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MasterAmp™ High Fidelity RT-PCR Kit

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1. Introduction

The MasterAmp™ High Fidelity RT-PCR Kit is an accurate and convenient RNA amplification system. First-strand synthesis is catalyzed by Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) Plus, the most reliable enzyme for full-length cDNA synthesis.[1] Subsequent PCR uses MasterAmp TAQurate™ DNA Polymerase Mix, a unique blend of thermostable DNA polymerases that dramatically reduces the error rate of PCR amplification.[2] The kit also includes a convenient MasterAmp 2X RT-PCR PreMix containing reaction buffer, dNTPs, and $MgCl_2$ that has been optimized for both reverse transcription and PCR amplification.

The MasterAmp High Fidelity RT-PCR Kit provides flexibility when performing RT-PCR reactions as either a one-step or two-step procedure. The one-step procedure is designed for performing first-strand synthesis and PCR in one tube using specific primers supplied by the user.[3-5] This continuous RT-PCR protocol minimizes sample handling and the possible introduction of RNases.

In the two-step procedure first-strand synthesis is performed using either random nonamers or oligo $d(T)_{18}$ primers supplied in the kit. Subsequent PCR is achieved in one of two ways. The modified two-step procedure allows the addition of specific primers and TAQurate DNA Polymerase Mix directly into the reaction mix after the RT step, eliminating the need to set up a separate PCR reaction. The standard two-step RT-PCR can also be performed. In this protocol, a small amount (5-20%) of the RT reaction is removed for subsequent PCR. This variation can be useful when several different primer pairs are used to analyze the same cDNA template.

MasterAmp 10X PCR Enhancer (with betaine)$^+$ has been provided to improve the yield and specificity of amplification reactions, especially those with high GC content or secondary structure.[6-10]

2. Product Specifications

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

Storage Buffers: MMLV-RT Plus is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 1% Triton® X-100. MasterAmp TAQurate DNA Polymerase Mix 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 DTT.

Contaminating Activity Assays: MMLV-RT Plus and MasterAmp TAQurate DNA Polymerase Mix are free of detectable non-specific DNase and RNase activities.

MasterAmp 2X RT-PCR PreMix: 100 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$, and 400 mM each dNTP.

Control Template/Primers Mix: each microliter contains 12.5 pmol of each primer and 100 ng of *E. coli* RNA (see page 7).

Quality Control: The MasterAmp High Fidelity RT-PCR Kit is function-tested by performance of the kit's control reaction (specific priming assay) and in two non-specific priming assays. The first using oligo $(dT)_{18}$ primers and the second using random nonamer primers. Both non-specific priming assays utilize human lung RNA as template.

3. Kit Contents

The MasterAmp™ High Fidelity RT-PCR Kit is available in 25- and 100-reaction sizes. The 100-reaction size kit contains:

Desc.	Concentration	Quantity
MMLV-RT Plus	@ 40 U/µl	4,000 Units
MasterAmp TAQurate DNA Polymerase Mix	@ 1 U/µl	100 Units
MasterAmp 2X RT-PCR PreMix		5 ml
MasterAmp 10X PCR Enhancer		1 ml
Random Nonamer Primer	@ 50 µM	100 µl
Oligo (dT)18 Primer	@ 12.5 µM	100 µl
Control Template/Primers Mix		40 µl
Sterile Nuclease-Free Water		3 ml

4. Related Products

The following products are also available:

- MasterAmp™ RT-PCR Kit for High Sensitivity
- MasterPure™ RNA Purification Kit
- MonsterScript™ Reverse Transcriptase
- MonsterScript™ 1st-Strand cDNA Synthesis Kit
- MMLV Reverse Transcriptase
- MasterAmp™ *Tth* DNA Polymerase
- CopyControl™ cDNA, Gene & PCR Cloning Kit
- 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 55°C or greater for maximum specificity. Researchers may also use oligo (dT) or random primers following either a **Modified or Standard Two-Step Protocol** 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. **Ribonuclease Inhibitors:** We recommend the addition of 20 units of RiboGuard™ RNase Inhibitor (Epicentre, not supplied in the kit) in the reaction when using less than 100 ng of total cellular RNA.
4. **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 without the MasterAmp PCR Enhancer. If reaction optimization is required, a final MasterAmp PCR Enhancer concentration of 1X to 3X may improve the amplification of some target sequences.
5. **Cycling Conditions:** RNA template denaturation before first strand synthesis (reverse transcription) is optional. However, a 5-minute incubation at 65°C may increase the cDNA yield from reverse transcription. If performing denaturation, heat only the RNA
and primers in water at 65°C for 2-5 minutes, then place on ice to cool, before the addition of the RT-PCR PreMix and enzyme. Likewise, template denaturation or enzyme inactivation, for 1 minute at 95°C, between first strand (RT) and second strand synthesis (PCR), is optional. Users may perform annealing and extension simultaneously (1 minute 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 minutes to maximize the amount of full-length product.
6. **Maximizing Sensitivity:** With extremely dilute RNA samples, the addition of 10 ng/µl carrier RNA 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.
7. **Cloning RT-PCR Products:** RT-PCR products generated with MMLV-RT Plus and MasterAmp TAQurate DNA Polymerase Mix have blunt-ends or a non-template encoded adenine residue at the 3' end. Hence, an efficient number of clones can be obtained with either blunt-end cloning or TA Cloning® strategies.

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 Step 4 (first-strand synthesis) in the protocol outlined below, or by performing a "No RNA" control by performing the RT incubation but omitting the MMLV-RT Plus in the reaction.

A. One-Step Continuous RT-PCR Protocol

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 but may be beneficial for some templates.*

Note: *this should be performed before the addition of RT-PCR PreMix and enzymes. For RNA denaturation suggestions see the General Considerations. If using oligo (dT) or random primers, follow the* ***Modified*** *or* ***Standard Two-Step Protocol*** *on page 5.*

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

x µl	Sterile Nuclease-Free Water	
x µl	RNA template	(50 ng-10 µg)
1 µl	12.5 µM gene specific primer 1	(12.5 pmol)
1 µl	12.5 µM gene specific primer 2	(12.5 pmol)
25 µl	MasterAmp 2X RT-PCR PreMix	(1X final concentration)
1 µl	MMLV-RT Plus	(40 Units)
1 µl	MasterAmp TAQurate DNA Polymerase Mix	(1 Unit)
50 µl	Total reaction volume	

2. Thoroughly mix reaction components and store on ice before starting the reaction.
3. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
4. Perform first strand synthesis (reverse transcription) in a thermal cycler preheated to 37°C for 30 minutes for target amplicons ≤1.5 kb in length. Add 10 minutes for each additional kb of target length.
5. Perform a denaturation step at 95°C for 1 minute.
6. 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°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.
7. A final extension step may be performed at 72°C for 4-7 minutes to ensure full primer extension.
8. After amplification, the samples may be kept at 4°C overnight or frozen at –20°C.

B. Modified Two-Step RT-PCR Protocol

Changes for Reaction Assembly and RT-PCR: We recommend using oligo (dT) or random nonamer primers supplied in the kit for synthesis of the first strand (reverse transcription). Users should add two gene-specific primers to the reaction before the start of PCR. Modifications to the one-step protocol are outlined below.

1. Use 25 pmol of oligo $(dT)_{18}$ primer or 100 pmol (~300 ng) of random nonamer primers in the amplification reaction mix.
2. Do not add the MasterAmp TAQurate DNA Polymerase Mix to the first strand synthesis reaction.
3. Adjust the amount of water included in the reaction mix to insure a final volume of 50 µl at the start of PCR.
4. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
5. If using random primers, preincubate reactions at room temperature for 10 minutes.
6. Perform first strand synthesis (reverse transcription) at 37°C-42°C for 30 minutes for RNA templates ≤1.5 kb in length. Add 10 minutes for each additional kilobase of target length.
7. Add 12.5 pmol each of two gene-specific primers.
8. Add 1 Unit of MasterAmp TAQurate DNA Polymerase Mix.
9. Denature at 95°C for 1 minute.
10. 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°C-5°C below the Tm 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.

C. Standard Two-Step RT-PCR Protocol

Changes for Reaction Assembly and RT-PCR: We recommend a standard two-step RT-PCR reaction protocol when multiple primer pairs will be used to amplify from cDNA generated with random, oligo (dT) or degenerate primers. The cDNA can be generated in the first step and then used as a template in subsequent PCR amplification reactions.

1. Use 25 pmol of oligo $(dT)_{18}$, 25 pmol of specific 3′ primer or 100 pmol of random nonamer primers in the first strand synthesis reaction (reverse transcription).
2. Do not add the MasterAmp TAQurate DNA Polymerase Mix to the first strand synthesis reaction.
3. Proceed with the reverse transcription as described in Part A, Step 4.
4. After reverse transcription, set up new reaction tubes for the PCR amplification step. Use 5%-20% of the reverse transcription reaction (cDNA) as template in 50-µl PCR reactions containing 12.5 pmol of each specific primer, 1X MasterAmp RT-PCR PreMix (final concentration), and 1 Unit MasterAmp TAQurate DNA Polymerase Mix.
5. Perform a sufficient number of cycles (30-50) depending on the abundance of the target.

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 GC-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.

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice.
   Combine all of the reagents outlined below on ice:

22	µl	Sterile Nuclease-Free Water	
25	µl	MasterAmp 2X RT-PCR PreMix	(1X final concentration)
1	µl	MMLV-RT Plus	(40 Units)
1	µl	MasterAmp TAQurate DNA Polymerase Mix	(1 Unit)
1	µl	Control Mix	(0.25 µM each primer final conc., 100 ng RNA)
50	µl	Total reaction volume	

2. Thoroughly mix reaction components and store on ice before starting the reaction.
3. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
4. Perform first strand synthesis (reverse transcription) in a thermal cycler preheated to 37°C for 30 minutes.
5. Perform a denaturation step at 95°C for 1 minute.
6. Perform 20 cycles of PCR:
   Denature at 92°C for 30 seconds.
   Anneal and Extend the primers at 68°C for 60 seconds.
7. Analyze 5 µl 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 an RNase inhibitor 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) **Alter the concentration of MasterAmp PCR Enhancer in the reaction.** Add increasing amounts of MasterAmp PCR Enhancer to a final concentration of 1X to 3X.
5) **Increase number of cycles.** Perform additional cycles in increments of five.
6) **Vary reaction components.** Vary the amount of MMLV-RT Plus, MasterAmp TAQurate 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 an RNase inhibitor 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.** Add increasing amounts of MasterAmp PCR Enhancer to a final concentration of 1X to 3X.
5) **Decrease concentration of reaction components.** Decrease the amount of enzyme, RNA template, or primer added to the reaction.
6) **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer dimers.
7) **Check primers for degradation.** Check by electrophoresis in a denaturing acrylamide gel.

9. References

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