qRT-PCR Protocol

RNA purification

Collecting RNA Sample

** RNA is very sensitive to environmental RNAses. Bench surfaces must be wiped down with 10% bleach as well as pipettes. Separate tubes, pipette tips, conicals, etc must be used. Separate buffers made with RNAse free water are also required.

1. Add 0.5 mL of bacterial culture to 1 mL of Qiagen Bacterial RNA Protect reagent
2. Vortex well and incubate at room temperature for 5 min
3. Spin down cells at 5,000 x g for 10 min at 4 °C
4. Decant supernatant and dab the open tube on clean paper towels to remove excess liquid
5. Freeze pellet at -80 °C overnight (or longer)

Alternatively, collect cells by centrifugation and snap freeze in liquid nitrogen.

Purifying RNA using the Qiagen RNA mini-spin columns

1. Resuspend pellet in 100 µL of lysis solution containing 1X TE buffer and 1 mg/ml lysozyme (125X lysozyme solutions are in aliquots in the -20 freezer in the RNA reagent box) and vortex
2. Incubate at room temperature for 5 min to lyse cells
3. During lysis, prepare buffer RLT by adding 10 µL beta-mercaptoethanol per 1 ml RLT. You will need 350 µL RLT per sample.
4. After lysis, add 350 µL RLT + BME to each sample and vortex to mix
5. Add 200 µL ethanol to each sample ** DO NOT VORTEX at this step to prevent shearing of genomic DNA
6. Mix the samples by pipetting and add 700 µL to a spin column
7. Spin for 15 sec at max speed and discard flow through
8. Wash column once with 700 µL RW1 (15 sec at max speed)
9. Put spin column in a new 2.0 ml collection tube and wash twice with 500 µL RPE. For the second wash, spin at 8,000 x g for 2 min to completely remove RPE buffer.
10. Transfer column to a clean 1.5 ml collection tube (supplied in kit) and elute RNA in 30 µL RNAse-free water

DNAse treatment and sample cleanup

1. To remove contaminating DNA, add 3.3 µL of RNase-free DNAse buffer to each sample
2. Add 1 µL DNAse
3. Incubate at 37 °C for 30 min
4. Dilute sample to 100 µL with RNase-free water
5. Purify RNA using the procedure above starting from step 4 (skipping the RW1 wash step)

Reverse transcription using the Thermo High Capacity RT kit (-20 freezer)

1. Quantify RNA on the nanodrop
2. Dilute a portion of each sample to a concentration of 50 ng/µL in RNase-free water

3. Prepare the RT master mix as follows:
    a. For each RNA sample you will need 1.5 µL of RT product for each PCR probe (0.5 µL per reaction, with each reaction run in triplicate). So, if we are probing for *ccna_01220*, *ccna_01212*, and *rpoD* (loading control), we would need 4.5 µL (round to 6 µl) of RT product per sample.
    b. If there are 5 samples, we would need a total of 30 µL of RT. The final volume is always lower than expected, so round up (i.e. 40 µL of RT).
    c. Also, it is critical to run a no-RT control to make sure the DNase treatment was effective in removing contaminating DNA. You do not need an -RT control for each probe—one is sufficient. So, in this example with 5 samples, we would need 15 µL of -RT product (each sample run in duplicate)..
    d. The final is a 1:1 ratio of master-mix and RNA. So, for our example, you would need to prepare 20 µL of master-mix for the RT samples and 10 µL of -RT master-mix.

RT Master mix (for 10 µL mix)

10X buffer: 2.0 µL
25X dNTPs: 0.8 µL
10X random primers: 2.0 µL
Reverse transcriptase: 1.0 µL
RNase-free water: 4.2 µL

No-RT control Master mix (for 10 µL mix)

10X buffer: 2.0 µL
25X dNTPs: 0.8 µL
10X random primers: 2.0 µL
Reverse transcriptase: ---
RNase-free water: 5.2 µL

4. Aliquot master-mix into RNase-free PCR tubes (in our example, 3 µL per tube) and add 3 µL of RNA
5. Place tubes in thermal cycler and run the RT program
    a. 25 °C, 10 min
    b. 37 °C, 120 min
    c. 85 °C, 5 min
    d. 4 °C, hold

qPCR with SYBR green

1. Prepare qPCR mastermix for each probe set
2. Each reaction is a total of 10 µL: 9 µL of mastermix and 1 µL RT product
3. Mastermix preparation (per sample)
    a. 5 µL Bio-Rad iTaq Universal Sybr Green mix (refrigerator)
    b. 300 nM forward primer
    c. 300 nM reverse primter
    d. Water to final volume of 9 µL
4. In our example, for *ccna_01220* we would need enough mastermix for 15 samples (5 samples each done in triplicate). For *rpoD*, we would need enough for 25 samples (5 samples, each in triplicate as well as the -RT controls in duplicate).
5. Add 9 µL of mastermix to the wells of a 384-well PCR plate. If you will be loading the RT samples using a multichannel pipette, add the mix to every other well so that the tips line up
6. Dilute the cDNA samples 1:1 in water and add 1 µL to the corresponding wells

7. Seal the plate with clear optical adhesive and spin the plates at 1,000 x g for 1 min
8. Use the rubber “spatula” to fully seal the cover to prevent evaporation
9. Load plate into qPCR machine for analysis