2-STEP RT-QPCR

Written by: Kevin Zhao
Date: February 2023
Updated by: Aunika Venables
Date: April 2023
Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

BACKGROUND

This protocol is intended as an introduction to qPCR primer operationalization, experimental design, and plate setup. This protocol has been written with the 2009 MIQE guidelines in mind to ensure maximal reliability.[1]

Two-step reverse transcription quantitative polymerase chain reaction, often abbreviated as 2-Step RT-qPCR or simply as qPCR, is a high-throughput assay design that allows the real-time measurement of the expression level of specific genes at the RNA level. qPCR is often broadly categorized into two categories: (1) Taqman probe-based assays or (2) SYBR green-based assays. Taqman probe assays rely on fluorescent probes matched for an amplicon's sequence, are generally more specific, and have better detection of amplification, however, are frequently more expensive. SYBR green-based assays rely on a nonspecific DNA dye that fluoresces upon intercalating between newly-formed DNA strands during amplification. SYBR green assays often demand more care in their initial setup and results interpretation, however, are more cost-efficient and are not amplicon sequence-specific. This protocol was written for SYBR green.

MATERIALS

- GoTaq qPCR Master Mix (Promega, Cat #: A6001)
- CXR Reference Dye (Promega, Cat #: C541A, included in Master Mix kit)
- Forward & reverse primers (10 µM, see primer design protocol)
- Nuclease-free water
- cDNA samples (see cDNA synthesis protocol)
- qPCR Workstation Laminar Flow Hood
- RNaseZap or 10% Bleach

- PCR tubes
- qPCR plate compatible with thermocycler
- StepOnePlus qPCR Thermocycler (Applied Biosystems)

PROTOCOL: Running a qPCR Plate

qPCR should be done with a plan for plate setup, and ideally a plate map with all wells mapped to (1) gene of interest (i.e. target) and (2) cDNA (i.e. sample) measured. Plate setup is discussed in the section following this.

<u>qPCR Station Setup</u>

1. Wipe down the work surface of the qPCR laminar flow hood with RNaseZap or 10% Bleach.
2. Spray down the work surface of the qPCR laminar flow hood with MilliQ water and dry with paper towels.
3. Turn on the HEPA/UV setting on the qPCR laminar flow hood and activate the UV sterilization cycle for 30 minutes.
    a. Take care not to thaw samples near the qPCR hood while it is UV sterilizing to avoid risking sample degradation.

<u>Reaction Setup</u>

1. Thaw the qPCR master mix, CXR reference dye, primers, and cDNA (if cDNA was frozen after cDNA synthesis).

If this is the first time doing qPCR on cDNA after reverse transcription, proceed to step 2, otherwise skip to step 3.

2. Aliquot 10 µL of cDNA into a PCR tube, and dilute 1/10 (100 µL total volume).
    a. Freeze other 10 µL undiluted cDNA at -20°C.
    b. Aliquot 1/10 diluted cDNA into additional aliquots, if several freeze-thaws are anticipated (i.e. if many genes need to be measured using the same set of cDNA samples).
3. Mix all reagents by flicking and spin down to collect.
4. Create a 1x qPCR master mix according to the following recipe:

Reagent	For 1x sample
2x GoTaq qPCR Master Mix	5 µL
Fwd Primer	1 µL
Rev Primer	1 µL
CXR Dye	0.1 µL
Nuclease-free water	0.4 µL

   a. Each gene of interest will require its own 1x qPCR master mix.

5. Pipette 7.5 µL of each target-specific 1x qPCR master mix into each respectively marked-out well on the plate map. Pipette directly into the well such that the master mix remains at the bottom of the qPCR plate well.
6. Pipette 2.5 µL of each cDNA sample into each respectively marked-out well. Pipette cDNA onto the side of the well such that it forms a bead.
   a. Avoid disturbing the plate so that the cDNA beads do not slide into the master mix.
7. Pipette 2.5 µL nuclease-free into the wells marked for no-template control (NTC).
8. Pipette 2.5 µL inter-run control (IRC) cDNA into the respective wells.
9. Repeat steps 5-8 for each 1x qPCR master mix (i.e. each target to be measured on this plate).
   a. Each target requires its own NTC and IRC, with the IRC run in technical duplicate.
10. Spin down the plate at 1,200 g for 1 minute.
    a. Balance with either: (1) a reusable blank 'balance' qPCR plate loaded with water or (2) an old qPCR plate with a similar number of wells containing samples.
11. Load the plate into the qPCR thermocycler with the following (default) thermocycler cycling conditions:

	Temperature (°C)	Time
Holding Stage	95	20s
Cycling Stage (40x)	95	3s
	60	30s; read
Melting Curve Stage	95	15s
	60	60s
	Transition: +0.3	Read
	95	15s; read

12. Open StepOne Software and select "advanced experimental setup".

13. Under the “Experiment properties” tab, select the following options:
    a. “StepOnePlus Instrument (96 Wells)”.
    b. “Quantitation – Comparative CT (ΔΔCT)”.
    c. “SYBR Green Reagents”.
        i. Ensure the “Include Melt Curve” checkbox is ticked.
    d. “Standard (~ 2 hours to complete a run)”.
14. Under the “Plate Setup” tab, name the targets and samples (under the “Define Targets and Samples” tab at the top of the page) and input the plate map (under the “Assign Targets and Samples tab”).
15. Under the “Run Method” tab change the “Reaction Volume Per Well” to 10 µL.

PROTOCOL: Plate Setup Notes

There are two general approaches to qPCR plate setup: (1) a primer maximization strategy and (2) a sample maximization strategy.

A primer maximization strategy seeks to measure all targets of interest on each plate, splitting samples across several plates when necessary. This strategy is most often employed if samples need to be measured on an ongoing basis for quality assurance purposes. This strategy is less preferred, as comparing relative gene expression between samples across plates may introduce plate-effects that can bias results. If a primer-maximization strategy is adopted, it is essential to use a high-quality inter-run control that expresses all targets to a high degree (low CT value) to correct for plate effects.

A sample maximization strategy seeks to measure all samples of interest on each plate, measuring different targets on each plate when necessary. This strategy is generally preferred when using the ΔΔCT method for quantifying relative gene expression between samples. The relative expression of genes between samples can be reliably assessed without plate effects, and gene expression is not typically compared between different targets, rendering irrelevant any plate effects. When employing the sample maximization strategy, it is still important to use a high-quality inter-run control in case any samples fail to amplify.

All qPCR plates must be run in technical duplicate (i.e. each sample being measured twice for the same gene), to assess inter-duplicate variability. High variability can indicate poor pipetting, mixing of master mix, or poor handling of the qPCR plate. Inter-run controls should be run in technical duplicate as well whenever possible, so as to ensure maximal reliability. All qPCR plates must include a no template control (NTC) that contains no cDNA per target measured (i.e. per master mix created), so as to assess for contamination. Each cDNA synthesis reaction's no reverse transcription control should be measured by qPCR for each target of interest, so as to determine whether there is any significant DNA contamination.

PROTOCOL: Primer Validation Notes

Primer efficiency for qPCR must always be validated in practice, as *in silico* predictions frequently fail to hold up to experimental validation.

To validate primers, serially dilute the IRC intended for this primer 1/10, five times to a final volume of 5 µL per each primer pair to be validated. Using the primers to be validated, create sufficient master mix for measuring the 5x serially diluted IRC in technical duplicate, plus one NTC. Each qPCR plate can validate eight primer pairs. The goal of primer efficiency measurements is to confirm that qPCR done with the unvalidated primers can measure changes in cDNA concentration accurately.