RT-PCR Protocols

1. DNase I Digestion of RNA Preparation

If amplification products are detected from the PCR in the absence of SuperScript™ II RT, it may be necessary to eliminate residual genomic DNA from the RNA sample. Use the following protocol to remove genomic DNA from the total RNA preparation.

1. Add the following to a 0.5-ml microcentrifuge tube on ice:

Component	**Amount**
total RNA	1–4 µg
10X reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]	1 µl
50 mM $MgCl_2$	0.4 µl
Amplification grade DNase I (1 unit/µl)	1 µl
DEPC-treated water	to 10 µl

2. Incubate at room temperature for 15 min.
3. Add 1 µl of 25 mM EDTA.
4. Incubate for 15 min at 65°C to heat inactivate the DNase I, then place on ice for 1 min. Collect the reaction by brief centrifugation. This mixture can be used directly for reverse transcription.

2. cDNA Synthesis

This procedure converts 1 to 4 µg of total RNA into cDNA. Poly(A)+ RNA may be used in this protocol, but is typically not necessary.

1. Mix and quickly centrifuge each component before use.
2. Combine 1-4 µg of total RNA or 50 ng of poly(A)+ RNA and DEPC-treated water to a final volume of 11 µl in a 0.5-ml microcentrifuge tube. Or you can use DNase I-treated RNAs directly from above.
3. Add 1 µl of the 10 µM RT primer, mix gently, and collect reaction by brief centrifugation.
4. Heat the mixture to 70°C for 10 min and chill on ice for at least 1 min. Collect the contents of the tube by brief centrifugation and add the following:

Component	**Volume (µl)**
10X PCR buffer	1
50 mM $MgCl_2$	0.6
2.5 mM dNTP mix	4
0.1 M DTT	2
RNasin (40 U/µl)	0.4

5. Mix gently and collect the reaction by brief centrifugation. Equilibrate the mixture to 42°C for 2 to 5 min.
6. Add 1 µl of SuperScript II RT. Incubate the tube in a 42°C water bath or heat block for 50 min.
7. Terminate the reaction by incubating at 70°C for 15 min.
8. Chill on ice. Collect the reaction by brief centrifugation. Add 1 µl of RNase H (2 U/µl) to the tube, mix, and incubate for 20 min at 37°C.
9. The reaction mixture may be stored at –20°C.

3. Amplification of the Target cDNA

Optimal conditions for amplification are dependent on the nature of each particular primer and target sequence used. Alteration of the magnesium ion, dNTP, or primer concentration, as well as the thermocycling protocol, may be required. The optimal free magnesium concentration for efficient amplification is reported to be between 0.7 to 0.8 mM. Since magnesium binds deoxyribonucleoside triphosphates, this factor is affected by both primer and dNTP concentration. In general, lower concentrations of dNTP (50 to 200 µM), $MgCl_2$ (1 to 1.5 mM), and primer (0.1 to 0.2 µM) promote higher fidelity and specificity. Higher nucleotide concentration, however, can be used to improve product yield as well as to promote 3´-terminal T-mismatches.

The addition of either Taq DNA polymerase, dNTPs, or $MgCl_2$ after reactions have been equilibrated at 75°C to 80°C has been reported to improve the specificity of the reaction. This “hot start” practice reduces nonspecific binding and the extension of primers during the initial denaturation process. A practical alternative to this classic “hot start” method is to set up reactions on ice then place complete PCR mixtures in a thermal cycler equilibrated to 80°C to 90°C.

1. To a fresh 0.5-ml microcentrifuge tube, add the following:

Component	**Volume (µl)**
10X PCR buffer	5
50 mM $MgCl_2$	1.5
autoclaved, distilled water	34
2.5 mM dNTP mix	5

Primer 1 (10 μM)..... 1
Primer 2 (10 μM) 1
TaqDNA polymerase (2 to 5 units/μl) 0.5

2. Add 2 μl from the cDNA synthesis reaction to the tube. Mix gently and collect the reaction briefly by centrifugation.
3. PCR amplication as follows;

94°C for 3 min

94°C for 30 sec
55°C for 30 sec
72°C for 1 min (depends on product size)
Repeat 25 times.

72°C for 10 min
4°C for 20 min

4. Analyze 5 to 10 μl of the amplified sample, using 1% agarose gel electrophoresis and ethidium bromide staining, and the appropriate molecular size standards.