United Nations
Educational, Scientific and
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Oceanographic
Commission

The Institute of
Applied Sciences

PacMAN PROJECT

Building Capacity in Pacific Small Island Developing States on Marine Bio-invasion

Report on Development of qPCR assay's

Covering the Period from Sept 1st – October 30, 2023

Date: 21 November, 2023

By

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Acronym

eDNA- Environmental DNA
LoD- Limit of Detection
OBIS- Ocean Biodiversity Information System
NEG- Negative
NTC- No template controls
PacMAN- Pacific Islands Marine Bio-invasion Alert Network
PNPRC- Pacific Centre for Natural Products Research
SAGEONS- School of Agriculture, Geography, Environment, Oceans and Environment Resource
USP – University of the South Pacific
UNESCO- United Nations Educational, Scientific and Cultural Organization

1.0 Executive overview

This extension period which covers the months of September to October has been earmarked primarily establish the limits of detection of *Didemnum perlucidum* (DP) as well as subject USP allotted samples through establish protocols for DP assay.

Table 1. Existing qPCR primers and probes to be utilized for assay development for USP

Target Species	Primer name	Forward sequence	T (C)	Reverse sequence	T (C)	Length	Probe sequence	Reference
*Perna viridis**	Fw A, Rev A	CTTAGTGGC ATTAATTCG DAATCC	59.2	CAAAGTACC AATATCTTT ATGATTRGT WGA	57.5	281	ACTCAAACAACAAAG TAAAC (lagging DNA strand)	Dias et al. 2013
Didemnum perlucidum	Dper new F/R	AGCTCCTGA TATAGCATT TCCTCGTTT AAA	63.3	AGATATTCC TGCTAAATG TAATGAAA AAATAGCTA	61.2		TAGCTCATTCAAATA GGGCAGTA	Simpson et al. 2017

The Pacific Natural Products Research Centre (PNPRC) continues to develop necessary protocols during this phase included:

- Developing qPCR assays to test *Didemnum Perlucidum* (DP) and *Perna Viridis* (PV)
- Calculate limits of detection for the DP assay
- Analyze 80% (n = 136) of samples accumulated from year analyses
- Identify the presence of high-risk species in all 136 samples
- Report all results with raw data and share materials and methods openly

The team at USP has made advances in the development of the D. *perlucidum* assays with the successful calculation of the assays limits of detection (LoD). All samples have also been subjected through the assay. The following sections describe the methods and results.

2.0 Protocol's

2.1 LoD development

The establishment of the LoD followed an extensive discussion and several meetings with scientific advisors at Deakin University. A protocol was developed through a series of trials as reported in Report 1 of this extension phase to establish the LoD. It is safe to say that the experience

has enabled the USP team to familiarize themselves with necessary matrices of analyses required to establish new qPCR assays.

- **Protocol**
    - Prepare 7 to 10 fold serial dilution for the positive standards. Each dilution is to have 10 replicates to generate a standard curve of known DNA quantities ranging from 10ng/µL to 0.1 ng/µL (standards). **For Didemnum, the initial start up concentration for pure standards was 0.05ng/uL with a 7 fold dilution series and a replicate of 10 per dilution.**
    - Place all reaction components on ice.
    - Mix and then briefly centrifuge to collect contents at the bottom of the tube
    - Prepare enough master mix to run all dilutions
    - Be sure to include duplicate no template (NTC) and NEG controls
    - Calculate amount of reagents to mix. Add 10% volume to allow for pipetting error
    - Mix well, avoiding bubbles.
- Mastermix (for qPCR ready mixes)
    - For each reaction calculate the following reagents and combine:
        - 10 µl of 2X qPCR mix
        - 0.6 µl forward primer (10 µM concentration)
        - 0.6 µl reverse primer (10 µM concentration)
        - 0.6 µl Probe
        - 4.8 µl PCR water
- Setup reactions:
    - For NTC reactions, add 4 µL of AE buffer to the empty reaction tube
    - For NEG reactions, add 4 µL of water to the empty reaction tube
    - For experimental reactions, add 4 µL of DNA solution to the empty reaction tubes.
    - Centrifuge all tubes briefly. Visually confirm that all tubes or wells contain sample at the bottom at the correct volume.
    - Carefully aliquot 16 µL of template master mix into each qPCR tube or plate well.
    - Mix reactions well and spin if needed.
    - Cap tubes or seal the PCR plate and label (according to instrument requirements). (Make sure the labelling does not obscure instrument excitation/detection light path.)
- Run samples as per instrument manufacturer recommendations. Examples of standard have been included below:
    - Standard cycling parameters:
        - Initial denaturation 94 °C for 2 min
        - 40 cycles:
            - Denaturation 94 °C for 15 sec
            - Annealing, extension, and read fluorescence 60 °C or 5 °C below lowest primer TM for 1 min
        - (Optional) Hold at 4 °C only if products will be run out on a gel

Add melting curve analysis to the end of the program

- Find out Limits of Detection (LoD) by using the curve fitting approach by (Klymus, et al., 2020) and using their R script in Rstudio.
- Average out Cq values across replicates and input average Cq values and concentration values of the LoD experiment into an excel file to generate a curve (see Figure 1)

- Convert the excel file to CSV format and run the R script (You will have to download the LoD-calculator.R package first which will be shared by the USP team)
- Determine LoD from the R script result and test LoD for confirmation

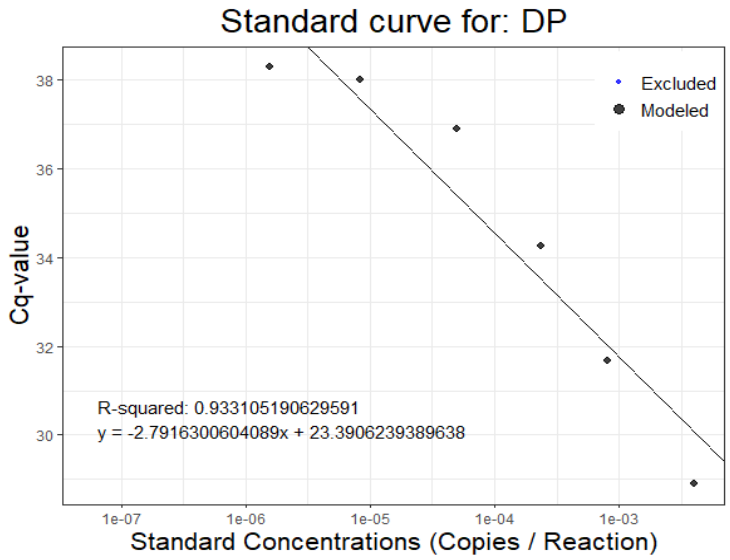

Figure 1. Standard curve of average Cq values generated in an excel

(conversion 10^7 copies per reaction = 10^{-3} ng/uL)

2.2 qPCR protocol

- Required materials:
  - qPCR SYBR Green Mix (IQ™ SYBR® Green Supermix)
  - DNA template - 10 ng to 100 ng gDNA
  - Target species DNA diluted for a standard curve
  - Forward and reverse primers diluted to working concentration (10µM working stocks are sufficient for most assays)
  - Sterile filter pipette tips
  - Sterile 1.5 mL screw-top microcentrifuge tubes
  - PCR tubes, select tubes to match desired format and amount of samples:
    - Individual thin-walled 200 µL PCR tubes
    - PCR strips if available
  - PCR grade water

- **Protocol**
  - Place all reaction components on ice.
  - Mix and then briefly centrifuge to collect contents at the bottom of the tube
  - Prepare enough master mix to run all samples in duplicate, and standard curve.
  - Be sure to include duplicate no template Negative Controls (NTC)
  - Calculate amount of reagents to mix. Add 10% volume to allow for pipetting error
  - Mix well, avoiding bubbles.
- Mastermix (for qPCR ready mixes)
  - For each reaction calculate the following reagents and combine:

        - 10 µl of 2X qPCR mix
        - 0.6 µl forward primer (10 µM concentration)
        - 0.6 µl reverse primer (10 µM concentration)
        - 4.8 µl PCR water
- Setup reactions:
    - For NTC reactions, add 4 µL of water to the empty reaction tube
    - For experimental reactions, add 4 µL of DNA solution to the empty reaction tubes.
    - Centrifuge all tubes briefly. Visually confirm that all tubes or wells contain sample at the bottom at the correct volume.
    - Carefully aliquot 16 µL of template master mix into each qPCR tube or plate well.
    - Mix reactions well and spin if needed.
    - Cap tubes or seal the PCR plate and label (according to instrument requirements). (Make sure the labelling does not obscure instrument excitation/detection light path.)
- Run samples as per instrument manufacturer recommendations. Examples of standard have been included below:
    - Standard cycling parameters:
        - Initial denaturation 94 °C for 2 min
        - 40 cycles:
            - Denaturation 94 °C for 15 sec
            - Annealing, extension, and read fluorescence 55 °C for DP
        - Hold at 4 °C only if products will be run out on a gel
        - Add melting curve analysis to the end of the program

3.0 Results

3.1 Confirmation of LoD assay

As a requirement under the protocol described by Klymus, 2020, limits of detections can be validated through testing controls at corresponding concentrations from R analysis results. Results from the LoD analyses and R analyses revealed that DP LoD is 1.6 X 10^{-6} copies per reaction. A standard with concentration of 0.005ng/ul was included as visible in Figure 2 below as a positive control for the assay.

The LoD was calculated by averaging all Cq values and plotting this data on an excel sheet. Data was transformed to csv format before loaded in LoD-calculator.R package. Commands were executed to run the data and resulting data generated a confirmation result for e.g.

```
For DP, the lowest standard with 95% or greater detection is: 1.6e-06 copies/
reaction. Report as 1.6e-06 copies/reaction.
```

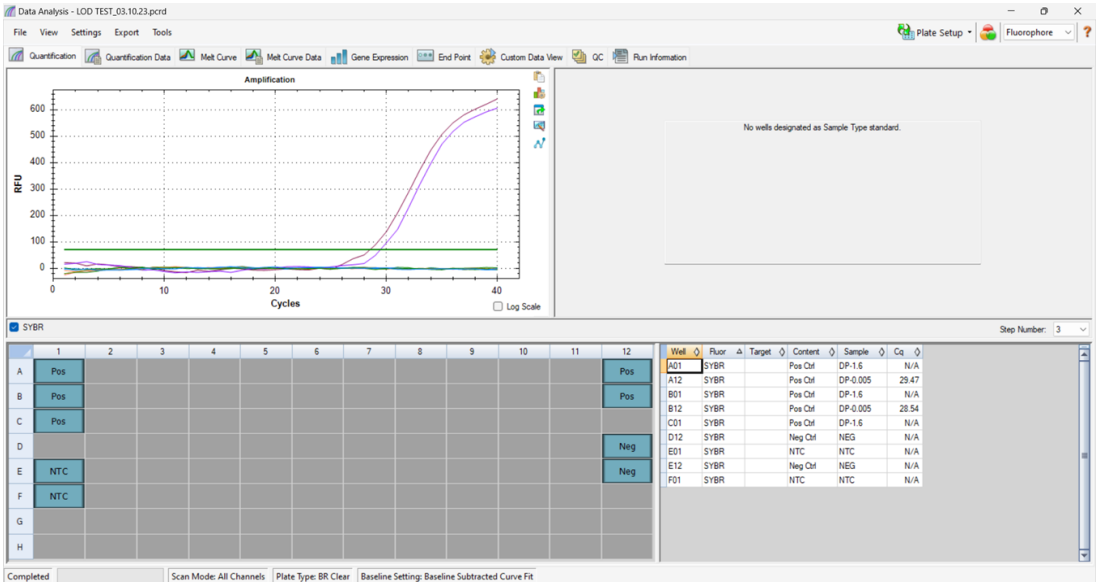

Figure 2. Confirmatory assay for the LoD of Didemnum perlucidum assay

3.2 Testing the positive eDNA detections on DP assay

To confirm eDNA results, respective samples were subjected to qPCR analyses to confirm detections. All samples testing produced positive signals as visible in Figure 3.

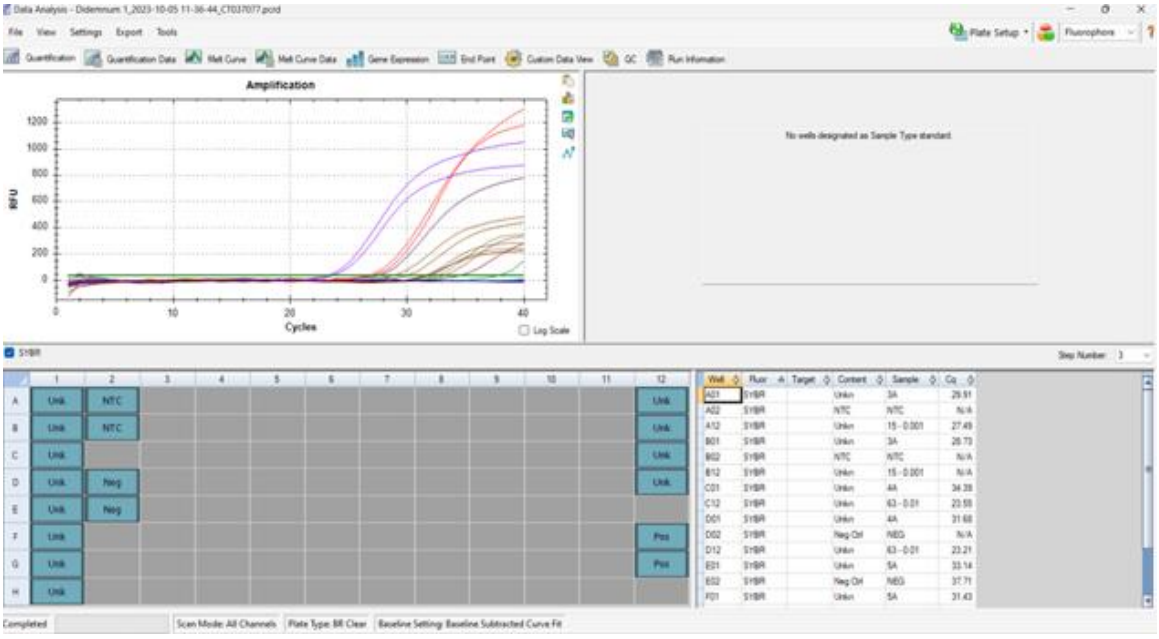

Figure 3. Positive DP detections from eDNA analyses analyzed in qPCR

From the team's analyses of samples which showed positive detections of DP on eDNA analyses, results corroborated eDNA screening data received earlier in the project trial phase. Further

evidence was provided by negative controls and non-template control displaying no signals during the analyses.

3.3 Raw eDNA sample analyses

After a stock take of sample's together which also included remaining volumes, there was a total of 129 eDNA samples which were identified. Factors identified in the shortfall n=7 samples has been attributed to low volumes indicating that these samples contained low DNA concentrations which may have been required more testing during confirmation stages through gel electrophoresis, Qubit and/or Nano drop. These samples may have also been used in the trial optimization of CO1 and 18S PCR assays. It must be noted that at this stage, sample volumes were very much depleted and a reduced sampling size for follow-up assays.

4.0 Acknowledgment

The USP PacMAN team wish to thank the Biosecurity Authority of Fiji for providing their facility and technical support for the development of the PacMAN project Didemnum perlucidum qPCR assays. Many thanks to the Dr. Craig Sherman and Dr. Morgan Ellis for their continued support of the PacMAN project activities.

Appendix 1. Table showing the total samples analysed in the Didemnum perlucidum assay (n=129)

FILE NAME : EDNA_qPCR (31/10/2023)		FILE NAME: qPCR2_EDNA_Didemnum_1/11/2023				FILE NAME: qPCR3_edna_didemnum_1/11/2023			File Name: qPCR_eDNA_Didemnum_2/11/2023
2nd Extraction Box	**31/08/22 BOX**	**28/09/2022 BOX**	**31/08/2022 BOX**	**06/08/2022 BOX**	**1ST EXTRACTION BOX**	**1st EXTRACTION BOX**	**14/09/2022 BOX**	**LAST & 2ND LAST EXTR BOX**	**LAST EXT**
1A	1A	1A	5A	1A	2A	14A	1A	S2S1P3 3/10	p site 1C 4/10
2A	2A	2A	14A	2A	3A	13A	2A	S2S3P1 3/10	p site 1A 4/10
3A	7A	3A	16A	3A	4A	15A	3A	S2S3P2 3/10	p site 1B 4/10
4A	8A	4A	3A	4A	5A		6A	S2S3P3 3/10	p site 2A 4/10
5A	9A	5A	4A	5A	6A		12A	S3S3P2 24/11	p site 2B 4/10
6A	11A	6A		6A	7A		13A	SITE 1C 4/10	p site 2C 4/10
7A	12A	7A		7A	8A		8A	p site 4B 24/11	p site 3A 24/11
8A	13A	8A			9A			p site 4A 24/11	p site 3B 24/11
9A	15A	9A			10A			p site 3C 24/11	p site 4C 24/11
10A	17A	10A			11A			S2S2P3 3/10	site 1A 4/10
11A	18A	11A			12A			S3S2P3 24/11	site 1B 4/10
12A	19A	12A							site 2A 4/10
13A	20A	13A							site 2B 4/10
14A	21A	14A							site 2C 4/10
15A	22A	15A							S3A 24/11
16A		16A							S3B 24/11
17A		17A							S4A 24/11
18A		18A							S4C 24/11
19A		19A							S2S1P1 3/10
20A		20A							S2S1P2 3/10
21A		21A							S1S3P1 3/10
22A		22A							S1S2P3 3/10
23A									S2S2P1 3/10
24A									S3S2P1 24/11
									S3S3P3 24/11
									S3S2P2 24/11
									1B (1st Extraction)
									s4s3p3 24/11

Appendix 2. Table showing run information

File Name	LOD TEST_03.10.23.pcrd
Created By User	admin
Notes	
ID	
Run Started	10/ 02/ 2023 22:45:57 UTC
Run Ended	10/ 03/ 2023 00:35:37 UTC
Sample Vol	20
Lid Temp	105
Protocol File Name	PacMAN P1.prcl
Plate Setup File Name	LOD TEST_03.10.23.pltd
Base Serial Number	CT037077
Optical Head Serial Number	785BR19138
CFX Maestro Version	5.3.022.1030.

Appendix 3. Table showing raw data for qPCR analyses

Well	Fluor	Target	Content	Sample	Melt Temperature	Peak Height	Begin Temperature	End Temperature
A01	SYBR		Pos Ctrl	DP-1.6	None	None	None	None
A12	SYBR		Pos Ctrl	DP-0.005	75.50	77.12	70.50	79.50
B01	SYBR		Pos Ctrl	DP-1.6	None	None	None	None
B12	SYBR		Pos Ctrl	DP-0.005	75.00	88.39	67.50	78.50
C01	SYBR		Pos Ctrl	DP-1.6	None	None	None	None
D12	SYBR		Neg Ctrl	NEG	None	None	None	None
E01	SYBR		NTC	NTC	None	None	None	None
E12	SYBR		Neg Ctrl	NEG	None	None	None	None
F01	SYBR		NTC	NTC	None	None	None	None