Real Time Quantitative PCR (for Suspension Cells)

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[Abstract] Real-time quantitative PCR (qPCR) is an efficient and sensitive method to detect gene expression levels. This protocol provides a complete and detailed procedure for qPCR using mammalian suspension cell samples, including mRNA extraction, genomic DNA removal, cDNA synthesis and targeted gene amplification steps.

Materials and Reagents

1. Suspension cells (such as K562 cells)
2. Chloroform
3. Isopropyl alcohol
4. 75% DEPC-ETOH
5. RNase-free H_2O
6. *E.coli* RNase H
7. Trizol (Life Technologies, Invitrogen™)
8. Turbo DNA-free kit (Life Technologies, Ambion®)
9. Superscript III first-strand synthesis supermix (Life Technologies, Invitrogen™)
10. SYBRgreen qPCR Supermix universal (Life Technologies, Invitrogen™)

Equipment

1. CFX96TM or CFX384TM real-time PCR detection system (Bio-Rad Laboratories)
2. NanoDrop

Procedure

A. Total RNA extraction (for suspension cells)
   1. Spin down cells (5~10 x 10^6) at 300 *x g* for 5 min at 4 °C. Add 1 ml of Trizol to lyse cells by repetitive pipetting. Incubate at room temperature (RT) for 5 min.
   2. Add 0.2 ml of chloroform and cap tubes securely. Shake tubes vigorously by hand for 15 sec and incubate at RT for 2 to 3 min. Centrifuge at 12,000 *x g* for 15 min at 4 °C.

3. Carefully transfer the top aqueous phase (about 0.6 ml) to a new clean tube.
4. (Second chloroform extraction) Add 0.6 ml chloroform to the aqueous phase and cap tubes securely. Shake tubes vigorously and incubate at RT for 2 min and centrifuge at 12,000 *x g* for 15 min at 4 °C.
5. Carefully transfer the top aqueous phase to a new clean tube.
6. Precipitate RNA by adding 0.5 ml isopropyl alcohol to the aqueous phase and mix well. Incubate at RT for 10 min. Centrifuge at 12,000 *x g* for 10 min at 4 °C.
7. Remove supernatant and wash RNA with 1 ml of 75% DEPC-ETOH. Mix the sample by vortexing, and centrifuge at 7,500 *x g* for 5 min at 4 °C.
8. Remove the supernatant and briefly air dry the RNA pellet (no more than 10 min). Dissolve RNA in RNase-free H_2O by pipetting. Incubate at RT for about 5-10 min. Measure RNA concentration using NanoDrop. Check RNA quality by A_{260}/A_{280} and A_{260}/A_{230} (RNA with good quality is close to 2 for both). Store RNA at -80 °C.

B. Remove genomic DNA (Turbo DNA-free kit)

9. Set up reaction (50 µl)

   RNA prep (up to 10 µg)

5 µl	10x buffer
1 µl	Turbo DNase
RNase free H_2O	make up to 50 µl

   Mix gently and incubate at 37 °C for 20-30 min.
10. Add 5 µl DNase inactivation reagent and mix well.
11. Incubate at RT (>22 °C) for 5 min and mix occasionally.
12. Centrifuge at 10,000 *x g* for 1.5 min and transfer the supernatant to a fresh tube. Spec again.

C. First-strand synthesis (Superscript III first-strand synthesis supermix)

13. Combine the following kit components in a tube on ice. For multiple reactions, a master mix without RNA may be prepared:

2x RT reaction mix	10 µl
RT enzyme mix	2 µl
RNA (up to 1 µg)	X µl
Nuclease free-H_2O	to 20 µl

14. Gently mix tube contents and incubate at 25 °C for 10 min.
15. Incubate at 50 °C for 30 min.
16. Terminate reaction at 85 °C for 5 min and then chill on ice.
17. Add 1 µl (2 U) of *E.coli* RNase H and incubate at 37 °C for 20 min.

18. Use diluted or undiluted cDNA in qPCR or store at -20 °C until use (Up to 10% of the qPCR reaction volume may be undiluted cDNA.).

D. qPCR reaction (SYBRgreen qPCR supermix universal)

19. Combine the following components in PCR tube. For multiple reactions, a master mix is strongly recommended to reduce pipetting errors.

SYBRgreen Supermix universal 2x	10 µl
Forward primer (4 µM)	1 µl
Reverse primer (4 µM)	1 µl
cDNA (generated from 1 µg of total RNA)	1 µl
Nuclease free- H_2O	to 20 µl

20. Centrifuge briefly to make sure every component at the bottom of the tube.
21. Place the reaction in the preheated real-time instrument and program as follows:
    50 °C 2 min (UDG incubation)
    95 °C 10 min (UDG inactivation and DNA polymerase activation)
    40 cycles of:
    95 °C 15 sec
    60 °C 60 sec
    Melting curve analysis: refer to instrument documentation.
22. Collect data and analyze the results.

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