Total RNA extraction, cDNA synthesis, and qPCR

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Abstract

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Protocol

Step 1.

Seed cells at a density of 1×10^5 cells/well in a 24-well plate. Culture cells under 5% CO_2, in a 95% humidified atmosphere at 37℃.

Step 2.

48 h post-transfection, the total cellular RNA is extracted using a Total RNA Extraction Kit (Promega, USA), according to the manufacturer’s protocol stated as below.

Step 3.

Wash cells three times with the PBS. Strip cells with cell scraper, collect cells in a nuclease-free EP tube by centrifugation at 1000 rpm for 5 minutes, and then discard the supernatant.

Step 4.

Add 100 µl of RNA lysate to the cell pellet, resuspend the cells with a pipette, transfer the lysate into a 1.5 ml nuclease-free EP tube, then add 100 µl of the dilution, and mix the mixture by inverting four times.

Step 5.

Incubate the lysate at 70℃ for 3 minutes to increase the RNA yield.

Step 6.

Centrifuge at 14,000 × g for 5 minutes. Carefully aspirate the supernatant into a 1.5 ml nuclease-free EP tube.

Step 7.

Add 0.5 times the supernatant volume of anhydrous ethanol, mix 4 times with a pipette.

Step 8.

Remove the centrifuge column/collection tube, and transfer the mixture to the centrifuge column. If the volume of the mixture is greater than 750 µl, add the mixture in batches.

Step 9.

Centrifuge at 14,000 × g for 1 minutes, discard the filtrate, and put the centrifuge column back into the collection tube.

Step 10.

Add 600 µl wash buffer to the column, centrifuge at 14,000 × g for 45 seconds, and discard the filtrate.

Step 11.

Take a nuclease-free tube, add 5 µl of 10 × DNase I buffer, 5 µl of DNase I, and 40 µl of nuclease-free water, and then gently mix them. The amount for extraction of a tube of RNA required by DNase I incubation is 50 µl.

Step 12.

Add 50 µl of freshly prepared DNase I incubation solution to the centrifuge tube and incubate at room temperature for 15 minutes.

Step 13.

Add 600 µl RNA wash buffer to the column, centrifuge at 14,000 × g for 45 seconds, and discard the filtrate.

Step 14.

Repeat step 13 once. And then centrifuge at 14,000 × g for 2 minutes.

Step 15.

Transfer the centrifuge column into the elution tube, add 50 µl of nuclease-free water to the spin-off membrane, incubate at room temperature for 2 minutes, and centrifuge at 14,000 × g for 1 minute.

Step 16.

Add the elution solution to the center of the column, incubate at room temperature for 2 minutes, and centrifuge at 14,000 × g for 1 minute to elute RNA again.

Step 17.

Determine the concentration of RNA by measuring the absorbance at 260 nm.

Step 18.

Prepare the reverse transcription reaction system (20 µl) as follows to synthesize cDNA:

5 × RT Master Mix (TaKaRa, Japan)	4 µl
RNA	2 µg
RNase-free ddH_2O	add up to 20 µl

Step 19.

Set up the RT-PCR reaction procedure as described below:

25℃	5 minutes
37℃	30 minutes
85℃	10 seconds
12℃	10 minutes

Step 20.

Prepare the following qPCR system (20 µl) on ice:

cDNA mixture	1 µl
2 × Top Green qPCR Super Mix (Transgen, China)	10 µl
upstream primer (10 mM)	1 µl
downstream primer (10 mM)	1 µl
ddH_2O	add up to 20 µl

Step 21.

Set up the qPCR reaction procedure as described below:

Step 1	95℃	5 minutes
Step 2	95℃	30 seconds
	55℃	30 seconds
	72℃	10 seconds
	40 Cycles in Step 2	
Step 3	72℃	5 minutes
Step 4	4℃	hold

Step 22.

Calculate relative gene expression by comparison of the CT value of the gene of interest with that of GAPDH which ia an internal control.