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Gene Expression Analysis by RT-qPCR

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We use this protocol and it's working

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Abstract

This protocol details the complete workflow for quantifying gene expression, beginning with total RNA isolation from cells or tissues, followed by reverse transcription to synthesize cDNA, and concluding with quantitative real-time PCR (qPCR) analysis.

Total RNA extraction



- 1 Important Note: During the whole procedure, always use RNase- and DNase-free supplies (barrier tips, tubes, water), and wear gloves, mask, and lab coat. RNA is fragile and Nuclease is everywhere including your skin, hair, dust, etc. Change gloves frequently, use disposable materials. You can use RNaseZap RNase Decontamination Solution (Thermo, Cat# AM9780) to remove RNase contamination from work surfaces.
- 2 Sample Preparation - Lung Tissue
 - 2.1 Harvest the tissues. If you want to save the tissues and do the RNA extraction later, save the tissues in RNeasy Lysis Solution. For cells, you can save the cell pellets (without solution) at -80°C.
 - 2.2 Weight the fresh tissues or tissues stored in RNeasy Lysis Solution and put tissues to the 5 beads-contained screw-top tube.
 - 2.3 Add 1 mL of TRIzol Reagent (cold) to 50 - 100 mg of tissue.

Try to use the same lobe of lung tissues for experiments.
30 to 50 mg of lung tissue is enough to perform Real-Time qPCR.
I use the 1 mL TRIzol Reagent : 100 mg of tissue ratio.
 - 2.4 Place the tissue-TRIzol Reagent on ice.
 - 2.5 Beads mill homogenization (BR4, Omni).

Speed: 3
Time: 60 sec
Pause: >60 sec on ice between cycles
Cycle: 2 cycles
 - 2.6 Transfer the lysed samples to E-tube.
 - 2.7 Centrifuge at 12,000 g, 4°C, 5 min
 - 2.8 Transfer supernatant to new E-tube. Do not disturb the pellet. You can save the samples at <-20°C



3 Sample Preparation - Cells

- 3.1 Remove growth media and wash with 1x PBS once
- 3.2 Add TRIzol Reagent directly to the culture dish to lyse the cells
HMVEL-L (at 90 – 100% confluence)
100 mm dish: 2 mL, 60 mm dish: 1 mL, 6-well plate: 500 μ L, 12-well plate: 300 μ L
- 3.3 Pipette up and down several times to lysis the cells.
- 3.4 Transfer the lysed samples to E-tube. Incubate at room temperature for 30 min (vortex samples every 10 min; total 4 times).
- 3.5 Centrifuge at 12,000 g, 4°C, 5 min
- 3.6 Transfer supernatant to new E-tube. Do not disturb the pellet. You can save the samples at $<-20^{\circ}\text{C}$

RNA Preparation

- 4 The protocol is modified based on the Direct-zol RNA Mini Kits manual. For details, see the original manual. Perform all steps at room temperature, unless specified.
- 5 Add an equal volume of ethanol (95 – 100%) to supernatant and mix thoroughly.
- 6 Transfer the mixture into a Zymo-Spin IC Column in a Collection Tube.
- 7 Centrifuge at 12,000 g, room temperature, 1 min.
- 8 Transfer the column into a new collection tube and discard the flow-through.



- 9 Add 400 μ L RNA Wash Buffer to the column and centrifuge at 12,000 g, room temperature, 1 min
- 10 (for Mini Kit) In an RNase-free tube, add 5 μ L DNase I and 75 μ L DNA Digestion Buffer and mix by gentle inversion.
- 11 Add the mix directly to the column matrix.
- 12 Incubate at room temperature for 15 min
- 13 Add 400 μ L Direct-zol RNA PreWash to the column and centrifuge at 12,000 g, room temperature, 1 min.
- 14 Discard the flow-through and repeat the PreWash step.
- 15 Add 700 μ L RNA Wash Buffer to the column and centrifuge at 12,000 g, room temperature, 2 min
- 16 Transfer the column carefully into an RNase-free tube.
- 17 To elute RNA, add 50 μ L of DNase/RNase-Free Water (you can warm the water to 37°C to increase yield) directly to the column matrix and centrifuge at 12,000 g, room temperature, 2 min

RNA Analysis

- 18 Measure the absorbance of RNA sample at 260 nm (RNA), 280 nm (DNA), and 230 nm (solvent).
Use the DNase/RNase-Free Water as blank
Use 2 μ L/droplet
- 19 Calculate RNA amount and check the purity of RNA
RNA concentration (ug/mL) = (OD₂₆₀) x (dilution factor) x 40
E.g., if OD₂₆₀ is 0.08, your RNA concentration is $0.08 \times 1 \times 40 = 3.2$ ug/mL
260/230 ratio: A ratio between 2.0 – 2.2 is considered pure.
260/280 ratio: A ratio of 1.7 – 2.0 is generally accepted.

cDNA Synthesis

- 20 The protocol is modified based on the iScript cDNA Synthesis Kit manual. For details, please see the original manual.
- 21 Decide the concentration of RNA template based on your RNA sample amount (ratio: 100 fg – 1 ug total RNA for 20 µL Volume of Reaction). I usually use 500 - 1000 ng total RNA for 20 µL Volume of Reaction.
- 22 Prepare the iScript mixture (this is based on 20 µL volume of Reaction).
5x iScript Reaction Mix (µL): 4 x (sample number + 2)
iScript Reverse Transcriptase: 1 x (sample number + 2)
- 23 Prepare an 8-strip qPCR tube and label the sample number
- 24 Add RNA sample (same amount among the sample), 5 µL of iScript mixture, and fill up to the 20 µL with
Nuclease-free water. Briefly spin down
- 25 Run a thermal cycler using the following protocol
5 min at 25°C
20 min at 46°C
1 min at 95°C
Hold at 4°C
- 26 You can save the cDNA at 4°C (do not freeze)
Youwei Chen (ywc@duke.edu) - Mar 21, 2022, 4:19 PM EDT
Sojin

qPCR - Running

- 27 Primer preparation

You can purchase the predesigned primers from many companies including IDT. Make sure you select the correct species (human = homo sapiens, mouse = mus musculus).

If you want to order the custom primer, check your sequence using Primer-BLAST.

Enter the forward and reverse primer

Select the organism

"Get Primers"

Results

Check primers target your interest gene.

Typical qPCR amplicons (product length) are 70 – 200 bp in length



If you target the specific exon region, you need to check it from the PubMed gene.

- 28 Prepare SsoFast mixture as below
SsoFast EvaGreen Supermix (μL): $5 \mu\text{L} \times (\text{sample number} + 2)$
Forward primers (μL ; $20 \mu\text{M}$): $0.5 \mu\text{L} \times (\text{sample number} + 2)$
Reverse primers (μL ; $20 \mu\text{M}$): $0.5 \mu\text{L} \times (\text{sample number} + 2)$
DNase free water: $1 - 3 \mu\text{L}$
The volume of DNase-free water can be changed based on your cDNA concentration.
- 29 Add $7 \mu\text{L}$ of SsoFast mixture to each 96-well CFX plate.
- 30 Add $1 - 3 \mu\text{L}$ of cDNA template to each well; final volume per well should be $10 \mu\text{L}$ (final concentration: $10 \text{ fg} - 10 \text{ ng}$)
- 31 Run qPCR. Always include Melt Curve Analysis
After running, always check Melt Curve. If your Melt Curve doesn't have a sharp peak or more than one peak, you need to optimize your primers or thermal cycling protocol.

Analyze the qPCR data

- 32 You need Ct value for your target gene as well as housekeeping gene (e.g., gapdh, 18s) to analyze the data

$$\Delta\text{Ct} = (\text{Sample Ct}) - (\text{housekeeping gene Ct})$$

Sample 1: $21 - 10$

$$\Delta\Delta\text{Ct} = (\text{Sample } \Delta\text{Ct}) - (\text{one of your control sample } \Delta\text{Ct})$$

Sample 1: $11 - 11$

Sample 2: $11 - 11.5$

Calculate $2^{-\Delta\Delta\text{Ct}}$

Divide $2^{-\Delta\Delta\text{Ct}}$ value with the average of $2^{-\Delta\Delta\text{Ct}}$ of the control group

Sample 1: $1 / \text{average of } 1 \text{ and } 1.4$

Sample 2: $1.4 / \text{average of } 1 \text{ and } 1.4$