Supplement 3. QPX qPCR Assay Detailed Methods Protocol

This file contains a detailed, step-by-step methods protocol for the QPX qPCR assay.

Outline

QPX Monitoring Clam Processing for Histology and qPCR

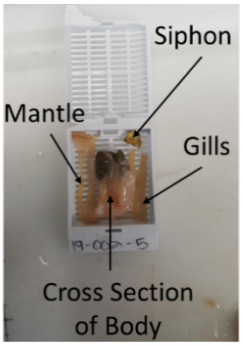
1. Each sampling batch should have an Accession Number (sampling year-sample set; ex. 15-025)
    a. Histo-cassettes and 2 sets of 1.5-2 ml microtubes labeled with accession number and sample number (ex. 15-025-1) must be prepared for each batch of clam samples

2. Wash and scrub clams keeping different batches separate. Label each clam with a number on both sides of the shell, usually #1-30. Measure and record clam length and width (mm) using digital calipers.

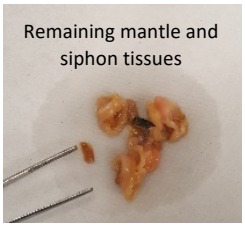
3. Shuck clams; it is usually best to leave them in order to match up histo-cassetes and in case the number wipes off

4. Dissect clams for a thin cross section of gills, mantle, siphon, and body. Do not discard rest of mantle and siphon.
    a. Place tissue in labelled histo-cassettes; place the histo-cassettes in a jar containing 10% buffered formalin and store in hood. Label the jar with the accession number.
    b. Disinfect all instruments (scalpels, forceps, etc.) between samples using water wash, bleach wash, ethanol wash and flame; wipe down cutting board with paper towels

    See Howard et al. 2004 (reference below) pg. 46-48 for additional information on gross anatomy and dissections. A cross-section can be taken or the individual tissues can be excised, as shown in the picture to the left.



5. Dry the rest of the mantle and siphon on a paper towel. Measure the weight of the tissue in a 50 ml falcon tube; record the weight on the tube. If the sample is > 2 g, then switch it to a specimen cup for homogenization.
    a. If there is not enough time to complete processing in one day, stop at this point and store dry tissue tubes/cups at -20°C to complete homogenization the next day



6. Add 10 times the weight of the tissue of 1X phosphate buffered-saline (PBS) and homogenize the sample. Make sure all tissue is homogenized (i.e. there should not be visible large pieces of tissue and should easily be pipetted).
    a. Make sure the homogenizer is clean, disinfect with ethanol and flame.
    b. Between samples, clean with water twice; followed by ethanol and flame; and cool with PBS.

7. Using the large pore tips, pipette out 1 ml and 200 µl aliquots of the homogenate to the labelled microtubes. The 200 µl aliquot will be used for DNA extraction and qPCR. Store the homogenate at -80°C.

NOTES:

- Mantle tissue homogenization can also be performed with a bead mill to expedite processing. Amendments to the above protocol: use 30 ml tubes, add 3 times the weight of the tissue of ice cold 1X PBS, steel beads (1 g of beads for every 1 g of tissue, and Fisherbrand Bead Mill 24 (speed = 4 m/s, cycle time = 20 sec, number of cycles = 2, pause dwell = 3 sec). We have tested this amendment to the protocol in the qPCR and did not find any signs of PCR inhibition.

Reference:

Howard, D. W., E. J. Lewis, B. J. Keller, and C. S. Smith. 2004. Histological techniques for marine bivalve mollusks and crustaceans. NOAA Technical Memorandum NOS NCCOS 5, 218 pp.

Procedure for DNA Extraction for Clam Samples for QPX qPCR

Kit = Genomic DNA from tissue, NucleoSpin Tissue (Macherey-Nagel, Inc. Bethlehem, PA)

Important Notes: DNA should not be extracted near the qPCR mastercycler. It should be prepared in a separate room; if not available, perform extractions in a separate area away from the qPCR. Do not use the same pipettes that will be used for qPCR; if using the same pipettes, disinfect thoroughly with ethanol. If contamination occurs, clean with bleach.

Day 1 – DNA Prep:

1. Defrost (thaw) the 200 µl (20 mg) homogenized clam sample preserved in 1X PBS. If you need to aliquot out the sample be sure to use the large pore pipette tips.
2. Centrifuge the samples for 10 minutes at 12,000 x g (or rcf) to pellet tissue and remove PBS.
3. While waiting turn on heat block on low setting to 56°C – while it warms up, check to make sure the temperature is stable; adjust the temperature setting as necessary.
4. After centrifugation, remove the PBS without disturbing the pelleted tissue (either pour out and blot or remove by pipetting).
5. Add **180 µl of BUFFER T1** and **25 µl of PROTEINASE K** (located in the freezer); re-suspend the tissue pellet and vortex to mix. Make sure the tissue is completely suspended in the lysis solution.
6. Place the sample in the heat block overnight at 56°C, for 16-18 hours. Alternatively, the samples can be incubated for at least 3 hours, while vortexing occasionally to make sure the tissue is lysed.

Day 2 – DNA extraction

1. Remove tubes from the heat block and vortex. Set heat block to high at 70°C and place the **ELUTION BUFFER (BE or EB)** on top of the heat block to warm buffer.
2. Add **200 µl of BUFFER B3** and vortex vigorously. Place tubes in heat block at 70°C for 10 minutes.
3. Prepare collection tubes and spin filter (label on the spin filter cap). Prepare sterile microcentrifuge tubes (must be autoclaved) with full label (accession and sample number i.e. 14-013-1). Save the microcentrifuge tubes for the end to be used for eluting DNA.
4. Remove tubes from the heat block and vortex briefly.
5. Add **210 µl of 100% ETHANOL** (200 proof; reagent grade – located in freezer) and vortex vigorously.
6. Transfer the sample to the spin filter (approximately 650 µl) and spin for 1 minute at 11,000 x g. Discard the flow through and place the spin filter back into the collection tube.
7. Add **500 µl of BUFFER BW** and centrifuge for 1 minute at 11,000 x g. Discard flow through and place spin filter back into collection tube.
8. Add **600 µl of BUFFER B5** and centrifuge for 1 minute at 11,000 x g. Discard flow through and place spin filter back into collection tube.
9. Centrifuge the column for 1 minute at 11,000 x g to dry the silica membrane and remove residual ethanol.
10. Place spin filter in labeled microcentrifuge tubes prepared at Step #4. Add **75 µl of pre-warmed BUFFER BE** and incubate at room temperature for 1-3 minutes.
11. Centrifuge for 1 minute at 11,000 x g. Add another **75 µl of pre-warmed BUFFER BE** and incubate at room temperature for 1 minute and centrifuge for 1 minute at 11,000 x g (modification for high DNA yield).

DNA Quantification using Nanodrop:

12. On the computer, click the ND-1000 icon located on the desktop. Click “Nucleic Acid” and check to see if sample type is on DNA.
13. Open and clean the pedestal with water and kimwipe.
14. Load 2 µl deionized, distilled water onto pedestal and close. Click “Okay.” Open and clean with kimwipe.
15. Load 2 µl BE (elution buffer from above) onto pedestal; close. Click “Blank.” Open and clean with kimwipe.
16. Write the sample ID (i.e. 14-013-1) in box located on the right side of the computer screen. Load 2 µl of sample DNA, close and click “Measure.” Open and wipe clean with kimwipe. Repeat this step for the rest of the samples.
17. After the last sample is measured, click “Show Report.” Save the file in Student Documents with accession no. and sample range (if subset).

18. Be sure to clean the pedestal after the last sample with water and kimwipe.
19. Store extracted DNA samples at -20°C.

Procedure for QPX qPCR Assay for Clam Tissue Samples

Prepare the qPCR plate away from the mastercycler and DNA extraction area, using a separate set of pipettes if available. Change gloves every time after handling QPX gDNA or plasmid standards to avoid contamination. Filter pipette tips should be used and all tubes used should be sterile (autoclaved).

****Disinfection Procedure** (perform every time):

- Disinfect the area with 10% bleach, let sit 20 minutes
- Remove bleach residue with distilled water
- Disinfect the area and all equipment (pipettes, tips, etc.) with 70% ethanol
- Let ethanol fully evaporate before beginning

This procedure is optimized for **Mastercycler realplex4 ep gradient S** (Eppendorf, Hamburg, Germany) and realplex software (version 2.2) using noiseband threshold and automatic baseline setting.

Plates: twin.tec PCR 96 well, semi-skirted, colorless plates (Eppendorf, Hamburg, Germany)
Optical film: TempPlate RT Optical Film (USA Scientific, Ocala, FL)

1. Prepare everything on ice to avoid DNA degradation (or keep in the fridge at 4°C until ready to use). Before using any reagent or sample, be sure that it is fully thawed, vortex to mix, and spin down.
    - Primers (should in 100-200 µl aliquots); synthesized by Integrated DNA Technologies (Coralville, Iowa)
        2 µM Forward = **5.8S24For** = 5'-TTTAGCGATGGATGTCT-3'
        2 µM Reverse = **QPXITS2-R2** = 5'-GCCCACAAACTGCTCTWT-3'
    - **Takyon No Rox SYBR MasterMix dTTP Blue** (should be in 2 ml aliquots; keep on ice, shield from light, wrap in tinfoil/tape)
    - Nuclease-free H_2O; PCR reagent grade (abbreviated at NFH_2O)
    - 50% glycerol; molecular grade, sterile aliquots stored at - 20°C
    - Dimethyl sulfoxide (DMSO) stored at room temperature in the dark
    - Clam Tissue DNA samples; 150 µl sample extracted from 200 µl clam tissue (20 mg) using NucleoSpin Tissue, Genomic DNA Tissue (Macherey-Nagel) stored at -20°C
    - **Inter-run calibrator (IRC)** is QPX gDNA at a concentration of 0.152 ng/µl
2. Record sample layout on plate using **"Clam QPX qPCR Assay Setup"** sheet. All samples must be run in triplicate, including the Negative Control (NTC; no template DNA) and IRC (QPX gDNA). See example plate layout.
3. Prepare Master Mix. The calculations below are for a full plate (leaving 1 column empty on the plate). Always prepare at least 10% more master mix than is needed. Add the Takyon last. Vortex and spin down.

Table S1: Preparation of qPCR mastermix.

REAGENT	AMOUNT (µl)	# Rxns	Total Volume (µl)
2X Takyon SYBR	6.25	X 96	600
NFH_2O	1.875		180
2 µM 5.8S24 For primer	0.625		60
2 µM QPX ITS R2 primer	0.625		60
50% Glycerol	2		192
DMSO	0.125		12
11.5 µl of Master Mix per reaction			

4. Aliquot 120 µl of master mix to each well of an 8-well tube strip for a full plate (reduce aliquot volume depending on plate layout). There should be leftover master mix for the NTC reactions. These will be aliquoted out at the end.
5. Using the multichannel pipette, aliquot out 11.5 µl of master mix to the plate for sample reactions and IRC. Change filter tips after dispensing each time to ensure that the proper amount is dispensed and avoid the production of air bubbles.
6. Vortex the sample DNA. Aliquot out 1 µl of sample template DNA to the designated wells using reverse pipetting to ensure that the sample was dispensed and to avoid the production of air bubbles and aerosols.
7. After all samples have been added, aliquot out 1 µl of the IRC to the designated wells. If running a standard curve with the plate, add plasmid DNA at this point (see QPX qPCR Assay Standard Curve). Again use reverse pipetting.
8. ****CHANGE OUT GLOVES****
9. Aliquot out 12.5 µl of master mix to the designated NTC wells.
10. Cover with optical film; use a kimwipe to secure the film.
11. Disinfect the setup area with 70% ethanol.
12. Centrifuge the plate for 30 seconds at 4°C at 280 x g (make sure the rotor setting is on 115). Check to make sure there are no bubbles; if bubbles are present, centrifuge again for an additional 30 seconds. If not running the plate right away, store in fridge at 4°C until use, but do not store plates for more than 24 hours.
13. Turn on the computer and mastercycler, and open RealPlex software. Log in to Ewelina's account (psd: realtime). Go to File → Open Assay → Select template: **"QPX qPCR Clam Assay_triplicate_no std".** Select semi-skirted and 12.5 µl. Make sure the labels on the plate match the computer (i.e. NTC, IRC samples and replicates).

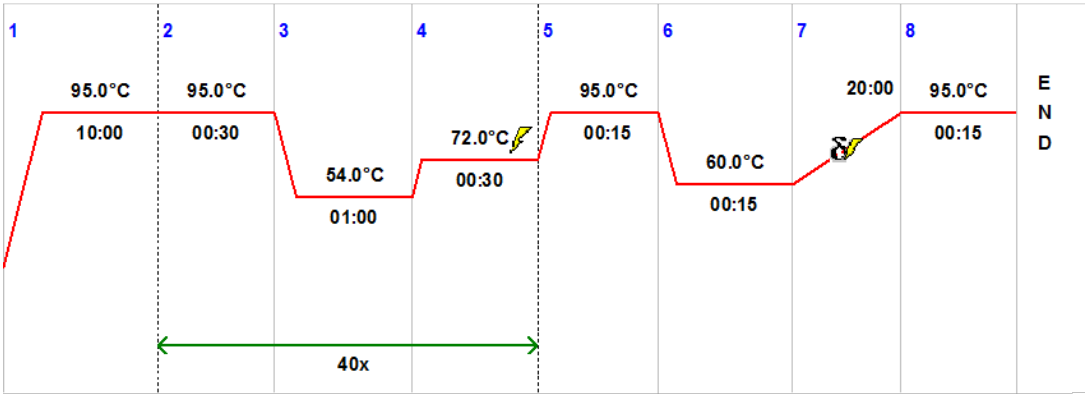


Figure S1: qPCR cycling program. 1 = Pre-denaturation; 2 = Denaturation; 3 = Anneal; 4 = Extension; 5-8 = Melt Curve; the lightning bolt symbol represents a fluorescence measuring point

14. Click the Green Arrow "Play" Button to start. Save assay with date, accession number, and sample numbers. The assay takes about 2.5 hrs. When complete, dispose of plate in waste bin; unless it is needed for gel electrophoresis or sequencing.
15. To save data, go to Edit → Save Results As. Click all samples and make sure everything is checked to export all data. Save the quantification data (i.e. Cq or Ct), make sure the Threshold is on Noiseband and Baseline Settings on Automatic Baseline. For the melt curve (Tm) data, set the threshold to 10% (although you can adjust depending on noise in the data but not > 33% which is the default). Save both as excel files.

EXAMPLE qPCR PLATE SET UP (DNA = QPX gDNA IRC/positive control)

(A) (B)

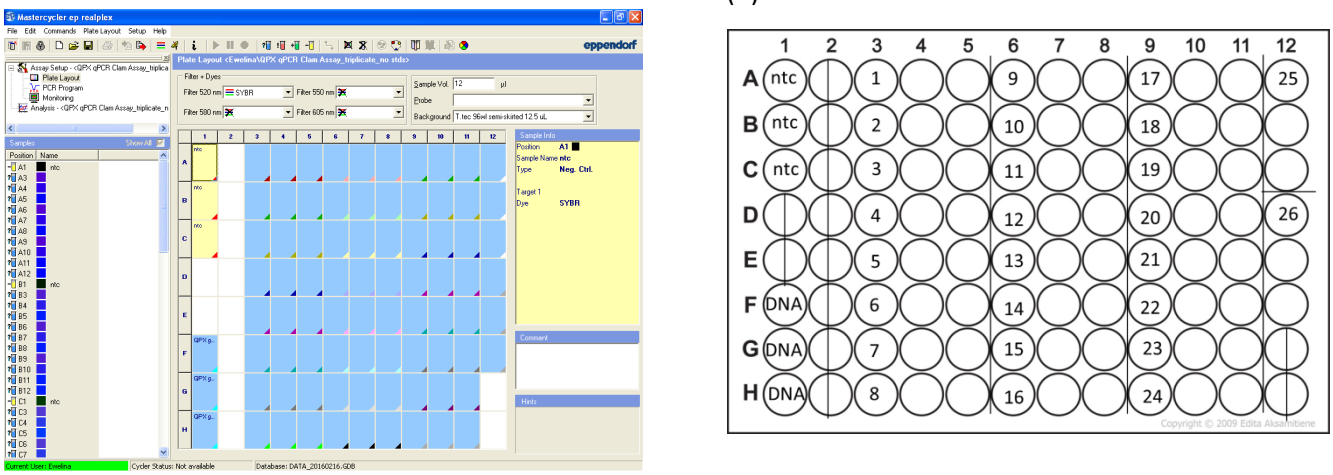


Figure S2: Assay Template on the Realplex software (A) and 96-well plate layout (B).

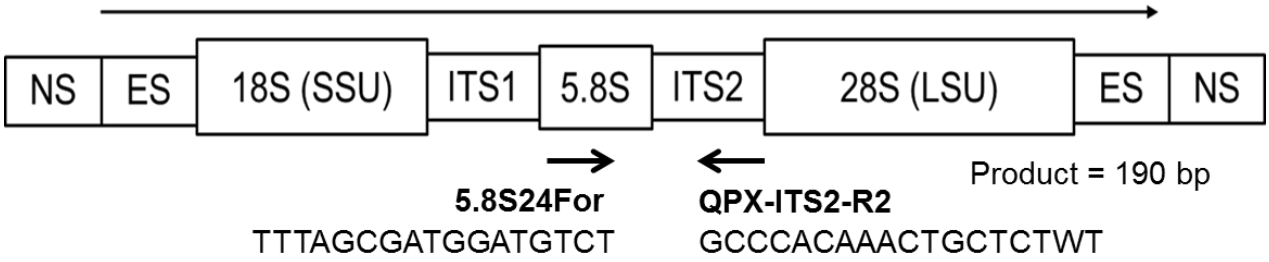


Figure S3: Schematic of the rRNA operon showing primers, respective locations and directions in which they prime. NS: nontranscribed region; ES: external transcribed spacer; SSU: small subunit or 18S rRNA gene; ITS1: internal transcribed spacer 1; 5.8S: 5.8S rRNA gene; ITS2: internal transcribed spacer 2; LSU: large subunit or 28S rRNA gene (modified from Figure 1 in Qian et al. 2007; primers are from Liu et al. 2009).

PCR Primer Preparation Working Stock:
Dilute the new Oligonucleotide (OGN) with 100 µl of sterile ddH_2O or nuclease-free H_2O for the stock solution of primer.

X.0 Amount of OGN (nmole) + 100 µl ddH_2O = X0 µM ← this is the concentration of the primer µM

Dilute to 2 µM for the working stock. Scale up to make several ml of working stock primers. Aliquot out in 100-200 µl fractions. Store at -20°C, prevent multiple freeze-thaw cycles.

Example:
A new OGN that is 32 nmole dissolved in 100 µl of ddH_2O = 320 µM
For a 2 µM stock (or 10X), use 2 µl of 320 µM stock plus 318 µl ddH2O, total volume = 320 µl

QPX qPCR Assay Data Analysis

1. **Check the NTCs.** If they crossed the threshold, the plate is invalid for absolute quantification and should be run again (note: it may be okay to use if there are no QPX Tms in the melt curve). Additionally, if the plate is being used to screen samples to select for histological evaluation, it may be okay, use your judgement about the potential level of contamination shown in the NTCs.

2. **Check the IRC.** The Cq value of the IRC should be between 19-21; however, the Cq of the IRC on all plates should be within a 1 Cq range. For example the Cq of all IRCs from several plates is between 19.5-20.5 or another other 1 Cq interval. If the Cq of the IRC varies significantly from expected, try using a new aliquot of IRC and check its Cq value. If the Cq value remains outside the desired range, then a new batch of IRC should be prepared (note: the stock QPX gDNA concentration should be redetermined by PicoGreen and fresh batch of IRC should be prepared). If the Cq still varies, then a new standard curve must be performed for the new IRC Cq value.

3. **Examine replicate Cq values.** Remove outliers (up to 1 replicate can be removed). The Cq standard deviation must be < 0.5 for at least two reactions to be accepted. If the Cq SD is > 0.5 then the sample is not accepted and must be rerun. If the sample did not cross the threshold (no Cq), then it is negative. Additionally, if two replicates are negative and the third replicate has a Cq < the first standard Cq value, then it is also negative.

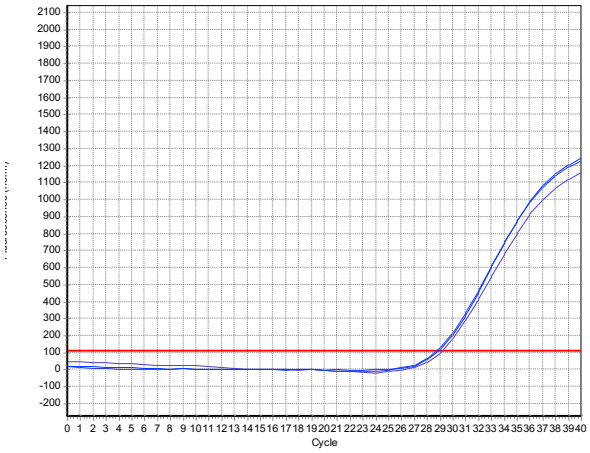
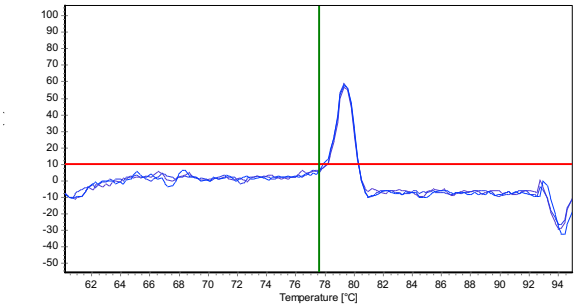


Figure S4: Example of the normalized **Fluorescence** graph of a positive clam sample with 3 replicates.

Cq raw = 28.96 Cq SD = 0.21

4. **Examine replicate Tm values.** The Tm should be 79.5°C (range: 79-80.5). Sometimes a peak may be called but it is noise, this is typically at high and low temperatures. If there are multiple peaks make a note and check the melt curve graph to see if it is noise. If there are true multiple peaks with a QPX peak, the sample is considered to be positive, but cannot be quantified because of the additional peak. This should be rare and if it occurs, run a gel and try to isolate the band and sequence to identify. All samples that cross the threshold (have a Cq value) should have a melt curve peak to be considered positive.



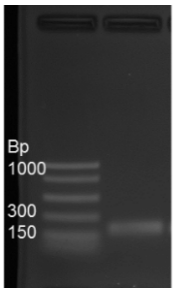


Figure S5: Example of the Melt Curve graph of a positive clam sample showing one peak at 79.5°C with corresponding gel electrophoresis confirms that there is 1 band at 190 bp.

5. For samples that are acceptable, correct the measured Cq value using the IRC to account for inter-plate variations. The standard curve IRC has a Cq value of 19.98, which should be included in the average Cq calibrator determination. The standard curve Cq values should also be corrected and a new equation should be made.

Cq corrected = Cq measured – Cq calibrator + Average Cq calibrators

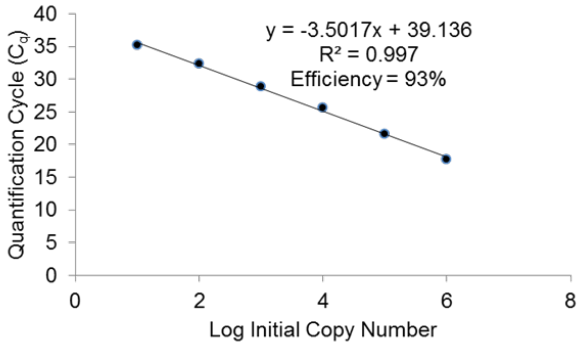
Table S2: Example of plasmid standard curve.

Standard Linear Plasmid	Copy #	Log Initial Copy #	Mean Cq	Cq SD
std1_10	10	**1**	**35.22**	0.5
std2_100	100	**2**	**32.29**	0.31
std3_1000	1000	**3**	**28.8**	0.32
std4_10000	10000	**4**	**25.61**	0.32
std5_100000	100000	**5**	**21.6**	0.18
std6_1E6	1000000	**6**	**17.76**	0.32

6. After Cq correction, graph Log Initial Copy # v. the Cq corrected values of the standard curve to determine the conversion equation to copy number.

QPX ITS copy # = 10 ^ (Cq corrected – Y-intercept) / -3.5017)

Figure 6: Example of the copy number equation determined from the standard curve.



Copy # = 10 ^ (Cq – 39.136) / -3.5017)

NOTE: The slope will not change because IRC calibration maintains the same PCR efficiency. Only the Y-intercept is affected by the calibration.

7. After conversion to copy number, use the following equation to determine QPX cells/mg tissue by multiplying by the DNA elution volume (150 µl) and dividing by the number of ITS copies/mononucleate cell (440) multiplied by the amount of clam tissue in each sample (20 mg).

QPX cells/mg tissue = (QPX ITS copy number x 150 µl) / (440 copies/cell x 20 mg)

8. Samples that cross the threshold and have proper Tm (79.5°C) but have less than 10 QPX ITS copies are denoted as below limit of detection (BLD). They are still considered positive but cannot be quantified.

QPX qPCR ITS LINEAR Plasmid Protocol

1. Transformed pGEM-T easy plasmid vector is stored in glycerol located in the MADL right -80°C freezer. There are several clones.
    a. PCR product = QPX-F and 28S46Rev = ~1505 bp
    b. Note: if transforming new plasmid, see next protocol
2. Make LB broth (sterilize by autoclaving) with ampicillin (100 mg/ml) – use 50 µl per 50 ml LB media. Some should be located in freezer.

3. Use a pipette tip to scrap a little of the frozen plasmid; add pipette tip to 3 ml of media in culture tube. Incubate at 37°C in shaker at ~230 rpm overnight.
    a. If more plasmid needs to be preserved, mix 600 µl culture with 400 µl of sterile 50% glycerol (for 20% glycerol final concentration). Let stand 20-30 minutes and freeze at - 80°C.
4. Purify culture (1.5 ml) using the **Wizard Plus SV Miniprep DNA Purification kit** (Promega). Elute purified plasmid in 100 µl of nuclease free water.
5. Quantify plasmid DNA using PicoGreen DNA quantification kit to determine how much to add to restriction enzyme reaction to linearize plasmid.
6. Use restriction enzyme **XmnI (from NEB, R0194S, time-saver protocol)** to linearize plasmid. Be sure to run a negative control with a small amount of circular plasmid and no enzyme.

Table S3: Restriction enzyme reaction preparation.

Reagents	Amount (µl)
10X NEBuffer	5
Plasmid DNA	~ 1 µg (=1000 ng) Determine the volume to use depending on circular plasmid concentration
Nuclease-free Water	50 – 6 – volume of DNA = amount of water
Enzyme (10u/ul)	1
	Total Volume = 50 µl

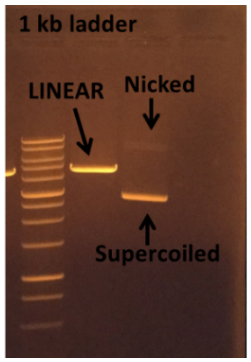


Figure S7: Example of linearized (cut) versus circular plasmid.

7. After adding enzyme, mix by pipetting and microcentrifuge.
8. Incubate at 37°C for 10 minutes.
9. Check linearization of plasmid by gel electrophoresis – 1 band at 4520 bp. Load all of the RE reaction into well.
10. Cut out the band and gel purify using **Wizard SV Gel Purification and PCR Clean Up** (Promega). Elute purified linearized plasmid in 75 µl nuclease-free water.
11. Re-quantify the plasmid using PicoGreen. Convert to # of QPX ITS copies/µl using the following conversion:
    1×10^9 QPX ITS copies = 4.96 ng/µl.
12. Dilute plasmid to 1×10^8 QPX ITS copies/µl and store at - 20°C in **Axygen MAXYMum Recovery microtubes** (Corning, 311-09-051) to minimize degradation.
13. The linearized plasmid is viable for 1 month and should be used to construct the QPX qPCR standard curve when it is fresh. If using old plasmid, you must re-assess the DNA concentration by PicoGreen; however if use of old plasmid results in a suboptimal standard curve, it is advised to make fresh linearized plasmid.

RESOURCES:
pGEM-T easy vector: https://www.promega.com/-/media/files/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol.pdf
Restriction Enzyme: https://www.neb.com/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions
DNA purification kit: http://2010.igem.org/wiki/images/e/e7/Tb225.pdf
Gel Purification kit: https://www.promega.com/-/media/files/resources/protocols/technical-bulletins/101/wizard-sv-gel-and-pcr-clean-up-system-protocol.pdf

For Transforming New Plasmid

1. Extract genomic DNA from QPX culture or if there is some QPX gDNA stored
2. PCR amplification of QPX ITS + partial 28S rRNA fragment using primer pair QPX-F and 28S46Rev
    a. QPX-F = 5'-ATCCTCGGCCTGCTTTTAGTAG-3' (Liu et al. 2009, Stokes et al. 2002)
    b. 28S46Rev = 5'-ATATGCTTAARTTCAGCGGGT-3' (Liu et al. 2009, Van der Auwera et al. 1994)
3. Insert PCR product to p-GEM T easy vector (Promega), follow kit instructions
4. Transform vector to *E.coli* cells and culture cells in a small amount of liquid medium
5. Spread medium culture on medium plate, culture overnight and pick positive colonies
6. Culture picked colonies in liquid medium tubes overnight
7. Purify plasmid DNA from *E. coli* culture using a kit
8. Examine and confirm target fragment using restriction enzyme EcoR1 and gel electrophoresis
9. Send plasmid DNA to sequence and check the length of the fragment

Calculations:

p-GEM T easy vector = 3015 bp

Typical inserted fragment by QPX-F and 28S46Rev = 1500-1508 bp, typically 1505 bp – determined by sequencing the plasmid

Plasmid Vector + Insert = 3015 + 1505 = 4520 bp

Double Stranded DNA (a pair of bases) = 660 dalton

A mole of vectors = $4520 * 660 * 10^9 = 2.9832 \times 10^{15}$ ng

1 copy weight = $(2.9832 \times 10^{15}$ ng) / $(6.02 \times 10^{23}) = 4.96 \times 10^{-9}$ ng → 10^9 copies/µl = 4.96 ng/µl

PicoGreen Protocol for DNA Quantification

Quant-iT PicoGreen dsDNA Reagent and Kits, Invitrogen

1. Stock Picogreen is 200X which must be diluted with 1X TE buffer to 1X (if there is no 1X TE buffer, it must be made from the 20X TE stock).
    a. The stock Picogreen should be thawed and kept out of light because it is light sensitive (photobleaching effect). Be sure to keep cold (in the fridge) and wrapped in tin foil.
    b. The amount of working solution of Picogreen per reaction is 125 µl.
    c. To determine the dilution use the following:

        # of Samples + 1 Blank + 1 extra = # of Reactions

        (# of Reactions x 125 µl) ÷ 200 = Amount of 200X Picogreen needed

        (# of Reactions x 125 µl) – Amount of 200X Picogreen = Amount of 1X TE buffer needed

    For Example: If there are 10 samples plus 1 blank, there are a total of 11 reactions. This should be rounded to 12 reactions to ensure there is enough Picogreen for all samples. Using 12 reactions the following calculations are made to determine the amount of 200X Picogreen and 1X TE buffer needed.

        12 x 125 µl = 1500 µl ÷ 200 = 7.5 µl of 200X Picogreen needed

        1500 – 7.5 = 1492.4 µl of 1X TE buffer needed

2. This is the diluted Picogreen 1X to be used as the working solution. Gently vortex the solution to make sure it is homogenized. Shield from light.

3. Use the 500 µl microtubes and prepare each sample reaction:
    a. First add 124 µl of 1X TE buffer
    b. Then add 1 µl of sample DNA, be sure the sample is thawed and mixed (vortex)
    c. Lastly add 125 µl of 1X Picogreen (working solution)
    d. The blank should be prepared with 125 µl of 1X TE and 125 µl of 1X Picogereen, this should be prepared first before introducing sample DNA into the setup area.
    e. Gently vortex to make sure the reaction mixture is homogenized.

4. Let reactions sit for 5-10 minutes out of light (usually place in a draw in the lab).

5. Transfer 200 µl of each reaction mixture into a cuvette and determine fluorescence using the MiniFluorometer (TBS 380, Turner Biosystems). Be sure that the holder is completely closed and in place before taking a reading.

6. To determine the DNA concentration in ng/µl, subtract out the blank from the sample fluorescence reading and divide the fluorescence reading by 4.

RESOURCE: https://tools.thermofisher.com/content/sfs/manuals/mp07581.pdf

QPX qPCR Assay Standard Curve

Perform the standard curve if a new batch of the IRC is being used (meaning a new extraction of QPX gDNA) or if there is an observed Cq shift, > 1 Cq from the average IRC value or the Cq is outside the desired range. The Cq IRC for this standard curve is 19.98, so it can be used for IRC Cq values between 19-21, within 1 Cq intervals.

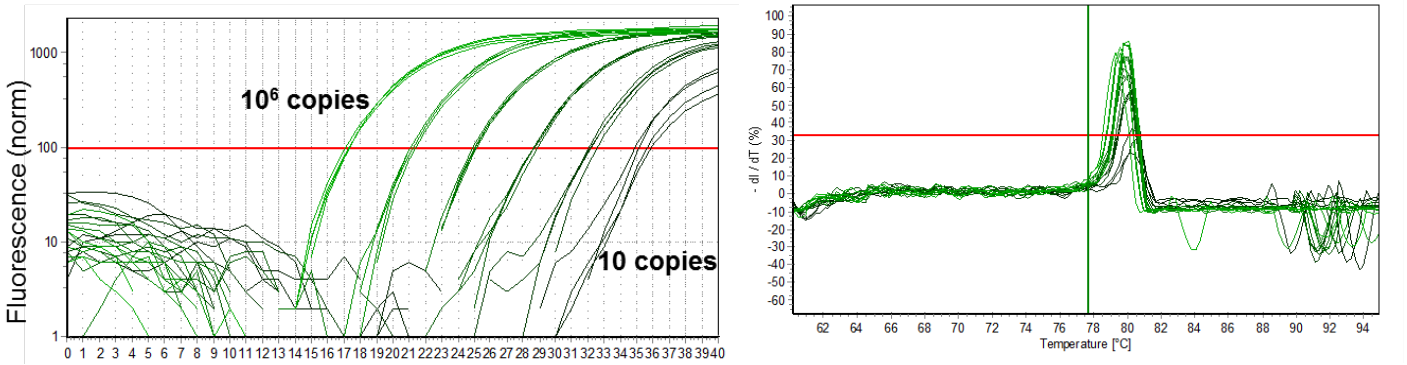


Figure S8: Example of the plasmid standard curve amplification (left) and melt curve plots (right).

- Prepare linear plasmid standard curve from aliquots of 1.0 x 10^8 QPX ITS freshly linearized plasmid. If using old linear plasmid (> 1 month) be sure to re-assess DNA concentration as degradation may alter copy number.

- Label 7 tubes from 10^1 to 10^7. Use 10 µl of 10^8 + 90 µl of NFH_2O to create 1.0 x 10^7. Vortex to mix for 30 seconds and by pipetting at least 10X, spin down. Repeat to make 1:10 serial dilutions to 10 copies.

- Perform at least 3 replicate reactions for each dilution from 10 to 10^6. Be sure to run with the IRC in at least triplicate reactions. The standard curve can be run with samples, but be sure to aliquot the plasmid DNA after dispensing sample DNA.

Inhibition Testing for the QPX qPCR Assay

- Inhibitors can be co-purified with DNA so it is important to test for their presence
    - To test for inhibition, perform a Cq dilution series on a sample that is spiked with cultured QPX to assess PCR amplification efficiency
- If the PCR efficiency of the sample Cq dilution series is within 10% of efficiency of the standard curve (85-105%), then inhibitors are considered minimal and the sample matrix is similar enough to the purified standards to be used for absolute quantification
- Use a representative set of samples; for example by sample type (positive, BLD or negative), site, season or any other aspect
    - From the 1 ml clam tissue homogenate stored at -80°C, aliquot out a new 200 µl sample to spike with QPX cells and perform DNA extraction and qPCR

Procedure:

1. Culture up QPX in Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS)
2. Centrifuge a 2 ml aliquot of culture at 12,000 x g for 10 minutes; remove media
    a. If using a large culture in a falcon tube, centrifuge at 300 x g for 20 minutes.
3. Re-suspend cells in 1-2 ml of 1X PBS
4. Count cells using a hemocytometer
5. Add 10^6 cells to the 200 µl clam tissue homogenate sample
6. Follow DNA extraction procedure in the same way as "unspiked" samples
7. Prepare a 1:10 dilution series in the same way that the standard curve is prepared spanning 6 magnitudes
8. Run in qPCR with triplicate replicates
9. Determine sample amplification efficiency and linearity by graphing Cq v. dilution (6→1)
    a. To convert slope to efficiency: http://www.genomics.agilent.com/biocalculators/calcSlopeEfficiency.jsp

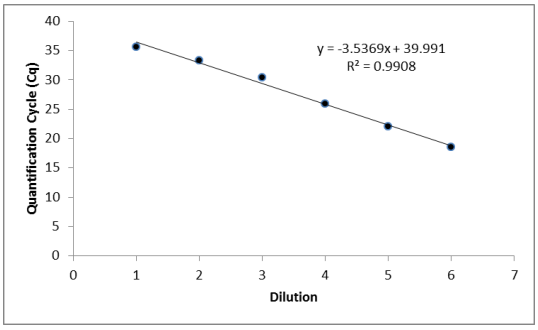


Figure S9: Example testing for PCR inhibition. The presence of inhibition was tested on a sample that was BLD. The efficiency was determined to be 91.75% based on the slope. This is within 10% of the standard curve (E=93%) so we can assume inhibition is minimal.

Recovery of QPX

- The recovery of QPX cells through the DNA extraction kit can be determined by comparing the DNA sample from inhibition testing that is spiked with 10^6 QPX cells to the unspiked sample (original DNA sample)
- Determine QPX cells/sample (not cells/mg tissue)
    - QPX cells/sample = QPX ITS copy number x 150 / 440
        - 150 = elution volume; 440 = ITS copy number/cell
- Determine the recovery rate
    - % DNA recovery rate = (QPX spiked – QPX unspiked / QPX added) x 100
        - QPX spiked = sample from inhibition testing spiked with 10^6 QPX cells
        - QPX unspiked = original DNA sample
        - QPX added = 10^6

Culturing QPX and Media Preparation

1. From stock QPX cultures, use 10% inoculum to Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (pen/strep)
    a. Example: 1 ml culture and 10 ml media
    b. QPX cultures produce a lot of mucus, mix the culture by pipetting to ensure that you get cells and not just media/mucus
2. Preparation of Media, makes 1 L (can scale up or down)
    a. Weigh out the salts (in the table to the left). Dissolve in 800 ml ddH_2O with stirring.
    b. Adjust pH to 7.2 with 1M NaOH. The pH should be acidic around 4.6 to start; add about 1 ml at a time, then slowly add 0.5 ml as the pH increases and the media turns pink.
    c. Add 10 ml of antibiotics (equivalent to 0.08 g or 80,000 µg): 10,000 µg/ml aliquots of streptomycin and penicillin are usually stored in the freezer. Be sure to thaw/mix before use.
    d. Bring the volume of the media up to 1000 ml by adding ddH_2O.
    e. Transfer the media to a sterile filtration unit and filter
    f. Disinfect the hood with ethanol. In the hood, aliquot 40 ml of the media from the collection container of the filtration unit to 50 ml falcon tubes. Store at -20°C.
    g. Before using for QPX culturing, supplement the MEM with 10% FBS. Add 4 ml FBS per 40 ml media.
3. If reduction of mucus is desired, place cultures on a shaker in the dark (covered).

Table S4: QPX medium.

Ingredient	Weight (g)
$CaCl_2 \cdot 2\ H_2O$	1.82
KCl	0.68
$MgCl_2 \cdot 6\ H_2O$	4.36
NaCl	24.26
$MgSO_4 \cdot 7\ H_2O$	3.16
HEPES	5
D+ glucose	0.5
MEM (fridge)	5.1

Counting Cells using a Hemocytometer

1. Make sure the slide and coverslip are clean. Put the coverslip on and load 10 µl of sample on each side. Let the slide sit for at least 10 minutes.
2. The central square millimeter is ruled into 25 groups (known as medium squares), made up of 16 small squares, with each group separated by triple lines, of which the middle line is the boundary for cell counts.
3. **FOR SMALL CELLS:** Count 4 small squares of a medium square. Repeat for 4 other medium squares, for a total of 20 small squares counted on the one side of the slide. Repeat for the other side of the slide. There should be 40 small squares counted.
    a. Take the average of the small square counts and multiply by 16, which is the average cell count for a medium square.
4. **FOR LARGE CELLS:** Count the medium squares and take the average. It is recommended that at least 8 medium squares on each side for a total of 16 medium square counts.
5. A medium square (16 small squares) is equivalent to 0.004 µl. Determine the cell concentration in cells/µl.