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qRT-PCR (SYBR Green)

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We use this protocol and it's working

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Abstract

Quantitative reverse-transcription PCR (qRT-PCR) to measure relative gene expression using TRIzol RNA extraction, PrimeScript™ RT Master Mix, and SYBR™ Green on a 96-well real-time instrument (e.g., StepOnePlus™). Analysis by 2^-ΔΔCT with melt-curve confirmation.

Attachments

DOCX

qRT-PCR_protocol_Bay...

37KB

Guidelines

Pause Points:
- RNA stable at −80 °C.
- cDNA stable at −20 °C.
- Pre-run plate: keep on ice.

Troubleshooting:
- Low RNA yield: ensure clean separation, avoid overdrying.
- Multiple melt peaks: increase annealing temp, redesign primers.
- High variability: mix reagents well, centrifuge plate.
- NTC amplification: replace reagents, check contamination.

Record Keeping:
Record RNA purity, input amounts, primer sequences, plate map, run file, and ΔΔCT calculations.

Materials

Instruments: NanoDrop, PCR thermal cycler, real-time PCR system (96-well), plate centrifuge.
Consumables: Pipettes/tips, RNase-free tubes, 96-well qPCR plates, optical seals.
Reagents: TRIzol™ Reagent; chloroform; isopropanol; 70% ethanol (RNase-free); RNase-free water; PrimeScript ™ RT Master Mix (TAKARA RR036A); SYBR™ Green Universal Master Mix (Applied Biosystems 4309155); forward/reverse primers.

Safety warnings

- Work RNase-free (gloves, RNase-free plastics).
- TRIzol™, chloroform, and isopropanol are hazardous—use in a fume hood.
- Keep samples on ice unless otherwise specified.

Before start

- Pre-cool a centrifuge to 4 °C.
- Thaw reagents on ice; mix and briefly spin.
- Design primers (amplicon 70–200 bp; Tm ~60 °C when possible).

Step 1 – Total RNA Extraction (TRIzol)

1 Lyse/Homogenize: Add 1 mL TRIzol per 50–100 mg tissue or 5–10×10^6 cells. Homogenize thoroughly; incubate 5 min at RT.

2 Phase Separation: Add 0.2 mL chloroform per 1 mL TRIzol, vortex 10 s, incubate 10 min at RT. Centrifuge 12,000×g, 10 min, 4 °C. Transfer aqueous phase.

3 RNA Precipitation: Add 0.5 mL isopropanol per 1 mL TRIzol, invert gently, incubate 10 min RT. Centrifuge 12,000×g, 10 min, 4 °C.

4 Wash: Wash pellet 3× with 70% ethanol, centrifuge each time. Air-dry 30 min RT.

5 Resuspend & QC: Dissolve in 50 µL RNase-free water, measure purity (A260/A280 > 1.8). Store at −80 °C.

Step 2 – Reverse Transcription (cDNA Synthesis)

6 Reaction (20 µL): 1 µg RNA, 4 µL 5× PrimeScript RT Mix, water to 20 µL. Program: 37 °C 15 min, 85 °C 5 s, 4 °C hold. Dilute to 100 µL, store at −20 °C.

Step 3 – qPCR Reaction Setup

7 Reaction mix (10 µL): 5 µL SYBR Mix, 1 µL cDNA, 0.5 µL primers each, 3 µL water. Set up triplicates, NTCs. Seal, centrifuge plate.

Step 4 — Cycling Conditions & Melt Curve

8 Option A (Tm ≥ 60 °C): 50 °C 2 min; 95 °C 2 min; 40× (95 °C 15 s, 60 °C 1 min).
Option B (Tm < 60 °C): 50 °C 2 min; 95 °C 2 min; 40× (95 °C 15 s, 55–60 °C 15 s, 72 °C 1 min).
Melt Curve: 95 °C 15 s; 60 °C 1 min; slow ramp to 95 °C.

Step 5 — Data Analysis (2^-ΔΔCT)

9 Export CT values.

10 ΔCT = CT(target) – CT(housekeeping).

11 ΔΔCT = ΔCT(experimental) – ΔCT(control).

12 Expression = 2^-ΔΔCT.

13 5. Confirm single melt peak. Plot in Prism.