Lazzara Lab
Evan Day & Brooke McGirr
Last updated by BAM November 26, 2018

Protocol for qRT-PCR

Lyse cells for mRNA collection

Note: Perform experiment using cells plated in 6-cm culture plates.

1. Clean bench and pipettes with RNase zap, and use only RNase-free tubes, tips, and other consumables.
2. Use the QIAGEN RNeasy kit (Cat# 74104), and follow directions for "Purification of total RNA from Animal Cells using Spin Technology." [See link to user manual posted at Lazzara lab protocols tab online.]
    - For disrupting cells, prepare a mixture of Buffer RLT and 2M DTT, with 20 µL of 2M DTT per 1 mL of Buffer RLT. Use 500 µL of mixture per 6-cm plate, and scrape with RNase zap treated scrappers.
    - To homogenize the lysate, use QIAshredder spin columns (Cat# 79654), by adding the lysate to a QIAshredder spin column and centrifuging for 2 min at full speed. Combine the flow-through with equal volume of 70% ethanol, and proceed with QIAGEN RNeasy kit instructions.
    - If your qPCR primers are likely to detect genomic DNA, perform the optional step in the kit directions to do a DNase digestion. If you design your primers to span two adjacent exons, this is step is not necessary.
3. Nanodrop to determine concentration of RNA. Ideal concentration is > 500 ng/µL.

Reverse Transcription

Protocol for using the Applied Biosciences "High-Capacity cDNA Reverse Transcription Kit" (Cat# 4368814)

1. Clean bench and pipettes with RNase zap, and use only RNase-free tubes, tips, and other consumables.
2. Thaw the kit components on ice. Keep the transcriptase in the freezer and only take it out to use it.
3. Mix up 2X mix (gently) – this is for one reaction:

Component	µL
10X RT Buffer	2.0
25X dNTP mix	0.8
10X RT primers	2.0
transcriptase	1.0
Nuclease-free water	4.2

4. Dilute your RNA to a concentration of 2-5 µg in 10 µL of nuclease-free water. Make sure to reverse transcribe the same amount of RNA across your experimental conditions.
5. Pipette 10 µL 2X master mix into each PCR tube.
6. Pipette 10 µL RNA to each tube to bring your reaction to a total volume of 20 µL. Pipette up and down twice to mix.
7. Seal the tubes, and briefly spin. Keep on ice until ready to put in thermal cycler.
8. Use the correct program on the PCR cycler machine (10 min at 25ºC, 120 min at 37 ºC, 5 min at 85 ºC, forever at 4 ºC).
9. Before proceeding to SYBR green qPCR, add 80 µL water to 20 µL from reaction (if 5 µg of RNA was used). Then, use 2.5 µL of this dilution per PCR reaction. If you reverse transcribe < 5 µg, you can scale the dilution of the final reaction appropriately.

SYBR Green qRT-PCR

Adapted from Michele Wozniak

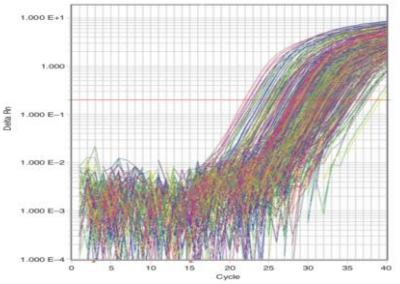
Design Primers:

- Check Lazzara Lab Inventory – qRT-PCR Primers to see the validated primers available in lab.
- If designing new primers, try to find validated primer sequences for your gene of interest in the literature. Confirm the specificity of the primers by entering the forward and reverse primer sequences into NCBI

Primer-Blast. If needed, you can also design your own, using online design programs such as primer3. To avoid detection of genomic DNA, design your primers to span two adjacent exons.

- Ensure the primers meet these criteria:
  - Tm = 58-61°, with 2° difference (or less) between each pair
  - 19-24 bp primer length
  - 100-200 bp amplicon length
  - 45-55% GC content
- In addition to primers for your targets of interest, be sure to include primers for an internal control (housekeeping gene), commonly *GAPDH*. This will be used to normalize the qPCR results.
- Ordering Primers:
  - Order from Thermo Fisher – Invitrogen
    - Account is linked to Molmart
  - Chose Standard Oligos
    - Enter name and oligo sequence (5' to 3')
    - Synthesis scale = 25 nmole
    - Purification = Desalted
    - Formulation = Dry
  - Oligos are shipped to Molmart within a couple days
  - When you receive primers, reconstitute them at 100 µM, based on the nmole value on bottle, and then dilute them to 10 µM for the reaction. Store primers at -20°C.
  - Log primers into Lazzara Lab Inventory – qRT-PCR Primers on GoogleDrive.

- You have to verify experimentally that the primers only detect one product. This can be done in two ways. The first is to add a dissociation stage to qPCR run. This will melt all the products generated. The dissociation graph should only have one peak that corresponds with your single product, as shown on the graph to the right. The other method is to run your finished PCR reaction on an agarose gel and confirm there is only one band.



Schmittgen and Livak. Analyzing real-time PCR data by the comparative C_T method (Nature Protocols, 2008)

Notes for optimizing the reaction:

- You can either run standard curves or use the ddCT method (or another method). The standard curve method requires you create standard curves with all primer sets. This method is good when you are interested in absolute amounts. The ddCT method is easier, but one concern is that the ddCT method assumes equal primer efficiencies for all primers used.
- You may have to try different amounts of cDNA to determine how much you need to use. If the cDNA is obtained from collaborators at an unknown concentration, the starting cDNA amount may need to be optimized. Additionally, you may want to optimize the amount of cDNA based on target abundance.
- This protocol is based on reverse transcribing 5 µg of RNA.
- You can also optimize primer concentrations. Typical protocols call for a range of primer concentrations from 0.2 - 2 µM for each primer. 0.5 µM (the concentration called for below) generally works well.

Setting up the reaction:

Notes:
- Everything should be kept on ice, with limited exposure to light.
- Set up your qPCR reactions in duplicate or triplicate.

1. Prepare a <u>target master mix</u> according to this recipe on a per well basis:

Component	µL per well
2X SYBR green master mix	12.5
Primer 1 (at 10µM)	1.25
Primer 2 (at 10µM)	1.25
Total	15

2. Prepare a template master mix according to this recipe on a per well basis:

Component	µL per well
Diluted cDNA	2.5
Nuclease-free water	7.5
Total	10

3. Add 15 µL of target master mix to each well of that target
4. Add 10 µL of template master mix to each well with that template.
5. Seal the plate with the clear adhesive lid.
6. Spin the plate briefly at max RPM for one minute using plate-holding rotor to get rid of bubbles.

Running reaction:

1. Thermocycling:
    - If using the StepOnePlus thermocycler, follow the directives on the Applied Biosystems software for 2X SYBR green and ddCt method.
    - If using other thermocyclers, consult common users for instructions on running the reactions.
2. Gather the Ct value for each well from the reaction to use for analysis.

Data Analysis:

Analysis approach based on:
Schmittgen and Livak, Analyzing real-time PCR data by the comparative C_T method, Nature Protocols 2008.
Read through this paper prior to performing analysis.

1. Average the Ct values across technical replicates.
2. Subtract the Ct value of the housekeeping gene from the Ct value of the target gene for each biological replicate.
3. Average the resulting value across biological replicates for your biological control condition (i.e., the untreated condition), since you are determining the fold change. This should be done separately for each target gene.
4. For each biological replicate, substrate the value from Step 3 from the value in Step 2.
5. For each biological replicate, take $2^{-(\text{Value from Step 4})}$. This is the value of the fold change per biological replicate. Take the mean and standard deviation across replicates, and plot the resulting values.

Example calculation from Schmittgen and Livak (2008), where *H0XD10* is the gene of interest, and 18S rRNA is the internal control gene used to normalize.

The mean C_T of the *H0XD10* gene in treated and untreated samples was 24.6 and 27.5, respectively. The mean C_T of the 18S rRNA in the treated and untreated samples was 9.9 and 9.8, respectively. What is the fold change in expression of the *H0XD10* gene due to treatment?

$$\begin{aligned}\text{Fold change due to treatment} &= 2^{-\Delta\Delta C_T} \\ &= 2^{-[(24.6-9.9)\ -\ (27.5-9.8)]} \\ &= 8\end{aligned}$$