

REAL TIME - POLYMERASE CHAIN REACTION (PCR)



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REACTION I: REVERSE TRANSCRIPTASE

1. To each sample tube, add the following reagents:

- Deoxynucleotide triphosphates (dNTPs), 2.5 mM mix (stock solutions = 100 mM; make 10 mM dilutions of each, then a 1:4 for a final concentration of 2.5 mM by combining Equal volumes of the 1:10 diluted dNTPs) **2.5 μ L**
- Reverse transcriptase (RT) buffer (supplied by manufacturer) **5.0 μ L**
- 0.1 M Dithiothreitol (DTT) **2.0 μ L**
- RNasin (RNase inhibitor from Promega) **0.5 μ L**
- Random hexamer oligonucleotides 20-40 units/ μ L; stock is 50 A260 units/vial) L. **2.0 μ L**
- Sample (RNA template) + ddH₂O **13.0 μ L**

The above unit volumes are based on an RNA sample of 1.8 μ g and 1.0 μ L reverse transcriptase II (RT). It is also less time consuming to make "master mixes" by combining the reagents cited above (minus the samples and perhaps the H₂O, if you need to make adjustments) in a single tube, and aliquoting the necessary amount per sample tube from the master mix.

2. Heat the samples in the reaction I mix at 70°C for 5 min, chill samples on ice for 5 min. (File 50 on PCR Machine 9600 is used for this.) Microfuge samples at maximum speed 2-3 sec, then put on ice.

3. Add 1 μ L of RT (vortexed) per tube, incubate at 37°C for 60 min, followed by 90°C for 5 min; chill samples on ice for 5 min. (File 51 on PCR Machine 9600 is used for this.) NOTE: At this point, the assay can be stopped and the cDNA frozen at -70°C indefinitely.



REACTION II: Amplification of cDNA by PCR

1. Prepare the PCR reaction mix for each sample in a separate well:

- Cytokine Primer/Probe **2.5 μ L**
- cDNA + dH₂O **22.5 μ L**

Volume of cDNA varies with the cytokine of interest:

IL-4 10 μ L cDNA

IL-13 10 μ L cDNA

IFN- γ 1 μ L cDNA

Ribosomal 1 μ L of 1:100 dilution of cDNA

- Mixed Buffer **25.0 μ L**

2. Cap the plate and mix by gentle inversion.

3. Centrifuge the plate.

4. Place the plate in the 7700 Sequence Detection System.

5. Program the detector as indicated in the manufacturer's directions.