Relative quantification of mRNA transcript levels by qPCR

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Abstract

This method describes isolation of RNA from cultured cells, generation of cDNA, and relative quantification of transcript levels by qPCR.

Keywords

RNA, cDNA, qPCR

Solutions to prepare

DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco).

A. Cell culture and treatments

1. HeLa-M cells were cultured at 37°C in 5% CO_2 and DMEM containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine (all from Gibco).
2. For any given experiment, cells were plated at such density so as to be approximately 90% confluent at the time of lysis.
3. For experiments using siRNA, 60 pmols of the indicated siRNA was transfected using 6µL Lipofectamine RNAiMax (ThermoFisher) in Opti-MEM (Gibco) per well according to manufacturer protocol. Cells were lysed 72 hours after siRNA transfection.

B. Cell lysis, RNA purification, and qPCR

1. Aspirate media from cells and rinse cells with PBS on ice.
2. Isolate RNA using RNeasy Micro Plus kit (Qiagen) according to manufacturer's protocol.
3. Generate cDNA from 1 µg purified RNA using iScript cDNA synthesis Kit (Bio-Rad) according to manufacturer's protocol.
4. Dilute the iScript reaction to a total of 400 uL Sterile Water (American Bio)
5. Combine 10 uL SYBR Green Master Mix (BioRad) with 6.78 uL Sterile Water (American Bio) per sample.
6. Combine 16.78 µL diluted SYBR Green Master Mix with 0.61 µL each of 10 µM forward and reverse primers per sample. Pipette this mixture into wells of 96-well qPCR plate. Perform at least two technical replicates for each sample.
7. Pipette 2 uL of diluted RNA from step 4 in well with SYBR Green Master Mix.
8. Cover plate with Optical Adhesive Covers (Applied Biosystems).
9. Spin down plate in table top centrifuge
10. Run qPCR in CFX96 Real-Time System (BioRad) using the following protocol:

95 ˚C	3 min	
95 ˚C	10 sec	Repeat 39x
55 ˚C	10 sec	
72˚C	30 sec	
95 ˚C	10 sec	
65 ˚C	5 sec	
95 ˚C	5 sec	

C. Data analysis

1. Subtract the housekeeping gene (β-actin) mean threshhold cycle (Ct) values from transcript of interest mean Ct values to calculate ΔCt.
2. Subtract the ΔCt of the control sample from each sample ΔCt to calculate the ΔΔCt value.
3. Calculate relative expression using the $2^{-\Delta\Delta Ct}$ method.