

REVERSE TRANSCRIPTASE - POLYMERASE CHAIN REACTION (RT-PCR)



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PRECAUTIONS:

When performing PCR assays, it is important to be aware of potential sources of contamination of the test samples with extraneous sources of DNA. To guard against this, one should treat any pipettes used to aliquot cDNA as "contaminated" - they either should be kept separate from pipettes used to handle source reagents, or if the same pipettes are to be used, they should be cleaned with a dilute solution of HCl (10 ml 12 N HCl : 1 liter H₂O) (racks can be soaked, pipettes should be sponged off). Gloves should be worn, and changed frequently throughout the procedure. Handle samples containing RNA or cDNA in separate work areas if possible; if not, at least change lab matting or wipe off lab bench top with the dilute acid solution.

REACTION I: REVERSE TRANSCRIPTASE

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

- Deoxynucleotide triphosphates (dXTPs), 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 dXTPs) **2.5 μL**
- Reverse transcriptase (RT) buffer (supplied by manufacturer) **5.0 μL**
- 0.1 M Dithiothreitol (DTT) **2.0 μL**
- RNasin (RNase inhibitor from Promega) **0.5 μL**
- Random hexamer oligonucleotides 20-40 units/μl; stock is 50 A260 units/vial) L. **2.0 μL**
- Sample (RNA template) **3.0 μL**
- H₂O (double-distilled, high quality) **8.8μL**

The above unit volumes are based on an RNA sample of 1.8 μg and 1.0 μL 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 μL 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. To the samples (2.5 μL cDNA) prepared in reaction I, add the following reagents:

- Deoxynucleotide triphosphates (dXTPs), 2.5 mM mix (prepared as in reaction I) **2.0 μL**
- PCR buffer **2.5 μL**
(our recipe: 500 mM KCl, 100 mM Tris-HCl pH 8.4, 15 mM MgCl₂, 1 mg/ml bovine serum albumin)
- Sense primers (0.2 $\mu\text{g}/\mu\text{L}$) **1.0 μL**
- Antisense primers (0.2 $\mu\text{g}/\mu\text{L}$) **1.0 μL**
- Taq polymerase (Promega, 5U/ μL ; need 1U) **0.15 μL**
- H₂O (double-distilled, high quality) **15.85 μL**
- Mineral oil (overlay as last step)

** As in reaction I, you can compensate for a smaller sample volume by increasing the amount of H₂O added per tube, and again, it is less work to make a master mix for the reagents listed above than to add them singly!

PCR PROGRAM:

1. Turn power on to the 9600 PCR Machine.
2. Press edit → enter → 59 → enter → enter → cycle → change cycle number → enter → enter → store → enter 59 → stop → run → enter → 52



THE NUMBER OF PCR CYCLES FOR MOUSE CYTOKINE cDNA AMPLIFICATION

CYTOKINE	# OF CYCLES	CYTOKINE	# OF CYCLES	CYTOKINE	# OF CYCLES
HPRT	13	IL-5	27	IFN- γ	21
IL-1 β	14	IL-6	22	TNF- α	24
IL-2	26	IL-9	28	TCR- α	20
IL-3	28	IL-10	20	TCR- γ 4	20
IL-4	24	IL-13	26	TGF- β	20
P40(IL-12)	22	P35(IL-12)	20	TCR- γ 1	21