

Protocol for RT-qPCR

Step 1: RNA isolation

Isolate RNA from you cells/tissues using the appropriate technique

- TRIzol for cell lines or organs. Manufacturer's guidelines from ThermoFisher can be followed (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf)
- For samples with a more limited number of cells, a RNA extraction kit can be used. We recommend the Total RNA Mini Kit (Blood/Cultured Cell) from Geneaid (cat. RB300). To extract RNA from primary pituitary cells, we used a slightly modified protocol from the manufacturer's (original protocol can be found here (<http://www.geneaid.com/sites/default/files/RB11.pdf>)).

- 1) Aspirate culture medium
- 2) Add 200 μ L of RB buffer solution containing 1% β -mercaptoethanol
- 3) Pass through a syringe (21G) to disrupt cells and homogenize
- 4) Transfer the contents to a RNase-free tube
- 5) Add another 200 μ L of RB buffer solution containing 1% β -mercaptoethanol
- 6) Pass through a syringe (21G) to disrupt cells and homogenize
- 7) Transfer the contents to the previous tube (total 400 μ L of RB buffer 1% β -mercaptoethanol per well)
- 8) Add 400 μ L of 70% ethanol prepared in RNase- and DNase-free water to each sample
- 9) Pipet up and down until the solution is homogeneous
- 10) Place a RB column in a 2 mL collection tube
- 11) Transfer 500 μ L of the mixture to the RB column and centrifuge 14-16,000 x g for 1 min
- 12) Discard the flow-through and transfer the remaining mixture to the same RB column
- 13) Centrifuge at 14-16,000 x g for 1 min
- 14) Discard the flow-through and place the RB column in a new 2 mL collection tube
- 15) Add 400 μ L of W1 buffer into the RB column and centrifuge at 14-16,000 x g for 30 s
- 16) Discard the flow-through and place the RB column back in the 2 mL collection tube
- 17) Add 600 μ L of Wash buffer (ethanol added) into the RB column and centrifuge at 14-16,000 x g for 30 s
- 18) Discard the flow-through and place the RB column back in the 2 mL collection tube
- 19) Add 600 μ L of Wash buffer (ethanol added) into the RB column and centrifuge at 14-16,000 x g for 30 s
- 20) Discard the flow-through and place the RB column back in the 2 mL collection tube
- 21) Centrifuge at 14-16,000 x g for 3 min to dry the matrix
- 22) Place the dried column in a clean 1.5 mL tube (RNase-free).
- 23) Add 25 μ L RNase-free water into the center of the matrix
- 24) Let stand for 5-10 min at 55°C
- 25) Centrifuge at 14-16,000 x g for 1 min to elute the purified RNA
- 26) Store the RNA at -80°C

Step 2: reverse transcription

Measure the amount of RNA contained in each sample (e.g. Nanodrop). For the following protocol, up to 1 µg of RNA can be reverse transcribed. However, we have reverse transcribed as little as 75 ng of RNA and obtained good quality results.

- 1) Aliquot the desired amount of RNA (we recommend 100 ng) in strip tubes [Corning PCR-0208-CP-C]
- 2) Add RNase-free water until 23 µL
- 3) Prepare master mix #1, with each tube receiving
 - a. 6 µL M-MLV Reverse Transcriptase 5X Reaction Buffer [Promega M531A]
 - b. 1 µL RQ1 DNase (RNase-free) [Promega M6101]

Note: 1 unit of DNase should be used for 1 µg of RNA. The solution is at a concentration of 1 unit/µL.

- 4) Add 7 µL of the master mix #1 to each tube
- 5) Incubate 30 min at 37°C
- 6) Prepare master mix #2, with each tube receiving
 - a. 3 µL RNase-free water
 - b. 2 µL random primers (random hexadeoxynucleotides) of a concentration of 50 ng/µL (diluted 1:10 from the stock) [Promega C1181]
- 7) Add 5 µL of the master mix #2 to each tube
- 8) Incubate 10 min at 70°C (random primer annealing and DNase inactivation)
- 9) Incubate 2 min on ice
- 10) Prepare master mix #3, with each tube receiving
 - a. 2 µL M-MLV Reverse Transcriptase 5X Reaction Buffer
 - b. 2 µL dNTPs (from a solution of 10 mM of each dNTP) [Wisent 800-401-TL]
 - c. 0.25 µL M-MLV Reverse Transcriptase [Promega M1701]
 - d. 0.5 µL recombinant RNasin ribonuclease inhibitor [Promega N2511]
 - e. 0.25 µL of RNase- and DNase-free water

Note: 200 units of M-MLV reverse transcriptase enzyme should be used for 1 µg of RNA. The solution is at a concentration of 200 units/µL.

- 11) Add 5 µL of the master mix #3 to each tube
- 12) Incubate 10 min at 23°C
- 13) Incubate 60 min at 37°C
- 14) Incubate 5 min at 70°C
- 15) Store the cDNA at -20°C

Step 3: qPCR

Samples should always be run in duplicates or triplicates. This protocol has been validated and used on RotorGene 6000 machine (Corbett Life Sciences)

Important:

A) Ensure to use a dedicated set of pipets. The working bench/area and all tools used should be cleaned with Eliminase [Decon labs 1102] prior to the start of the experiment.

B) All qPCR primers used should be extensively validated. This includes:

- checking primer efficiency (by doing a standard curve → one can use a cDNA sample and run a qPCR with 1:1, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions)
- checking that a single peak is obtained on the melting curve
- running the qPCR product on a gel → a single band should be seen at the appropriate length
- sending the qPCR product for sequencing and confirming that the appropriate target was amplified

- 1) Prepare the master mix, with each qPCR tube receiving
 - a. 0.2 µL forward primer (from a 20 µM stock)
 - b. 0.2 µL reverse primer (from a 20 µM stock)
 - c. 10 µL EvaGreen mix 2X [Diamed, MasterMix-S]
 - d. RNase-free water

The amount of water to add depends on how much sample volume will be added. The final volume in each qPCR tube should be 20 µL for the 72-well rotor (other rotors are available, ensure to use the appropriate volume/condition for each by referring to the manufacturer's guidelines

http://corbettlifescience.com/control-Rotor-Gene_6000_6-0196.html). For highly expressed genes, 1 µL of sample can be used per tube (i.e. 8.6 µL water); for less expressed genes, use higher volumes [we have worked with volumes up to 5 µL, yielding satisfactory results].

- 2) Make sure the RotorGene tube holder is cooled down (leave it in the fridge)
- 3) Place your tube strips (Axygen® 0.1mL Polypropylene PCR Tube [Corning PCR-0104-C) in the holder
- 4) Add the appropriate amount of master mix in each tube
- 5) Add your samples (final volume in each tube: 20 µL), changing tip for every tube
- 6) Close the tubes with the lids provided (Axygen® 0.1mL Polypropylene PCR Tube [Corning PCR-0104-C)
- 7) Place the tubes in the RotorGene 6000 machine
- 8) For optimal results, use the following cycling program
 - 95°C for 10 min (enzyme activation)
 - 95°C for 15 s (denaturation)
 - 60°C for 60 s (annealing/extension)

Repeat denaturation and annealing for a minimum of 35 cycles. More cycles should be used if the target has a low expression. Always perform a melting curve at the end of the program.