Real Time PCR Protocol

(For scientific research purposes only, not for clinical diagnosis!)

Isolate RNA

1.1 Homogenization

1.1.1 Tissues: Homogenize tissue samples in 1 mL of RNA Extraction per 20 mg of tissue using Tissue homogenizer. The sample volume should not exceed 10% of the volume of RNA Extraction used for the homogenization.

1.1.2 Cells grown in Monolayer: Rinse cell monolayer with ice cold PBS once. Lyse cells directly in a culture dish by adding 1 ml of RNA Extraction per 3.5 cm diameter dish . Pass the cell lysate several times through a pipette. Vortex thoroughly. The amount of RNA Extraction added is based on the area of the culture dish (1 mL per 10 cm2) and not on the number of cells present. An insufficient amount of RNA Extraction may result in DNA contamination of the isolated RNA.

1.1.3 Cells Grown in suspension: Spin cells for 10 min at 3000rpm. Remove media and resuspend cells in ice cold PBS. Pellet cells by spinning at 3000rpm for 10 min. Lyse cells with RNA Extraction by repetitive pipetting . Use 1 mL of the reagent per 1X 10^6 of animal cells.

1.1.4 Blood: Take 1 mL of whole blood, centrifuge at 3000 rpm for 5 min, and discard the supernatant. Add 3mL of red blood cell lysate and mix with full oscillation. After 10 min at 4℃(or room temperature), centrifug at 3000rpm for 5min, and discard the supernatant. Add the red blood cell lysate and process 1-2 times until the liquid is clear. The precipitation was collected by centrifugation, and add 1mL of RNA extraction and mixed by shock.

1.2 Phase Separation

Centrifuge the samples at 12000 rpm for 10 min at 4℃ to remove cell debris. Transfer the supernatant to a new tube. Add 0.25 mL of chloroform per 1 mL of RNA Extraction. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate them at room temperature for 2 to 3 minutes. Centrifuge the samples at 12,000 rpm for 10 minutes at 4℃. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh tube.

1.3 RNA Precipitation

Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0.5 mL of isopropyl alcohol per 1 mL of RNA Extraction used for the initial homogenization. Incubate samples at -20℃ for 15 minutes and centrifuge at 12,000 rpm for 10 minutes at 4℃. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

1.4 RNA Wash

Remove the supernatant completely. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 mL of RNA Extraction used for the initial homogenization. Mix the samples by vortexing and centrifuge at 12,000 rpm for 10 minutes at 4 ℃. Remove all leftover ethanol.

1.5 Redissolving RNA

Air-dry RNA pellet for 5-10 minutes. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility. Then add 15μL RNA Storage Solution.

1.6 SPECTROPHOTOMETRIC ANALYSIS

Take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.8.

First Strand cDNA Synthesis

After thawing, mix and briefly centrifuge the components of the kit. Store on ice.

2.1 Add the following reagents into a sterile, nuclease-free tube on ice in the indicated order: Template RNA 2μg , 5×SweScript All-in-One SuperMix for qPCR 4 μL , gDNA Remover1 μL, nuclease-freeWater to 20 μL , Total volume 20 μL.

2.2 Optional. If the RNA template is GC-rich or contains secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5 min. Chill on ice, spin down and place the vial back on ice.

2.3 Mix gently and centrifuge briefly.

2.4 Incubate for 5 min at 25°C ,30 min at 42°C.

Note. For GC-rich RNA templates the reaction temperature can be increased up to 50°C.

2.5 Terminate the reaction by heating at 85°C for 5 seconds.

Preparation of PCR Master Mix

3.1 For each 15μL reaction, prepare the following reation mix:

2×SYBR Green qPCR Master Mix (None ROX)	7.5 μL
F/R Primers(2.5μM)	1.5 μL
cDNA	2.0 μL
Water Nuclease-Free	4.0 μL

3.2 PCR amplification

Stage1	Stage2 （40 cycles）	Stage3 （Melt Curve）
95℃,30s Pre-denaturation	95℃,15s Denaturation 60℃,30s Anealing/Extension	65℃→95℃

Results of Processing

ΔΔCT method:

A = CT (target gene, sample) - CT (internal standard gene, sample)

B = CT (target gene, control) - CT (internal standard gene, control)

K = A-B

RNA Expression = 2^{-K}