United Nations
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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

Final Report on Development of qPCR assay's

Covering the Period from October 1st – December 15, 2023

Date: 25 November, 2023

By

Joape Ginigini and the USP team

PacMAN Local Project Manager (UNESCO Contract No. 4500491484.)

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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 contract covering the period from November 1st to December 15th has been utilized to establish the limits of detection of *Perna viridis* (PV) as well as subject USP allotted samples through established protocols for the PV 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

*Uses the same probe as *Perna Perna*

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

- Completion of the development qPCR assays to test *Didemnum Perlucidum* (DP) and in this report the completion of the development of *Perna Viridis* (PV) assay
- Calculate limits of detection for both DP and PV assays
- Analyze 80% (n = 136) of samples accumulated from 2022-year analyses. This total has been reviewed to cater for vial volumes. The total has again been reduced by 24 due to low volumes inadequate to run an assay. The existing total is now n=112
- Identify the presence of high-risk species in all 112 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) of as well as the analyses of all samples through the DP assay but now look to complete all work including developing the for PV qPCR assay, establishing its LoD and subjecting all samples to the PV assay.

2.0 Protocol's

2.1 LoD development for PV

The establishment of the LoD followed on from developed protocols for DP. An initial series of analyses were conducted to decide on the optimum annealing temperatures, primer and probe combinations as well as cycle length. The amended protocol for the PV assay is as seen below.

- **Protocol**
    - Prepare 8 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)
    - 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 (8 µM concentration)
        - 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 52.5 °C for 1 min

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

We conducted different reactions of 20, 10 and 8 µM of primers and probe. It was then decided that having the reaction of 8 µM probe and 10 µM primers gave the most optimized results.

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 Establishment and Confirmation of LoD assay

The limits of detection for the PV assay was achieved by subjecting the standard through a 7 step 10 fold serial dilution with each dilution replicated to a factor of 10. The curves for all standard concentrations and replicates can be seen in Figure 1. A representative curve for one dilution at 10^{-4} is captured in Figure 2.

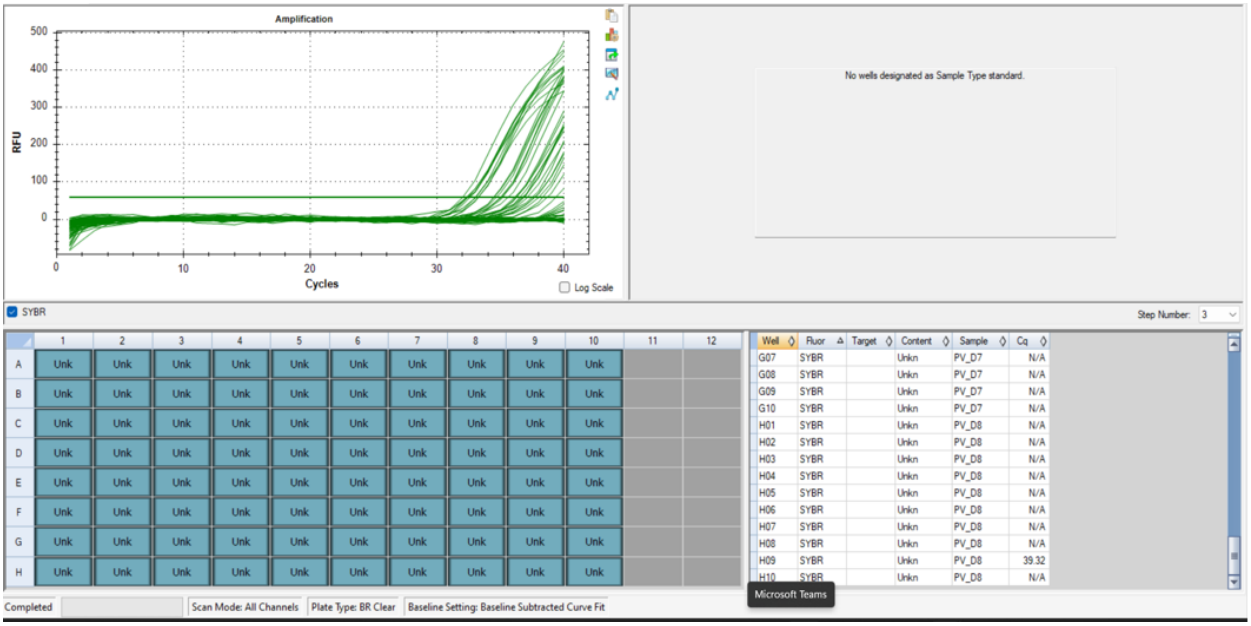

Figure 1. LoD analysis for PV assay. Curve shows all dilutions and replicates

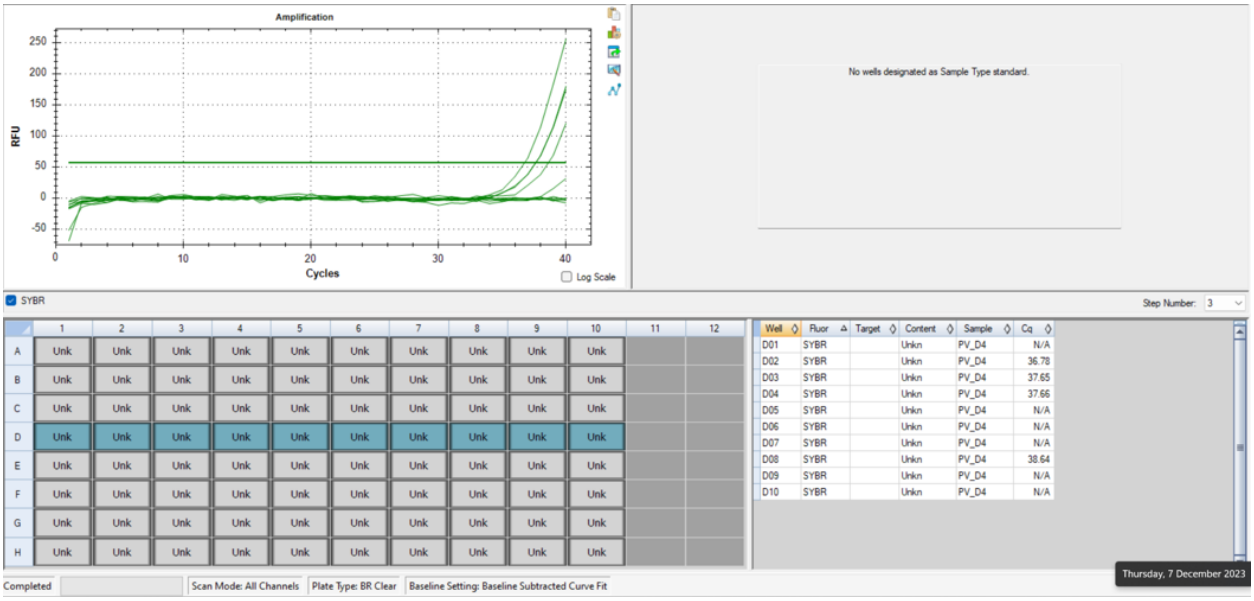

Figure 2. qPCR standard curve showing a single concentration at 1 x 10^{-4}

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 qPCR LoD analyses and follow up R analyses revealed that PV LoD is 6.4 X 10^{-5} ng/µL. A positive control [0.008 ng/ µL] was included and can be seen as red coloured in the curve on Figure 4. Appendix 2 shows sample results from the LoD analysis while Appendix 3 shows average results used to calculate the LoD for the PV assay.

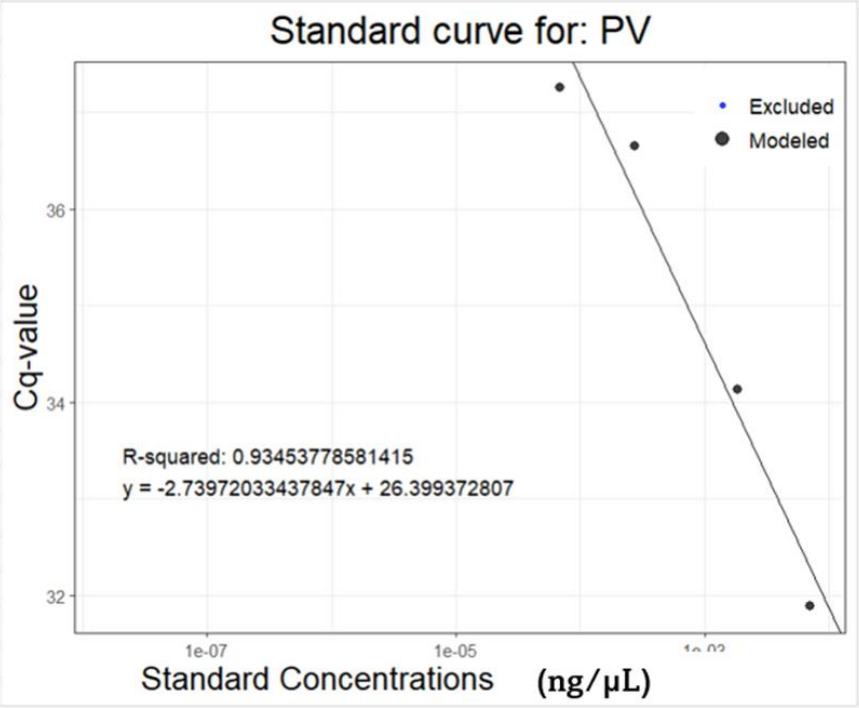

Figure 3. Standard curve of average Cq values generated with the LoD-calculator R package

3.2 Testing eDNA samples on established PV 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 4. Appendix 1 shows the dataset for all samples that were subjected through the PV qPCR assay.

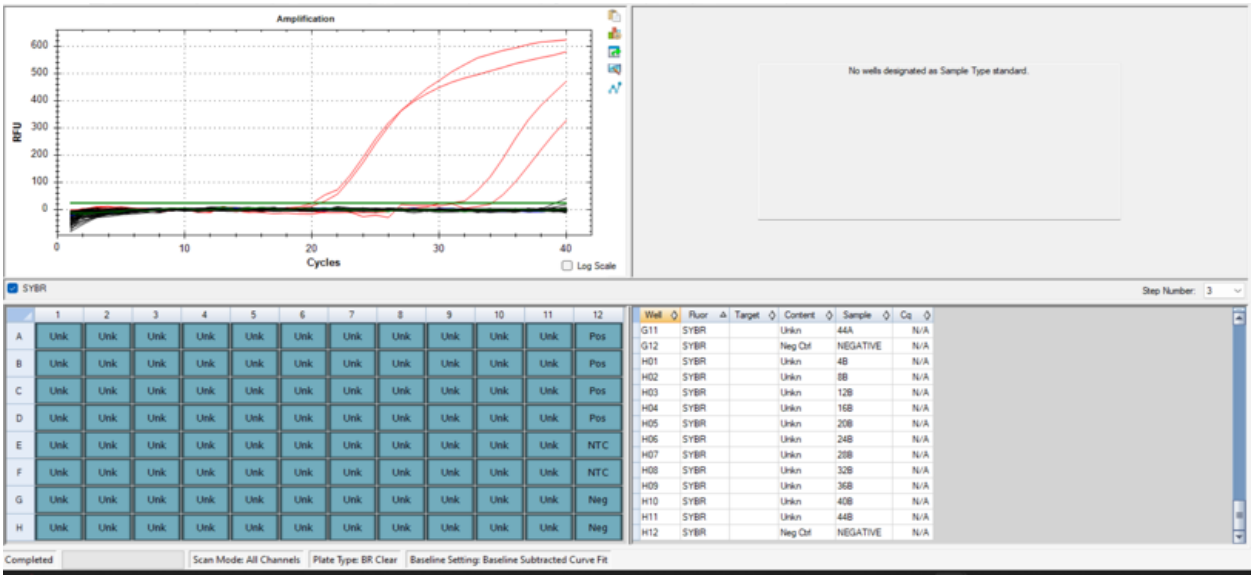

Figure 4. eDNA samples analyses in PV qPCR assay

From the team's analyses of samples which no positive detections for PV 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.

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 assay and the *Perna viridis* qPCR assay. Many thanks to the Dr. Craig Sherman and Dr. Morgan Ellis for their continued support of the PacMAN project activities.

Appendix 1. Table showing all samples subjected to the PV qPCR assay

6/08/2022 Extraction	Project ID	qPCR	1st Extraction 2022	Project ID	qPCR
1A	SUVA3_20211207_PB	NA	2A	SUVA2_20220201_PC	NA
2A	SUVA3_20211207_PA	NA	3A	SUVA1_20220201_PA	NA
3A	SUVA3_20211207_PC	NA	4A	SUVA1_20220201_PA	NA
4A	SUVA4_20211207_PA	NA	5A	SUVA1_20220201_PC	NA
5A	SUVA3_20211207_PB	NA	6A	SUVA2_20220201_PB	NA
6A	SUVA2_20211207_PC	NA	7A	SUVA4_20220216_PC	NA
7A	SUVA4_20211207_PA	NA	8A	SUVA2_20220201_PC	NA
			9A	SUVA4_20220216_PB	NA
			10A	SUVA4_20220216_PA	NA
			11A	SUVA2_20220201_PB	NA
			12A	SUVA2_20220201_PA	NA
			13A		
			14A		
			15A		
22/11/2023 1st qPCR for Perna Viridis (38 samples)					
6/08/2022 Extraction (7samples)					
1st Extraction of 2022 (11 Samples)					
31/08/2022 Extraction (20 Samples)					
23/11/2023 qPCR eDNA for PV (44 Samples)					
2nd Extration of 2022 (24 Samples					
1st Extraction of 2022 (3 Samples)					
14/09/2022 Extraction (7 Samples)					

Last Extration of 2022 (10 Samples)
Second Run of the day (23/11/2023) wih 41 qPCR eDNA Samples
28/09/2022 Extraction (22 Samples)
3/10& 4/10 & 24/11 & 6/08/22 Extraction (19 Samples)

31/08/2022 Extraction	Project ID	qPCR	2nd Extraction 2022	Project ID	qPCR	9/14/2022	Project ID	qPCR
1A	SUVA3_20220620_WA	NA	1A	SUVA1_20211207_WA	NA	1A	SUVA1_20211207_PC	NA
2A	SUVA3_20220620_WC	NA	2A	SUVA1_20211207_WB	NA	2A	SUVA2_20211207_PA	NA
3A	SUVA4_20220620_WB	NA	3A	SUVA1_20211207_WC	NA	3A	SUVA1_20220620_PB	NA
5A	SUVA4_20220620_WA	NA	5A	SUVA2_20211207_WB	NA	6A	SUVA4_20220620_PB	NA
4A	SUVA4_20220620_WC	NA	6A	SUVA2_20211207_WC	NA	8A	SUVA4_20211207_PC	NA
7A	SUVA2_20220201_WC	NA	7A	SUVA3_20211207_WA	NA	12A	SUVA4_20211207_PB	NA
8A	SUVA2_20220201_WC	NA	8A	SUVA3_20211207_WB	NA	13A	SUVA4_20220620_PA	NA
9A	SUVA3_20220620_WB	NA	9A	SUVA3_20211207_WC	NA			
11A	SUVA2_20220201_WA	NA	10A	SUVA4_20211207_WA	NA			
12A	SUVA3_20220216_WC	NA	11A	SUVA4_20211207_WB	NA			
13A	SUVA2_20220216_WD	NA	12A	SUVA2_20211207_WC	NA			
14A	SUVA4_20220216_WB	NA	13A	CONTROL_10/12/2022	NA			
15A	SUVA2_20220525_WA	NA	14A	SUVA1_20220201_WA	NA			
16A	SUVA2_20220525_WB	NA	15A	SUVA1_20220201_WC	NA			
17A	SUVA2_20220525_WC	NA	16A	SUVA2_20220201_WA	NA			
18A	SUVA2_20220525_WB	NA	17A	SUVA2_20220201_WB	NA			
19A	SUVA1_20220525_WA	NA	18A	CONTROL_16/02/2022	NA			
20A	SUVA1_20220525_WB	NA	19A	SUVA3_20220216_WA	NA			
21A	SUVA1_20220525_WC	NA	20A	SUVA3_20220216_WB	NA			
22A	SUVA1_20220525_WB	NA	21A	SUVA3_20220216_WC	NA			
			22A	SUVA4_20220216_WA	NA			

			23A	SUVA4_20220216_WB	NA			
			24A	SUVA4_20220216_WC	NA			

Last Extraction of 2022	Project ID	qPCR	28/09/2022 Extraction	Project ID	qPCR	Last Extraction	Project ID	qPCR
S2S1P3 3/10	SUVA2_20221003_S1C	NA	1A	SUVA1_20220525_S2A	NA	p site1 C 4/10	SUVA1_20221003_PC	NA
S2S3P1 3/10	SUVA2_20221003_S3A	NA	2A	SUVA1_20220525_S3A	NA	p site1 A 4/10	SUVA1_20221003_PA	NA
S2S3P2 3/10	SUVA2_20221003_S3B	NA	3A	SUVA1_20220525_S2B	NA	p site 1B 4/10	SUVA1_20221003_PB	NA
S2S3P3 3/10	SUVA2_20221003_S3C	NA	4A	SUVA1_20220525_S3B	NA	p site 2A 4/10	SUVA2_20221003_PA	NA
S3S3P2 24/11	SUVA3_20221123_S3B	NA	5A	SUVA1_20220525_S2C	NA	p site 2B 4/10	SUVA2_20221003_PB	NA
Site 1C 4/10	SUVA1_20221003_WC	NA	6A	SUVA1_20220525_S3C	NA	p site 2C 4/10	SUVA2_20221003_PC	NA
P SITE4B 24/11	SUVA4_20221123_PB	NA	13B (old label)	SUVA2_20220525_S3A	NA	p site 3B 24/11	SUVA3_20221123_PB	NA
P SITE4A 24/11	SUVA4_20221123_PA	NA	8A	SUVA2_20220525_S2A	NA	site1B 4/10	SUVA1_20221003_WB	NA
P SITE 3C 24/11	SUVA3_20221123_PC	NA	9A	SUVA2_20220525_S2B	NA	site2A 4/10	SUVA2_20221003_WA	NA
			10A	SUVA2_20220525_S3B	NA	site 2C 4/10	SUVA2_20221003_WC	NA
			11A	SUVA2_20220525_S2C	NA	s3-A 24/11	SUVA3_20221123_WA	NA
			12A	SUVA2_20220525_S2C	NA	s2s1p1 3/10	SUVA2_20221003_S1A	NA
			13A	SUVA2_20220525_S3C	NA			
			14A	SUVA2_20220525_S3C	NA			
			15A	SUVA4_20220620_S2B	NA			
			16A	SUVA4_20220620_S2B	NA			
			17A	SUVA3_20220620_S2B	NA			
			19A	SUVA3_20220620_S3C	NA			
			20A	SUVA3_20220620_S2A	NA			
			21A	SUVA3_20221123_S3A	NA			
			31B	SUVA3_20220620_S3B	NA			

Appendix 2. Table showing raw data from LoD analysis of PV on qPCR

DILUTIONS	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	AVERAGE Cq	Concentration
1	31.73	32.21	31.96	31.71	32.49	31.56	31.54	31.58	31.05	31.23	NA	NA	31.75861572	0.008
2	33.94	37.02	34.45	35.07	34.49	35.07	35.66	33.94	33.08	33.16	NA	NA	34.58717637	0.0016
3	NA	37.67	NA	NA	NA	35.88		NA	NA	NA	NA	NA	36.77526427	0.00032
4	NA	NA	39.32	NA	NA	37.23	NA	NA	35.26		NA	38.69	37.26946778	0.000064
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.0000128
6	NA	NA	NA	NA	NA	NA	38.50		NA	NA	NA	NA	38.50474339	0.00000256
7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	38.88		NA	0.000000512
8	NA	NA	NA	NA	NA	39.24	NA	NA	NA	NA	NA	NA	39.24411553	1.024E-07

Appendix 3. Raw data for the LoD calculation on R package

DILUTIONS	AVERAGE Cq	DNA Concentration Sq
1	35.10	0.008
2	36.78	0.0016
3	37.27	0.00032
4	38.69	0.000064
5	NA	0.0000128
6	38.50	0.00000256
7	NA	0.000000512
8	39.24	1.024E-07