

MasterAmp™ RT-PCR Kit for High Sensitivity

Cat. Nos. RT71225 and RT712100

Connect with Epicentre on our blog (epicentral.blogspot.com),
Facebook (facebook.com/EpicentreBio), and Twitter ([@EpicentreBio](https://twitter.com/EpicentreBio)).

1. Introduction

The MasterAmp™ RT-PCR Kit for High Sensitivity is a single-enzyme, single-tube continuous system for the amplification of target sequences from an RNA template. Measuring the level of gene expression by traditional methods generally involves either extensive sample preparation or substantial quantities of RNA. RT-PCR, conversely, is a simpler and more sensitive means for determining patterns of gene expression and for the cloning of expressed sequences. Of the possible RT-PCR protocols, continuous RT-PCR is more efficient and minimizes sample contamination by combining cDNA synthesis and PCR, and eliminating component addition following cDNA synthesis.¹⁻³

The MasterAmp Kit includes RetroAmp™ RT DNA Polymerase, a highly efficient, thermostable enzyme and MasterAmp 10X PCR Enhancer (with betaine).⁴ The use of a thermostable polymerase allows reverse transcription to take place at an elevated temperature, minimizing the effects of RNA secondary structure. The presence of betaine (trimethyl glycine) in the MasterAmp 10X PCR Enhancer likewise substantially improves the yield and specificity of amplification of many target sequences, especially those containing a high G+C content or secondary structure.⁴⁻⁸ Betaine lowers the melting temperature of G+C-rich regions to a temperature more similar to that of A+T(U)-rich regions.⁹ This results in the destabilization of double-stranded regions which limits polymerase pausing,⁶ thereby increasing the yield of full-length product. In addition, betaine also may enhance PCR by protecting DNA polymerases from thermal denaturation.¹⁰

2. Product Specifications

Storage: Store only at -20°C in a freezer without a defrost cycle.

Storage Buffer: RetroAmp RT DNA Polymerase is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.5% Tween® 20, 0.5% NP-40, and 1 mM dithiothreitol.

Unit Definition: One unit converts 10 nmoles of deoxyribonucleoside triphosphates into acid-insoluble material in 30 minutes at 74°C using standard assay conditions.

Activity Assay: The activity assay is performed in a reaction containing 25 mM TAPS (pH 9.3), 50 mM KCl, 2.0 mM MgCl_2 , 8.5 μg of activated calf thymus DNA, 0.2 mM of each dNTP, and 0.02-0.1 unit of enzyme.

Contaminating Activity Assays: RetroAmp RT DNA Polymerase is free of detectable non-specific DNase and RNase activities.

20X RT-PCR Buffer: 1.0 M Tris-HCl (pH 9.0), 250 mM NaCl, and 400 mM $(\text{NH}_4)_2\text{SO}_4$. Separate solutions of MasterAmp 10X PCR Enhancer, 25 mM MgCl_2 , and 25 mM MnSO_4

*⁴ See page 10 for patent and licensing information.

are also provided to allow optimization of individual reactions.

Control Template/Primers Mix: each microliter contains 12.5 pmol of each primer and 100 ng of *E. coli* RNA (see page 6).

Quality Control: The MasterAmp RT-PCR Kit for High Sensitivity is function-tested by performance of the kit's control reaction. Kit sensitivity is assayed in a similar fashion, using serial dilutions of *E. coli* RNA. A 479-bp PCR product (see page 6) must be visible when using the kit provided primers and as little as 10 pg of *E. coli* RNA as template.

3. Kit Contents

Desc.	Concentration	Quantity
The MasterAmp™ RT-PCR Kit for High Sensitivity is available in 25- and 100-reaction sizes. The 100-reaction size kit contains the following reagents:		
RetroAmp™ RT DNA Polymerase	@ 5 U/μl	250 Units
20X RT-PCR Buffer		500 μl
MasterAmp 10X PCR Enhancer		750 μl
dNTP Mix (2.5 mM each)		1 ml
25 mM MgCl ₂ Solution		750 μl
25 mM MnSO ₄ Solution		200 μl
Control Template/Primers Mix		20 μl
Sterile Nuclease-Free Water		3 ml

4. Related Products

The following products are also available:

- MasterAmp™ High Fidelity RT-PCR Kit
- MasterPure™ RNA Purification Kit
- MMLV Reverse Transcriptase
- MasterAmp™ *Tth* DNA Polymerase
- RNase-Free DNase I
- dNTP Solutions
- RiboGuard™ RNase Inhibitor

5. General Considerations for Optimization of RT-PCR

1. **Template Preparation and Storage:** Template RNA must be of high quality and purity; using partially degraded RNA will significantly limit the yield of the RT-PCR product. Determine RNA quality by agarose gel electrophoresis. We further recommend that users remove contaminating genomic DNA by enzymatic treatment (e.g., RNase-Free DNase I) followed by organic extraction to eliminate both exogenous and endogenous nucleases. Store RNA preparations either as an aqueous solution or as ethanol precipitates at -70°C to minimize degradation.
2. **Primer Design:** We recommend using gene-specific primers with melting temperatures of 60°C or greater for maximum sensitivity. Researchers may also use oligo (dT) or random primers (not supplied in the kit) following the Modifications

outlined on page 5. To distinguish amplification products originating from primers annealed to RNA rather than contaminating genomic DNA, design primers that anneal to exons flanking intronic sequences. Take care to design primers that do not form hairpin loop structures or are not self-complementary. The 5' end of a primer may contain bases not complementary with the template; however, the 3' end of the primer must be complementary with the template.

3. **MasterAmp 10X PCR Enhancer:** The appropriate concentration of MasterAmp 10X PCR Enhancer may vary with each template-primer pair combination. We recommend performing initial experiments using a final concentration of 1X MasterAmp PCR Enhancer. A final concentration of 0X to 3X may improve the amplification of some target sequences.
4. **Magnesium and dNTP Concentration:** Although most templates will amplify using the final concentrations of $MgCl_2$ (3 mM) and dNTPs (400 μM each nucleotide) outlined in the protocol on page 4, optimization may be necessary. If optimization is required, we suggest a range of magnesium concentration from 1.5-3.5 mM, and 200-400 μM each dNTP.

Note: *The optimal amount of Mg^{2+} may change if the concentrations of dNTPs and primers are altered.*

5. **Manganese Concentration:** Although most templates will amplify using the final concentration of $MnSO_4$ (0.5 mM) outlined in the protocol on page 4, optimization may be necessary. If optimization is required, we suggest a range of manganese concentration from 0.5-0.75 mM. Our observations indicate that 0.5 mM is more likely to be optimal with templates <2 kb, and 0.75 mM is optimal with templates \geq 2 kb. Use of higher concentrations is not recommended because of the potential for manganese-dependent degradation of the RNA template.
6. **Ribonuclease Inhibitors:** We recommend the addition of 20 units of RNase inhibitor in the reaction when using less than 1 ng of total cellular RNA.
7. **Cycling Conditions:** RNA template denaturation before first strand synthesis (reverse transcription) is not required and not recommended. Likewise, template denaturation between first strand and second strand synthesis (PCR) is not required. Users may perform annealing and extension simultaneously (1 min. per kb of product at 65°C-68°C) if the primers have a melting temperature greater than 60°C. If cloning the RT-PCR product, users may add a final extension step at 72°C for 4-7 min. to maximize the amount of full-length product.
8. **Maximizing Sensitivity:** With extremely dilute RNA samples, the addition of carrier RNA to a concentration of 10 ng/ μl may improve sensitivity by serving as an alternative target for contaminating ribonucleases. The addition of an RNase inhibitor may also aid in maintaining RNA integrity. If possible, optimize the RT-PCR using an abundant target message and then increase the number of PCR cycles to 40-50 for the detection of rare, low-copy target messages.
9. **Fidelity of RetroAmp RT DNA Polymerase:** In general, the error rates of reverse transcriptases (RTs) are high because the enzymes lack a 3'→5' proofreading exonuclease activity. Fidelity is largely dependent upon the ability of the RT to limit both the incorporation of a noncomplementary nucleotide and the extension of a mismatched 3'-primer nucleotide. Thermostable *Taq* DNA polymerase, which displays a weak Mn-dependent RT activity, is 10- to 100-fold more likely

to limit these two events than is AMV RT.¹¹ Likewise, because RetroAmp RT is a thermostable, Mn-dependent RT, we expect the enzyme to catalyze the reaction similar to *Taq*. Several factors affect the fidelity of reverse transcription *in vitro*, including buffer composition, and pH, the concentrations of Mg²⁺, Mn²⁺, and nucleotides, and the sequence of the template.¹¹ If maximum fidelity is desired, users should decrease the amount of nucleotide included in the reaction to 200 μM.

Note: *Decreasing the amount of nucleotide significantly lowers the reaction sensitivity.*

6. Suggested RT-PCR Protocol for Experimental Templates

Use the following protocol as a guideline for establishing the parameters necessary for RT-PCR of experimental templates. Assembly of reactions in a clean area, or using positive displacement pipettors with aerosol-barrier tips, will minimize contamination from extraneous DNA and RNA templates. In addition, users may wish to perform a control reaction for the presence of a homologous DNA template by omitting Part B, Step 3 in the protocol outlined below.

A. Assembly of the Amplification Premixes

Preparation of a reaction mix simplifies the assembly of multiple amplification reactions. The volumes listed below are for one 50-μl RT-PCR reaction. Assemble an amount of mix corresponding to the total number of reactions. Extra mix may be required to offset losses caused by pipeting.

Note: *RNA template denaturation before first strand synthesis (reverse transcription) is not required and is not recommended. If using oligo (dT) or random primers, follow the Modifications to the RT-PCR Protocol on page 5.*

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Prepare the two Premixes on ice in the order outlined below:

Premix 1:

10	μl	Sterile Nuclease-free Water
2.5	μl	20X RT-PCR Buffer
6	μl	25 mM MgCl ₂ *
5	μl	MasterAmp 10X PCR Enhancer*
1	μl	25 mM MnSO ₄ *
0.5	μl	RetroAmp RT DNA Polymerase
<hr/>		
25	μl	Total reaction volume

Premix 2:

x	μl	Sterile Nuclease-free Water
8	μl	2.5 mM dNTP Mix
1.25	μl	10 μM gene-specific primer 1
1.25	μl	10 μM gene-specific primer 2
x	μl	RNA Template
<hr/>		
25	μl	Total reaction volume

* See details on page 2, General Considerations for Optimization of RT-PCR.

B. Reverse Transcription and PCR

1. Just prior to starting the reaction, combine both Premixes in an RNase-free PCR tube, on ice.
2. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
3. Perform first strand synthesis (reverse transcription) in a thermal cycler preheated to 60°C for 20 min. for target amplicons ≤ 2 kb in length. Add 10 min. for each additional kb of target length.
4. Perform a sufficient number of cycles (30-50) depending on the abundance of the target:
Denature at 92-95°C for 30 seconds.
Anneal the primers at a temperature 2°C-5°C below the T_m of the primers for 30 seconds.
Extend the annealed primers at 68°C-72°C for 45-60 seconds for every kb of expected product.
5. A final extension step may be performed at 72°C for 4-7 min. to ensure full primer extension.
6. After amplification, the samples may be kept at 4°C overnight or frozen at -20°C.

Note: *RetroAmp RT DNA Polymerase exhibits terminal transferase activity and it is therefore likely that PCR products will contain uncoded nucleotides at the 3' termini.*

C. Modifications to the RT-PCR Protocol

Use of Oligo (dT)₁₈₋₂₀ Primer (not supplied) **for Reverse Transcription:** We recommend using oligo (dT)₁₈₋₂₀ to prime the synthesis of the first strand (reverse transcription). Users should add two gene-specific primers to the reaction before the start of PCR. In addition, the yield of final product will be substantially improved by performing the reverse transcription reaction with greater amounts of RNA template than described on page 4; we recommend using 1 μ g of total RNA. Other modifications to the protocol are outlined below.

1. Use 75 pmol oligo (dT)₁₈₋₂₀ primer in Premix 2. Adjust the amount of water included in Premix 2 to compensate for the addition of two gene-specific primers at the start of PCR.
2. The RNA, water, and primers can be preheated to 65°C for 1 minute to denature the RNA prior to reverse transcription. Place the tubes on ice, then add dNTPs to Premix 2, on ice. (This step is optional.)
3. Just prior to starting the reaction, combine both Premixes in an RNase-free PCR tube, on ice.
4. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
5. Anneal the oligo (dT) primer to the RNA template, and allow extension of the primers by incubating the reaction at 42°C for 10 min. Then perform first strand synthesis (reverse transcription) at 60°C for 20 min. for RNA templates ≤ 2 kb in length. Add 10 min. for each additional kb of target length.
6. Add 12.5 pmol each of two gene-specific primers.

7. Perform a sufficient number of cycles (30-50) depending on the abundance of the target:
Denature at 92°C-95°C for 30 seconds.
Anneal the primers at a temperature 2-5°C below the T_m of the primers for 30 seconds.
Extend the annealed primers at 68°C-72°C for 45-60 seconds for every kb of expected product.

Use of Random Hexamer or Random Nonamer Primers (not supplied) **for Reverse Transcription:** We recommend researchers add two gene-specific primers at the start of the PCR as described above. Although it is not strictly necessary, the yield of specific product may be greater with this addition. Other modifications to the protocol are outlined below.

1. Use 0.5 µg of random primers in Premix 2. Adjust the amount of water included in Premix 2 to compensate for the addition of two gene-specific primers at the start of PCR.
2. The RNA, water, and primers can be preheated to 65°C for 2-5 minutes to denature the RNA prior to reverse transcription. Place the tubes on ice, then add dNTPs to Premix 2, on ice. (This step is optional.)
3. Just prior to starting the reaction, combine both Premixes in an RNase-free PCR tube, on ice.
4. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
5. Anneal the random primers to the RNA template, and allow extension of the primers by incubating the reaction at 37°C for 10 min. Then perform first strand synthesis (reverse transcription) at 60°C for 20 min. for RNA templates ≤ 2 kb in length. Add 10 min. for each additional kb of target length.
6. Perform a sufficient number of cycles (30-50) depending on the abundance of the target:
Denature at 92-95°C for 30 seconds.
Anneal the primers at a temperature 2-5°C below the T_m of the primers for 30 seconds.
Extend the annealed primers at 68°C-72°C for 45-60 seconds for every kb of expected product.

7. RT-PCR Protocol for Use with the Control Mix

The Control Mix contains *E. coli* RNA including the 23S, 16S, and 5S ribosomal species, and two primers (rRNA.5 & rRNA.3). Ribosomal RNAs are G+C-rich and possess a high degree of secondary structure. The use of these primers in RT-PCR yields a 479-bp product from the *E. coli* 16S rRNA that can be detected by agarose gel electrophoresis followed by ethidium bromide staining and UV illumination.

A. Assembly of the Amplification Premixes

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Prepare the two Premixes on ice in the order outlined below:

Premix 1:

10 μ l	Sterile Nuclease-free Water
2.5 μ l	20X RT-PCR Buffer
6 μ l	25 mM MgCl ₂
5 μ l	MasterAmp 10X PCR Enhancer
1 μ l	25 mM MnSO ₄
0.5 μ l	RetroAmp RT DNA Polymerase
<hr/>	
25 μ l	Total reaction volume

Premix 2:

16 μ l	Sterile Nuclease-free Water
8 μ l	2.5 mM dNTP Mix
1 μ l	Control Mix (0.25 μ M each primer final conc., 100 ng RNA)
<hr/>	
25 μ l	Total reaction volume

B. Reverse Transcription and PCR

1. Just prior to starting the reaction, combine both Premixes in an RNase-free PCR tube, on ice.
2. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
3. Perform first strand synthesis (reverse transcription) in a thermal cycler preheated to 60°C for 20 min.
4. Perform 20 cycles of PCR:
Denature at 92°C for 30 seconds.
Anneal and Extend the primers at 68°C for 60 seconds.
5. Analyze 5 μ l of the reaction by agarose gel electrophoresis on a 1% gel. The size of the product is 479 bp.

8. Troubleshooting RT-PCR

Little or no amplification detected

- 1) **Check template quality.** Check the integrity and purity of the template RNA by agarose gel electrophoresis. Organic extraction followed by ethanol precipitation may remove some inhibitors of amplification. Include RiboGuard RNase Inhibitor (Epicentre) (not supplied in the kit) in the RT-PCR to prevent template degradation (see General Considerations).
- 2) **Adjust RNA template concentration.** Increase the amount of RNA template in the RT-PCR.
- 3) **Lower annealing temperature.** Lower the annealing temperature in 2°C increments.
- 4) **Optimize Mg²⁺ concentration.** Perform reactions with varying concentrations of Mg²⁺ starting at 1.5 mM up to 3.5 mM, in 0.5 mM increments. Heat the vial of MgCl₂ at 70°C for 15 minutes and mix vigorously to resuspend any microcrystalline precipitates that may have formed.
- 5) **Alter the concentration of MasterAmp PCR Enhancer in the reaction.** While 1X MasterAmp PCR Enhancer works well for many templates, concentrations of 0X to 2X may work better.
- 6) **Increase number of cycles.** Perform additional cycles in increments of 5.
- 7) **Vary reaction components.** Vary the amount of RetroAmp RT DNA polymerase and primers.

Multiple products or a smear detected

- 1) **Check template quantity and quality.** Check the integrity and purity of the template RNA by agarose gel electrophoresis. Organic extraction followed by ethanol precipitation may remove some inhibitors of amplification. Include RiboGuard RNase Inhibitor (Epicentre) (not supplied in the kit) in the RT-PCR to prevent template degradation (see General Considerations).
- 2) **Decrease concentration of reaction components.** Decrease the amount of enzyme, RNA template or primer added to the reaction
- 3) **Increase annealing temperature.** Increase the annealing temperature in 2°C increments.
- 4) **Alter the concentration of MasterAmp PCR Enhancer in the reaction.** While 1X MasterAmp PCR Enhancer works well for many templates, concentrations of 0X to 2X may work better.
- 5) **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer dimers.
- 6) **Check primers for degradation.** Check by electrophoresis in a denaturing acrylamide gel.

9. References

1. Myers, T.W. and Gelfand, D.H. (1991) *Biochem.* **30**, 7661.
2. Young, K.K.Y. *et al.*, (1993) *J. Clin. Microbiol.* **31**, 882.
3. Mallet, F. *et al.*, (1995) *BioTechniques* **18**, 678.
4. Schanke, J.T. and Grunenwald, H.L. (1997) *Epicentre Forum* **4** (1), 2.
5. Grunenwald, H.L. and Schanke, J.T. (1997) *Epicentre Forum* **4** (1), 4.
6. Mytelka, D.S. and Chamberlin, M.J. (1996) *Nucl. Acids Res.* **24**, 2774.
7. Henke, W. *et al.*, (1997) *Nucl. Acids Res.* **25**, 3957.
8. Weissensteiner, T. and Lanchbury, J.S. (1996) *BioTechniques* **21**, 1102.
9. Rees, W.A. *et al.*, (1993) *Biochemistry* **32**, 137.
10. Santoro, M.M. *et al.*, (1992) *Biochemistry* **31**, 5278.
11. Cline, J. *et al.*, (1996) *Nucl. Acids Res.* **24**, 3546.

*Use of Betaine for DNA Polymerase Reactions, including, but not limited to use for PCR or DNA Sequencing, is covered by, U.S. Patent No. 6,270,962, European Patent No. 0742838, German Patent No. DE4411588C1 and other issued or pending applications in the U.S. and other countries that are either assigned or exclusively licensed to Epicentre. These products are accompanied by a limited non-exclusive license for the purchaser to use the purchased products solely for life science research. Contact Epicentre for information on licenses for uses in diagnostics or other fields.

*Notice to Purchasers, Limited License: Use of this product is covered by one or more of the following US patents and corresponding patent claims outside the US: 5,079,352, 5,789,224, 5,618,711, 6,127,155 and claims outside the US corresponding to US Patent No. 4,889,818. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim (such as the patented 5' Nuclease Process claims in US Patents Nos. 5,210,015 and 5,487,972), no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel. This product is for research use only. Diagnostic uses under Roche patents require a separate license from Roche. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

MasterAmp, MasterPure, RetroAmp, and RiboGuard are trademarks of Epicentre, Madison, Wisconsin.

Tween is a registered trademark of ICI Americas Inc., Wilmington, Delaware.

Visit our technical blog: epicentral.blogspot.com