cornishblue-qpcr-assays

qPCR Protocol - Genner Group

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Updated for project repo for eDNA in Cornish Blue project, by Molly Kressler, Lucy Whitelegg and Andrew Saxon :: July 2023

Consumables required

- PCRmax Eco48 plate
- PCRmax Eco48 plate seals
- qPCRBIO Probe Mix Lo-ROX
- Species-specific Primers & Probes
- Pipettes and filtered pipette tips (10-20 µL, 200 µL, 1000 µL)
- 1.5 mL microcentrifuge tubes
- Bench-top vortex
- Bench-top tube spinner
- Plastic tube racks
- Plastic sample boxes
- Lab note book
- 10% bleach in squeezy wash bottle
- 70% ethanol in squeezy wash bottle
- Paper towels
- Disposible gloves

Reagents and aliquots required (suppliers and part numbers are presented in Appendix 1):

- [IMPORTANT NOTE] These reagents need to be stored frozen in dedicated boxes at -20°C when not in use, and to avoid cross contamination the eDNA samples and amplification standards need to be stored in separate boxes from the assay reagents and from each other.
- Chai Bio Sahara Hot Start PCR Master Mix
- Nuclease free "ultrapure" water in 1 mL aliquots
- TE buffer in 1 mL aliquots

- Premixed primer+probe (PPM) in 50 µL aliquots
- Assay quantification standards at a one billion copy/µL stock solution with step-dilutions down to 1 copy/microL

Mastermix	Colour
qPCR Bio	Black
Promega	Blue
AmpliTagGold	Green

See Table 4 for a full list of qPCR parts and prices.

Step 1 - Preparing reagents

1. Step 1 must be conducted in the eDNA extraction lab.
2. Clean working surfaces with 70% ethanol, followed by 10% bleach, and then 70% ethanol solution.
3. Remove your probe, primers and qPCR Biosystems mastermix from the freezer.
4. Add distilled water to the tubes your probe and primers were delivered in as instructed on your order form in order to dilute the precipitates to a concentration of 100 nmol/µl.
5. Once the water has been added to your primers and probe, spin them at 13,000 g for 1.5 mins to mix.
6. In a new 1.5 mL tube, add your probe, primers and water to make a Primer Probe Mix (see Table 1).

Table 1. Reagents required to make 50 µl of Primer Probe Mix. Vortex all.

Reagent	Concentration (nmol/µl)	Vol (µl)
Probe	100	2
Forward primer	100	4
Reverse primer	100	4
Distilled water	N/A	40

7. Put the reagents that you're not using back in the freezer.
8. Vortex all primers first. Make a megamix by adding your mastermix, primer probe mix and distilled water together in a 1 mL tube (see Table 2).

Table 2. Reagents required to make the Megamix

Reagent	Vol for 1 reaction (µl)	Vol for 53 reactions (µl)
Mastermix	5	265
Primer Probe Mix	1	53
Distilled water	3	159

9. Take your megamix to the pre-PCR side of the main lab (131).

Step 2 - Loading the qPCR plate

10. Clean working surfaces with 70% ethanol, followed by 10% bleach, and then 70% ethanol solution.
11. Take your sample(s) out of the freezer.
12. Turn the qPCR machine on.
13. Place a qPCR plate into a plate holder and turn the light on.
14. Add 9 µl of megamix to each qPCR well from right to left (see Table 3). Positive control (1µl of the standard) always goes in the top right well, and always added outside the eDNA room, in the qPCR lab. Negative control (distilled water from field) always in the bottom right well.

Table 3. Reagents to be pipetted into each qPCR well

Reagent	Vol (µl)
Megamix	4
Template	1

(skip steps 14, 16-18 from original protocol)
19. In each well, add 9µl of megamix and 1µl of sample. Vortext, and divide each sample (e.g. WBT1.1) into 3 wells.
20. Add 1 µl of distilled water to negative control wells.
21. Gently tap the plate to cause the megamix to fall to the bottom of the wells.
22. Add a plastic seal (without cracks in it) to the top of the qPCR plate making sure that all the sides are tightly sealed by using the grey plastic tool.
23. Vortex the qPCR plate to remove any bubbles that have formed in the bottom of the wells. Bubbles are OK at the top of the wells but not at the bottom.
24. Spin the qPCR plate in the spinner if bubbles persist to remove them.

Step 3 - Running the qPCR

25. Place qPCR plate in the machine while setting up the plate layout on the laptop (to, hopefully, let the bubbles time to burst in the heat). Put the qPCR plate in the qPCR machine.

26. Open the laptop: Eco (not Eco Study) --> file --> open --> select eco.t files in the drop down list.
27. Open EcoStudio and find EcoD file.
28. Press run.

Table 4. qPCR parts and prices

Item	Quantity	Supplier	Part No.	Cost (£)	
OneStep PCR Inhibitor Removal	50	Cambridge Bioscience	D6030	116.00	
PCRmax Eco48 plate seals	50	SLS (Scientific Laboratory Supplies)	MOL6350	33.57	
PCRmax Eco48 plates	50	SLS (Scientific Laboratory Supplies)	MOL6352	30.00	
qPCRBIO Probe Mix Lo-ROX, 500 reactions	500 reactions	Insight Biotechnology	PB20.21-05	132.00	
1.5ml TubeOne Microcentrifuge Tube (Sterile)	5 x 100	StarLab UK Ltd	S1615-5510	14.28	
10/20µl XL Graduated TipOne Filter Tip (Sterile), Filter Refill	10 boxes	StarLab UK Ltd	S1120-3710	36.98	
1000µl XL Graduated TipOne Filter Tip (Sterile), Filter Refill	10 boxes	StarLab UK Ltd	S1122-1730	36.98	
200µl Graduated TipOne Filter Tip (Sterile), Filter Refill	10 boxes	StarLab UK Ltd	S1120-8710	36.98	

Suppliers and part numbers

Type	Item	Supplier	Part no.
equipment	Open qPCR machine	Chai Bio	Single channel
equipment	Pipette starter pack (4x pipettes)	Starlab UK	STARTERPACK42.5
equipment	Vortamix Mini Vortexer	SLS	ARG1878
equipment	Mini Fuge tube spinner	SLS	N2631-0017

Type	Item	Supplier	Part no.
equipment	250 mL wash bottle	SLS	BOT9000
equipment	96-well 1.5 mL microcentrifuge tube rack	Starlab UK	I2396-5048
equipment	96-well 0.2 mL PCR tube rack	Starlab UK	E2396-5240
equipment	81-tube freezer storage box	Starlab UK	I2381-5040
consumable (plastic)	10/20 µL pipette filter tips	Starlab UK	S1120-3710
consumable (plastic)	200 µL pipette filter tips	Starlab UK	S1120-8710
consumable (plastic)	1000 µL pipette filter tips	Starlab UK	S1122-1730
consumable (plastic)	1.5 mL microcentrifuge tube	Starlab UK	S1615-5510
consumable (plastic)	0.1 mL qPCR 8-well tube strips	Chai Bio	S02132B
consumable (reagent)	Chai Bio Sahara Hot Start PCR Master Mix	Chai Bio	R02151M
consumable (reagent)	Nuclease-free ultrapure water	Thermo Fisher Scientific	10977035
consumable (reagent)	qPCR assay primers and probes	Eurofins Genomics	as required
consumable (reagent)	Zymo OneStep PCR Inhibitor Removal kit	Cambridge Bioscience	D6030
consumable (reagent)	TE Buffer	Thermo Fisher Scientific	12090015