

qPCR Protocol - Genner Group

created by Jack A. Greenhalgh :: November 2020

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

Updated by Molly Kressler :: April 2024

Consumables required

- MicroAmp Fast 96-Well Reaction PLate (0.1 mL)
- MicroAmp OPTical Adhesive Film
- qPCRBIo Probe Mix Lo-ROX
- Species-specific Primers & Probes (Eurofins Genomics)
- Pipettes and filtered pipette tips (10-20 µL, 200 µL, 1000 µL)
 - pre-qPCR tips
 - plating tips
- 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
- Disposable gloves
- Samples, in freezer

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

Reagents and aliquots required:

- [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.

- PCR Biosystems aPCR BIO Probe Mix Lo-ROX
- 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

Step 1 - Preparing reagents

1. Step 1 must be conducted in a DNA Clean Space (preferably a dedicated room)
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.
Steps 4 & 5 only need to be done the first time you use the probes and primers
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.

These volumes are for a 10µL total reaction volume, adjust accordingly for greater reaction volumes.

qPCR Probe Mix requires the PPM mix to have a 2:2:1 ratio of primer:primer:probe. So if you scale Table 1, keep this in mind.

You would make one PPM per species, since the Primers and Probes are species specific.

Reagent	Concentration (nmol/µL)	Vol for 50 reactions (µL)	Vol for 96 reactions (µL)
Probe	100	2	3.84
Forward primer	100	4	7.68
Reverse primer	100	4	7.68
Distilled	N/A	40	76.8

Reagent	Concentration (nmol/µl)	Vol for 50 reactions (µl)	Vol for 96 reactions (µL)
water			
Total volume	N/A	50	96

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

These volumes are for a 10µL total reaction volume and a template volume of 1µL, for one reaction and 96 reactions; adjust accordingly for greater reaction volumes.

You would make one Megamix per species, since the Primers and Probes are species specific.

Reagent	Vol for 1 reaction (µl)	Vol for 96 reactions (µl)
Mastermix	5	480
Primer Probe Mix	1	96
Distilled water	3	288

9. Take your megamix to the pre-PCR side of the main lab. If using the ESI labs, you can do either of the following options:

1. Plate on Level 1 lab bench, steps:
 1. Put away all reagents.
 2. Clean workspace with EBE cleaning protocol, including pipettes.
 3. Change gloves.
 4. Retrieve samples from freezer, these will need 10 minutes to defrost.
Only take out the samples you will process immediately.
 5. Retrieve Megamix from freezer.
 6. Get well plate from Project store, get pipette tips labelled for plating.
 7. Proceed to Step 2 Loading the qPCR Plate.

OR

2. Plate upstairs, steps:
 1. Clean pipettes with EBE method on Level 1.
 2. Retrieve samples from freezer, these will need 10 minutes to defrost.
Only take out the samples you will process immediately.
 3. Clean lab bench on Level 2 with EBE method.
 4. Take megamix and plating tips to the area adjacent to the qPCR machine (Level 2), along with the sign indicating active working.

- Remove gloves
5. Retrieve well plate(s) from Project store, get pipette tips labelled for plating.
 6. Proceed to Step 2 loading the qPCR plate, on Level 2.

Step 2 - Loading the qPCR plate

10. Turn the Quant 7 Studio Flex qPCR machine on using the button on the screen.
11. Make sure you have the right size pipette tips and are using the tips labelled 'Plating Tips'
12. Add 9 µl of megamix to each qPCR well from right to left (see Table 3). You can use the same pipette tip for all wells.
13. Pipette control and standards into plate well as follows. Change pipette tip between each well.
 1. Positive controls (1µl of the standard) always goes in the top right well and the well directly below it (A12 & B12).
 2. Negative control (1µL distilled water, NTC) always in the bottom right well (H12).
14. Gently tap the plate to cause the megamix to fall to the bottom of the wells.

Table 3. Reagents to be pipetted into each qPCR well

Reagent	Vol (µl)
Megamix	9
Template	1

15. In all wells except A12, B12, and H12, add 9µl of megamix and 1µl of sample. Before pipetting, vortex each sample. Samples (e.g. WBT1.3) are then replicated into three cells arranged in a column, i.e. A1, B1 and C1 have 9µL of megamix and 1µL of template from WBT1.3. Change pipette tips between EACH well.
16. 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 gently but firmly rubbing your thumb across the plate being extremely careful not to smudge or move the film as this will contaminate the plate.
17. Once you are sure the film is secure, 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. You can also tap the plate gently against the bench top.

Step 3 - Running the qPCR

18. Turn the Quant 7 Studio Flex qPCR machine on using the button on the screen.
19. Hit the eject button and load the plate into side arm, which swings out on the right hand side of the machine. 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).
20. Open the laptop: select New Experiment
21. Name the new experiment using this formula: YYYY-MM-DD-KRESSLER-ASSAY#-Genus-species
 1. Assays are numbered, refer to the plate layout document.
 2. Where a plate is a single species, label accordingly; where the plate is multiple species, label as follows: MIXED-GenusSpecies1-GenusSpecies2-GenusSpecies3...etc
 3. For example, an Engraulis encrasicolus assay (Let's say assay 3) run on 2 April 2024 would be labelled: 2024-04-02-KRESSLER-ASSAY3-Engraulis-encrasicolus.

22. Select relevant options on Experiment Properties tab:

What instrument? QuantStudio 7 Flex

Which block...? Fast 96-Well (0.1mL)

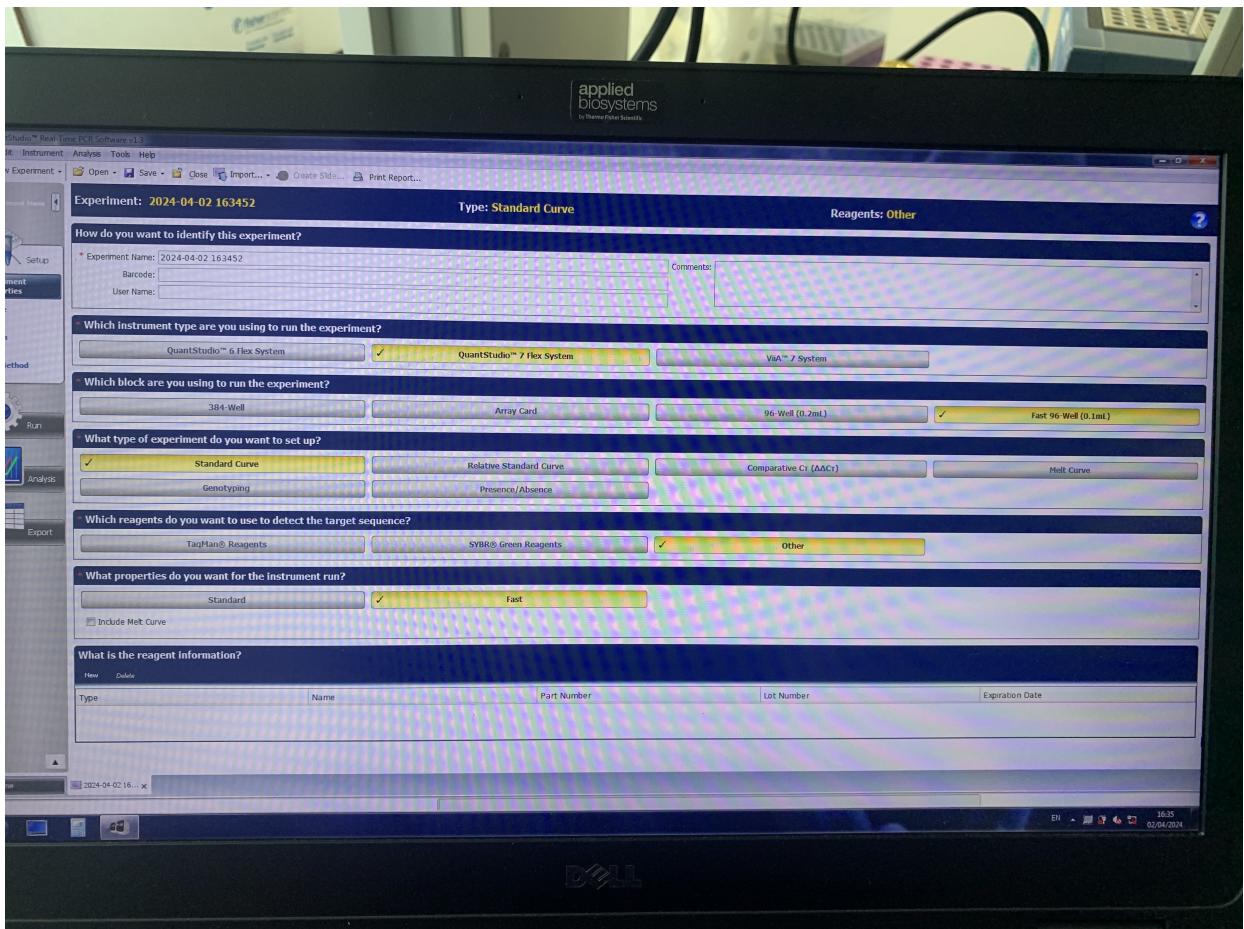
What type of experiment do you want to set up? Standard Curve

Which reagents...? OTHER

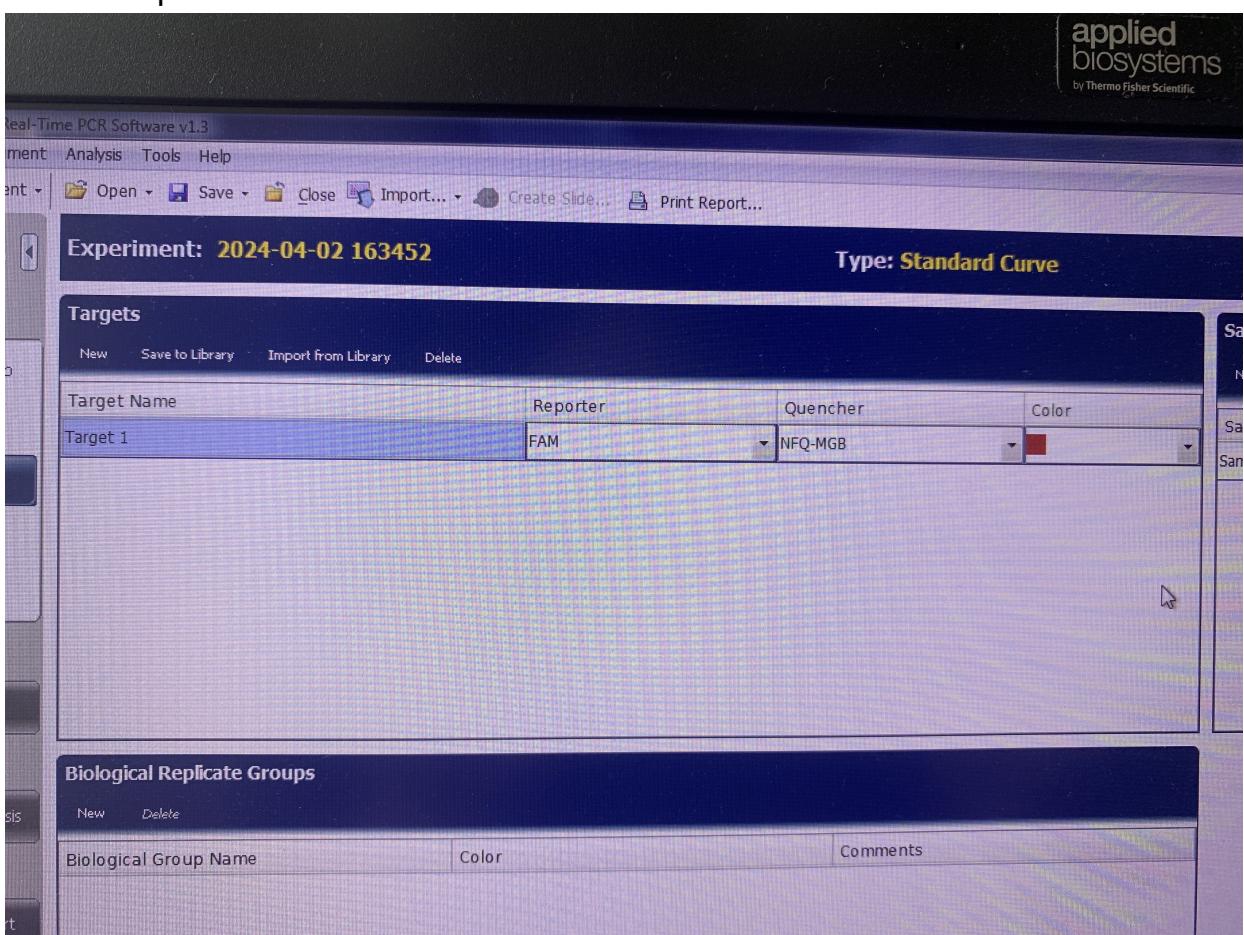
What properties...? Fast

The reagent information may be available still in the machine. But you should

input this if not.



23. Under the Define tab, define your targets and standards. Here, for all wells you need to set the **Reporter to FAM** and the **Quencher to NFQ-MBG**. This is a critical step.



24. Format the plate using the Assign Tab. Label each well as either Unknown, Standard, or NTC. Input sample name into each well (this is tedious but will save you time later). **MAKE SURE you identify that the wells have FAM (non-SYBR) reagents and BHQ**
25. Set thermal conditions (Table 4)

Table 4. Thermal conditions for qPCR with qPCRBIO Probe Mix Lo-Rox

Cycles	Temperature	Time
1	95°C	3 minutes
40 (2 steps)	95°C 60°C to 65°C	5 seconds 20-30 seconds

25. In the Run tab, press run. You may have to 'find' the machine again.
26. If at Step 9 you chose to plate upstairs, clean the work bench using the EBE method. Return consumables to the projects store or to the Lab bench on Level 1, return reagents and aliquots to the freezer.
27. After heating, the machine screen will show an estimated time to completion (typically, this is around 35 minutes).
28. When done, export the Experiment file to the local device > Documents > Experiments > MKRESSLER, as an XLSX file (not xls).
29. Then retrieve the flash drive from Molly's desk, and copy the file to the flash drive.
30. If conducting further assays, return to Level 1 and repeat the protocol.

Table 4. qPCR parts and prices

Item	Quantity	Supplier	Part No.	Cost (£)
Sterile water	1 kg	Fischer Scientific	10245203	37.45
MicroAmp Fast 96-well Reacton plate	10	Fischer Scientific	10670986	38.65
MicroAmp Optical Adhesive Film	100	Fischer Scientific	10299204	133.38
qPCRBIO Probe Mix Lo-ROX, 500 reactions	500 reactions	PCR Biosystems	PB20.21-05	132.00
1.5ml TubeOne Microcentrifuge Tube (Sterile)	5 x 100	StarLab UK Ltd	S1615-5510	14.28
TE Buffer	500mL	Melford	T32200-500	56.25

Item	Quantity	Supplier	Part No.	Cost (£)
Fisherbrand SureOne Filter Tip 0.1-5 µL	10 boxes	Fischer Scientific	11977724	32.43
Fisherbrand SureOne Filter Tip 100 µL	10 boxes	Fischer Scientific	11977724	23.32
Storage Boxes	4 boxes (800 tubes)	Merck	BR114866-4EA	48.80