiveness of the approach. Small, portable fluorometric devices are becoming increasing available (e.g. 17, 18, 19) making it easier for fluorescent detection to be carried out at the point-of-care.

The lack of analytical sensitivity coupled with resource constraints and other program priorities curtailed further work with these LAMP assays. This extended to testing these LAMP assays with eDNA samples. A limited test for Undaria was performed and suggested compatibility with eDNA samples. However, low sensitivity coupled with generally expected low target concentrations in eDNA samples makes it difficult to draw conclusions from negative LAMP assay results and questions the usefullness of the assays described here.

Advice to future researchers wishing to develop species/sequence specific LAMP assays would be to explore a variety of loci, be open to testing primer modifications such as hinges and expect to use detection methods other than pH linked approaches.

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Recipes

Concentrations for 10x Primer Pool mix

F3	2µM
B3	2µM
FIP	16µM
BIP	16µM
LF	4µM
LB	4µM

LAMP reaction mixes

Reagent	**1 x standard mix**	**1x fluoro mix**
2x Warmstart Master Mix (NEB M1800)	12.5µl	12.5µl
X10 LAMP Primer Pool	2.5µl	2.5µl
50x Fluoro (NEB B1700)	-	0.5µl
H2O	7.5µl	7µl
Template	2.5µl	2.5µl
Total	**25µl**	**25µl**

Incubation: 65°C for 30 minutes

Calculating molecules/reaction for gBLOCK experiments:

https://sg.idtdna.com/pages/education/decoded/article/calculations-converting-from-nanograms-to-copy-number

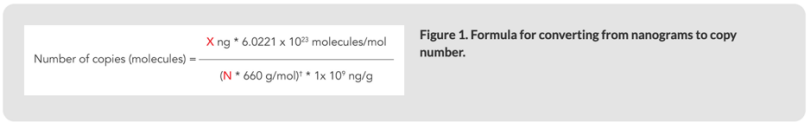

Figure 1. Formula for converting from nanograms to copy number.

Where:
X = amount of amplicon (ng)
N = length of dsDNA amplicon
660 g/mol = average mass of 1 bp dsDNA
6.022 x 10^{23} = Avogadro's constant
1 x 10^{9} = Conversion factor

† The actual oligonucleotide MW (in Daltons or g/mol) is provided on the IDT Spec Sheet for each oligonucleotide and can be substituted for this arithmetic phrase, which only provides an average MW for a sequence of this length.