RT-PCR protocol

RNA preparation

1. Grow cells to confluence in a single well of a 6-well plate.
2. Lyse the cells with 1 ml Trizol reagent. Pass the lysate through a pipette several times.
3. Incubate the homogenized samples for 5 minutes at 15 to 30°C.
4. Add 0.2 ml of chloroform per ml of Trizol and cap sample tubes securely.
5. Shake tubes vigorously by hand for 15 seconds and incubate them at 15 to 30°C for 2 to 3 minutes.
6. Centrifuge the samples at no more than 12,000*g* for 15 minutes at 2 to 8°C.
7. Transfer the colourless upper aqueous phase to a fresh tube.
8. Precipitate the RNA from the aqueous phase by mixing with 0.5 ml isopropyl alcohol. Incubate samples at 15 to 30°C for 10 minutes and centrifuge at no more than 12,000*g* for 10 minutes at 2 to 8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.
9. Remove the supernatant. Wash the RNA pellet once with at least 1 ml of 75% ethanol (prepared using RNase-free water). Mix the sample by vortexing and centrifuge at no more than 7,500*g* for 5 minutes at 2 to 8°C.
10. Briefly dry the RNA pellet (air-dry or vacuum-dry for 5-10 minutes). Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely as this will greatly decrease its solubility.
11. Dissolve RNA in RNase-free water or 0.5% SDS solution by passing the solution a few times through a pipette tip, and incubating for 10 minutes at 55 to 60°C.

RT reaction

1. Before performing the RT reaction, heat 5 μg of total RNA in a 10μl volume at 65°C for 5 to 10 minutes and then quench on ice.
2. Set up the following components in a 1.5 ml Eppendorf tube:

    10.0 μl heat denatured RNA
    3.0 μl 10 x PCR buffer
    2.5 μl 10mM dNTPs
    6.0 μl 25mM $MgCl_2$
    1.0 μl random primers (1.0 μg)
    0.5 μl SuperScript II reverse transcriptase
    17.0 μl water

!!!Warning: do not use DEPC-treated water for the RT reaction as DEPC will inhibit the RT and PCR reactions!!!

3. Leave the samples at 25°C for 10 minutes then incubate at 42°C for 1 hour.
4. Denature the cDNA at 95°C and place on ice.

PCR reaction

1. Set up the following components in a 0.5ml PCR tube:
   6.0 µl cDNA product
   1.5 µl 10 x PCR buffer
   0.2 µl Taq polymerase
   0.5 µl primer 1 (100 ng)
   0.5 µl primer 2 (100 ng)
   10.3 µl water
2. Perform PCR with 30 cycles of denaturation: 30 seconds at 95°C; annealing: 45 seconds at 60°C; and extension 60 seconds at 72°C.