



# One-Step RT-PCR Master Mix Kit

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One Step RT-PCR Master Mix Kit

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## About the Kit

### Description

The One Step RT-PCR Master Mix Kit allows rapid, sensitive analysis of gene expression from tissues and cells. One Step RT-PCR Master Mix Kit can replace methods for detecting and quantifying gene expression such as Northern blots, in situ hybridization, dot blots, S1 nuclease assays and conventional two step RT-PCR (i.e., two enzyme/two buffer system). The kit utilizes recombinant *Thermus thermophilus* (*rTth*) DNA Polymerase, which acts both as a thermostable RNA-dependent DNA polymerase and a DNA-dependent DNA polymerase. The thermostable property of *rTth* DNA Polymerase allows reverse transcription at 60°C minimizing problems from RNA secondary structure and high GC content.

The *rTth* DNA Polymerase is provided in a 2X master mix with an antibody for antibody-mediated hot start, optimized buffer, and ultra pure deoxynucleotides. Antibody-mediated hot start enhances specificity of both reverse transcription and PCR. The kit enables cDNA synthesis from input RNA followed by PCR amplification of the cDNA in a single reaction, with no additional hands-on requirement for buffer changes or adding reagents. Typically, detection of a specific transcript requires only 2 hours.

We recommend using either two gene-specific primers or oligo (dT) and one gene-specific 5'-primer with the kit. Each kit provides sufficient reagents to perform 50 RT-PCR reactions. Positive Control RNA and Control Primers are also included.

Although *rTth* adds 3' dA overhangs, it is generally not recommended for PCR product cloning because the *rTth* error rate is higher than standard *Taq* DNA Polymerase. This kit is ideally designed for the rapid screening of gene expression.

### Components

#### One Step RT-PCR Kit

- 2 × 625 µl 2X One-Step RT-PCR Master Mix
- 1 × 200 µl 50 mM Mn(OAc)<sub>2</sub>
- 1 × 1.1 ml RNase-free water
- 1 × 50 µl Positive Control RNA (5 × 10<sup>5</sup> copies/µl)
- 1 × 50 µl Primer F (10 pmol/µl, 5'-TCCACCACCCTGTTGCTGTA-3')
- 1 × 50 µl Primer R (10 pmol/µl, 5'-ACCACAGTCCATGCCATCAC-3')

### Storage

Store all components at -20°C.

## One Step RT-PCR Protocol

**One-Step RT-PCR Master Mix Kit is a unique RT-PCR system. Using reaction components or protocols designed for any other polymerase may result in poor amplification.** Reaction conditions listed below will provide satisfactory amplification for most primer/template combinations. Additional guidelines and troubleshooting sections provide details for optimizing reaction conditions.

### Standard reaction setup

- Combine all the following reagents according to the table below. **Setting up the control reactions is optional, but highly recommended:**

	Positive Control Rxn	Negative Control Rxn	Sample Rxn
2X One Step RT-PCR Master Mix	25 µl	25 µl	25 µl
50 mM Mn(OAc) <sub>2</sub>	2.5 µl	2.5 µl	2.5 µl
Anti-Sense Primer, 10 pmol/µl	2 µl (Primer R)	2 µl (Primer R)	1–3 µl
Sense Primer, 10 pmol/µl	2 µl (Primer F)	2 µl (Primer F)	1–3 µl
RNA (1–1000 ng total RNA)	2 µl (Positive Control RNA)	none	X µl (sample RNA)
RNase-free water	16.5 µl	A µl	Y µl
Total	50 µl	50 µl	50 µl

*Note:* The Positive Control RNA is a transcribed product from the human G3PDH (Glyceraldehyde 3-Phosphate Dehydrogenase) gene. When using Primers R and F as described here, a 450 bp product will be amplified.

- Use the following program for the RT-PCR reaction and PCR amplification:

CYCLING STEP	Time/temperature
1. Polymerase Activation	30 s at 90°C
2. Reverse Transcription	30 min at 60°C
3. Denaturation	1 min at 94°C
4. Denaturation	30 s at 94°C
5. Annealing	30 s at 60°C
6. Extension	1 min at 72°C
<b>Repeat steps 4–6 for 40 cycles</b>	
7. Final Extension	7 min for 60°C

*Note:* The annealing temperature for positive control Primers F and R is 60°C. For other primers, choose an annealing temperature about 5°C below the  $T_m$ . See p 4 for additional guidelines.

- Analyze the results on a 1–2% agarose gel stained with ethidium bromide.

## Additional Guidelines

### Avoiding ribonuclease contamination

Successful RT-PCR is dependent on the quality of the RNA. RNases are ubiquitous in the laboratory, and precautions should be taken to eliminate the risk of contamination. We recommend pretreatment of reagents and equipment to avoid RNase contamination. Heat glassware to 180°C for 60 min. Alternatively, place glassware in 0.1% DEPC (Diethylpyrocarbonate) solution at 37°C for 10 min with vigorous mixing followed by heating at 70°C for 1 h or autoclaving at 120°C for 30 min to eliminate residual DEPC. DEPC should always be used in a fume hood.

Use high quality sterile RNase-free plastic tubes. Wear gloves when handling reagents, equipment and samples. Wipe pipettes with 80% ethanol or isopropanol before working with RNA. Reserve reagents and equipment exclusively for RNA work and store them separately from those potentially contaminated with RNases.

## RNA template

The RNA template can be total RNA, messenger RNA, or viral RNA. Use the highest purity RNA possible ( $A_{260}/A_{280}$  ratio of 1.7 or higher) to amplify PCR fragments efficiently. The RNA sample should be DNA-free. Gel analysis of mRNA should reveal a smear ranging from 300 bp up to and beyond 4 kbp. The greatest concentration of mRNA will be between 1–2 kbp.

The quality of the template RNA can be assessed by setting up an additional control with the supplied positive control Primers R and F and your RNA sample. Primers R and F will amplify the housekeeping gene G3PDH (Glyceraldehyde 3-Phosphate Dehydrogenase). The G3PDH gene is expressed constantly in mammalian cells except when the expression level is altered by cytokine and forbol ester inducers. If the total or mRNA template is from mouse, rat, swine or human cells and tissues, the G3PDH gene will be amplified from high quality RNA.

## Cycling conditions

Use 20–50 amplification cycles, as appropriate. Note that if the template RNA is limited, increasing the number of cycles may increase the yield of nonspecific products, rather than the desired product.

## Primers

Use either two gene specific primers or an oligo-dT primer and one gene-specific primer with the One Step RT-PCR Master Mix Kit. Due to the high annealing temperature used, random hexamers are incompatible with this kit.

In order to distinguish between amplification from RNA or genomic DNA, design primers from different exons that are separated by at least one intron. Genomic DNA amplification will thus generate a larger fragment than RNA amplification. It is very important that the primers be complementary to the template, especially at the 3' end. The primer GC content should be approximately 40–60%.

It is important to determine the proper annealing temperature for any pair of primers. Primer pairs that exhibit similar melting temperatures and are completely complementary to the template are recommended. Contact the oligonucleotide manufacturer for an accurate estimate of oligo  $T_m$ .

## Troubleshooting

Problem	Probable Cause	Suggestion
Target cDNA band is not observed	Enzymes are inactivated due to incorrect storage.	Use the positive control RNA and primers to test the enzyme performance.
	The annealing temperature is not optimal	Change the annealing temperature to 5°C below the $T_m$ value.
	Primer sequence is incorrect	Redesign primers to be more complementary to the target gene. The 3' end of the primers should be completely complementary to the template.
		The primers should have a GC content of 40–60%. Check that the primer sequences are not self-complementary, especially at the 3' end.
	The reaction conditions and/or cycling times are not optimal	The amplification conditions described on p 3 are optimal for 0.1–1 kbp products. For a longer product increase reverse-transcription time and the PCR extension time. Note that the limit for robust amplification is ~1 kbp.
		Perform gel analysis of the template RNA to determine the RNA quality. The $A_{260}/A_{280}$ ratio should be $\geq 1.7$ . SDS, NaCl, heparin and guanidine thiocyanate from RNA purification methods can interfere with RT-PCR. Reduce the volume of RNA, perform additional purification steps or change purification method to avoid interference. Use RNase-free reagents and equipment. Prepare new template RNA.
Template RNA is poor quality and/or degraded		
Insufficient template RNA	Increase the amount of template RNA in the reaction (up to 1000 ng).	
Multiple products are observed from the RT-PCR reaction	The primer sequence is not specific to the desired target	Redesign the primers.
	The annealing temperature is not optimal	Change the annealing temperature to 5°C below the $T_m$ value.
	The $T_m$ value of the primers is low	Use primers with higher $T_m$ value.
	Number of cycles is too high	Try reducing the number of cycles; optimal range is 20–50 cycles.
		Increase the amount of template RNA.
	Mn(OAc) <sub>2</sub> concentration is insufficient	Usually 2.5 mM is optimal for RT-PCR but a range between 1–4 mM may be used.
	Genomic DNA contamination indicated by higher molecular weight product than expected.	Note that genomic DNA may amplify with gene specific primers. Perform a control reaction using a DNA polymerase without reverse transcriptase activity (e.g. NovaTaq™ DNA polymerase) instead of <i>rTth</i> DNA Polymerase. If amplification from the NovaTaq reaction occurs, then the template is contaminated with DNA.
	Multiple cDNA products may be obtained from multigene families or alternatively spliced genes	Redesign primers to ensure specificity, as appropriate. Sequence products to verify identity.

## References

Thein, S. L., and Wallace, R. B. (1986) in “Human Genetic Diseases: A Practical Approach”, IRL Press.