Manual

MasterAmp T*th* DNA Polymerase

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GENOMIC ANALYSIS BY LGC

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1. Introduction

MasterAmp T*th* DNA Polymerase is a thermostable DNA Polymerase from *Thermus thermophilus*. The enzyme displays optimal activity at temperatures between 70 °C and 74 °C, and it is useful for performing the polymerase chain reaction (PCR)* or other reactions requiring DNA synthetic activity at high temperatures. T*th* DNA polymerase has an intrinsic 5′→3′ exonuclease activity, but lacks a 3′→5′ (proofreading) nuclease activity. Furthermore, T*th* has a potent Mn-dependent reverse transcriptase activity.[1] The enzyme is provided with MasterAmp T*th* 20X PCR Buffer, MasterAmp 10X PCR Enhancer (with betaine), and 25 mM $MgCl_2$, and $MnSO_4$ Solutions to allow optimization of individual template-primer pair combinations.The presence of betaine (trimethyl glycine) in the MasterAmp 10X PCR Enhancer substantially improves the yield and specificity of amplification of many target sequences, especially those containing a high G+C content or secondary structure.[2-4] Betaine, a natural osmoprotectant, lowers the melting temperature of G+C rich regions to a temperature more similar to A+T rich regions.[5] This results in destabilization of double-stranded DNA which limits polymerase pausing,[2] thereby increasing the yield of full-length product. In addition, betaine also may enhance PCR by protecting DNA polymerases from thermal denaturation.[6] The effects of betaine seem to be independent of the polymerase used, though the concentration of betaine required for amplification varies with the target sequence.[2,3]

2. Product designations and kit components

Product	Kit size	Catalog number	Reagent description	Part numbers	Volume
MasterAmp T*th* DNA Polymerase	250 Units	TTH72250	MasterAmp T*th* DNA Polymerase (5 Unit/µL)	E0097-5D1	50 µL
			MasterAmp 10X PCR Enhancer (with Betaine)	SS000026-D1	3 mL
			$MgCl_2$, 25 mM	SS000136-D1	750 µL
			$MnSO_4$, 25 mM	SS000144-D1	500 µL
			20X T*th* DNA Polymerase Reaction Buffer	SS000322-D1	1.5 mL

3. Product specifications

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

Storage Buffer: MasterAmp T*th* 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.

Contaminating Activity Assays: MasterAmp T*th* DNA Polymerase is free of detectable non-specific DNase and RNase activities.

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.

20X T*th* DNA Polymerase Reaction Buffer: 400 mM $(NH_4)_2SO_4$ and 1.0 M Tris-HCl (pH 9.0). Separate solutions of MasterAmp 10X PCR Enhancer, 25 mM $MgCl_2$, and 25 mM $MnSO_4$ are also provided to allow optimization of individual reactions.

Enzyme Characteristics:

5′→3′ Exonuclease Activity	Yes
3′→5′ Exonuclease Activity	No
Terminal Transferase Activity	Yes
Extension Rate (72 °C)	2-4 kb/min
Processivity	50-60 bases
Half-life	10 min (97 °C), 40 min (95 °C)
Reverse Transcriptase Activity (Mn^{2+} dependent)	Yes

4. General considerations

1. **Template:** DNA prepared using standard isolation techniques is a suitable substrate for amplification. Nevertheless, numerous compounds inhibit amplification including ionic detergents, some gel loading dyes, phenol, and hemin. When purifying templates from agarose gels, minimise exposure to UV irradiation to prevent formation of pyrimidine dimers. Assembly of reactions in a clean area or using positive displacement pipettors with aerosol-barrier tips will minimise the risk of contamination from extraneous DNA templates. The optimal amount of template for a single-copy gene is between 10^4-10^6 copies (i.e., approximately 0.1-10 ng of *E. coli* genomic DNA), though this may vary depending on the source and quality of the template.[7,8]
2. **Primer design:** Primers typically are 15-30 bases in length and contain approximately 50% G+C residues; the annealing temperatures of primer pairs should be nearly identical. Care must be taken to design primers that do not form hairpin loop structures or are self-complementary. The 5′ end of a primer may contain bases that are not complementary with the template; however, the 3′ end of the primer must be complementary with the template.
3. **Reaction components:** DNA polymerases vary in their requirements for salt and pH, and therefore enzymatic activity will also vary depending on the reaction buffer. Mg^{2+} concentration is particularly critical for amplification of a specific target. The ratio of primer to template is important for controlling the specificity and efficiency of amplification; an excess of primer ensures that the denatured template molecules bind to the primers instead of binding to the complementary DNA strand.[9] Nevertheless, too much primer may lead to the formation of nonspecific products or primer dimers.
4. **Choice of enzyme:** Not all templates will be synthesised with equal efficiency by the various DNA polymerases. If amplification is not achieved with one enzyme, and after attempts to optimise fail, another enzyme should be chosen. Furthermore, use of a polymerase containing proofreading activity may be useful where fidelity or length of product is important.

5. **Cycling parameters:** Many parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing and elongation; ramp speed; and total cycle number. Amplification beyond ~10^{12} molecules may also result in the appearance of nonspecific products; if the starting number of template molecules is 10^5, 28-30 cycles will yield 10^{12} molecules.[7] Additional variations such as use of a hot start[10] or touchdown/stepdown PCR[11,12] can dramatically improve specificity and yield (see Troubleshooting Amplification Reactions).

5. Suggested PCR protocol

Use the following protocol as a guideline for establishing the parameters necessary for amplification of experimental templates. The goals of the individual experiment (e.g., fidelity versus yield) will influence many aspects of the reaction, including the amount of various reaction components, primer design, cycling parameters, and choice of DNA polymerase[7,9,13] (see General Considerations). Assembly of amplification reactions in a clean area and using positive displacement pipettors with aerosol-barrier tips will minimise the risk of contamination from extraneous DNA templates.

A. Assemble the Reaction Master Mix

Preparation of reaction "master mixes" simplifies the assembly of multiple amplification reactions. The volumes listed below are for one 50 µL amplification reaction. Assemble an amount of mix corresponding to the total number of reactions. Extra mix may be required to offset losses caused by pipeting. We recommend duplicate reactions (+ and − MasterAmp 10X PCR Enhancer) for new template-primer pair combinations.

1. Thaw and thoroughly mix all of the reagents listed below before dispensing; place on ice. Combine on ice, all of the following except for the DNA template (see Note below) in tubes labeled "+" and "−" MasterAmp Enhancer:

x	µL sterile water	
2.5	µL 20X T*th* DNA Polymerase Reaction Buffer	(1X final concentration)
2-10	µL 25 mM $MgCl^2$ Reaction Buffer	(1-5 mM final concentration)
0 or 15	µL MasterAmp 10X PCR Enhancer	(3X final concentration)
4	µL 2.5 mM dNTP Mix	(200 µM final concentration each)
0.5-1.25	µL 20 µM Primer 1	(0.2-0.5 µM final concentration)
0.5-1.25	µL 20 µM Primer 2	(0.2-0.5 µM final concentration)
0.25	µL MasterAmp T*th* DNA Polymerase	(1.25 Units)
y	µL DNA Template (see Note)	(1-1000 ng)
50	µL Total reaction volume	

Note: The DNA template is added directly to the individual PCR tubes and is not included in the tubes of reaction mix. (See step 2 below.)

B. Assemble the reactions

1. Thoroughly mix the tube of reaction mix and aliquot into the individual PCR tubes.
2. Add the DNA template to the PCR tubes and mix.
3. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
4. Program the thermal cycler following the recommendations provided by the manufacturer. A suggested program is outlined below.
   a) Initially denature the template at 92-95 °C for 3-5 minutes.
   b) Perform a 2-step or 3-step cycling program, for 20-50 cycles as required:
      Denature at 92-95 °C for 0.5-1.0 minutes.
      Anneal the primers at a temperature 5 °C below the Tm of the primers for 0.5 minute.
      Extend the annealed primers at 68-72 °C for 1 minute for every kb of expected product.
   c) A final extension may be performed at 68-72 °C for 10-30 minutes to ensure full extension and to increase the likelihood of 3′-uncoded nucleotide addition (3′-terminal A overhang).
5. Place the tubes in the thermal cycler and begin cycling.
6. After amplification, the samples may be kept at 4 °C overnight or frozen at -20 °C.

6. Suggested RT-PCR protocol

A. Assemble the Reaction Mix

Preparation of a reaction mix simplifies the assembly of multiple amplification reactions. The volumes listed below are for one 50 µL amplification reaction. Assemble an amount of mix corresponding to the total number of reactions. Extra mix may be required to offset losses caused by pipeting. We recommend using 1X MasterAmp PCR Enhancer (final concentration) with new RT-PCR template-primer pair combinations. If successful amplification is not achieved, then perform subsequent reactions using either a final concentration of 0X or 2X MasterAmp PCR Enhancer. Note that the optimal concentrations of MasterAmp PCR Enhancer (0-2X), $MnSO_4$ (0.5-0.75 mM), $MgCl_2$ (1.5-3 mM), and dNTPs (200-400 µM) may vary with each template.

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

x	µL sterile water	
2.5	µL 20X T*th* DNA Polymerase Reaction Buffer	(1X final concentration)
6	µL 25 mM $MgCl_2$	(3 mM final concentration)
8	µL 2.5 mM dNTP Mix	(400 µM final concentration each)
5	µL MasterAmp 10X PCR Enhancer	(1X final concentration)
1	µL 25 mM $MnSO_4$	(0.5 mM final concentration)
1.25	µL 20 µM Primer 1	(0.5 µM final concentration)
1.25	µL 20 µM Primer 2	(0.5 µM final concentration)
y	µL RNA Template	(1-10 µg)
0.5	µL MasterAmp T*th* DNA Polymerase	(2.5 Units)
50	µL Total reaction volume	

B. Assemble the reactions

1. Thoroughly mix the tube of reaction mix and aliquot into the individual PCR tubes.
2. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
3. For RNA templates up to 2 kb, synthesise the first strand by incubating the tubes at 60 °C for 20 min. Add 10 minutes of incubation time for each additional kb.
   If using a primer with a T_m of less than 60 °C (e.g., $oligo(dT)_{18}$), incubate the reactions at an appropriate annealing temperature for 5 min before performing the synthesis at 60 °C.
4. Program the thermal cycler following the recommendations provided by the manufacturer.
   Perform a 2-step or 3-step cycling program, for 30-50 cycles as required:
   Denature at 92-95 °C for 0.5-1.0 minutes.
   Anneal the primers at a temperature 5 °C below the Tm of the primers for 0.5 minute.
   Extend the annealed primers at 68-72 °C for 1 minute for every kb of expected product.
5. Place the tubes in the thermal cycler and begin cycling.
6. After amplification, the samples may be kept at 4 °C overnight or frozen at -20 °C.

7. Troubleshooting amplification reactions

Little or no amplification detected

1) **Lower annealing temperature.** Lower the annealing temperature in 2 °C increments.
2) **Optimise Mg^{2+} concentration.** Perform reactions with varying concentrations of Mg^{2+} starting at 0.5 mM up to 5.0 mM, in 0.5 mM increments. Heat the vial of $MgCl_2$ at 70 °C for 15 minutes and mix vigorously to resuspend any microcrystalline precipitates that may have formed.

3) **Perform hot start.**[10] The final assembly of amplification reactions at temperatures above the reaction annealing temperature improves both the yield and specificity. Assemble the reactions without Mg^{2+}; subsequently place the reactions in a thermal cycler heated to >80 °C, then add the appropriate amount of $MgCl_2$ and begin the cycling protocol. Alternatively, a modified hot start may be performed in which reactions assembled on ice are added directly to a thermal cycler pre-heated to 92-95 °C.
4) **Perform touchdown (TD)/stepdown (SD) PCR.**[7,11,12] TD or SD PCR consists of a series of cycles that are performed at decreasing annealing temperatures. This protocol favors amplification of the target at temperatures greater than or equal to the optimum annealing temperature, while enhancing target yield in later cycles at annealing temperatures below the T_m of the reaction. Perform the initial series of cycles at an annealing temperature a few degrees above the calculated T_m of the primers; annealing temperature is lowered by 1-4 °C every other cycle to ~10 °C below the calculated T_m. A hot start must be performed if using a TD or SD cycling protocol.
5) **Alter the concentration of MasterAmp PCR Enhancer in the reaction.** While 3X MasterAmp PCR Enhancer works well for many templates, concentrations of 1X, 2X, or 4X may work better.
6) **Increase initial template denaturation time or temperature.** Increase the temperature of initial denaturation up to 95 °C. Increase the length of initial template denaturation up to five minutes. Alternatively, denature the template by heating at 72 °C for 10 minutes in the presence of 50 mM NaOH before amplification.
7) **Increase number of cycles.** Perform additional cycles in increments of five.
8) **Vary reaction components.** Vary the amount of DNA polymerase and primers. Try using an alternative DNA polymerase.
9) **Check template quantity and quality.** Check the concentration of template DNA by agarose gel electrophoresis or fluorimetry. Use 10^4-10^6 molecules of template for each reaction (e.g., up to ng amounts for cloned templates or µg amounts for genomic DNA).[7,12] Organic extraction followed by ethanol precipitation may remove some inhibitors of amplification.
10) **Increase extension time.** Increase the extension time, generally 1 minute for every kb of product.

Multiple products or a smear detected

1) **Decrease concentration of reaction components.** Check the concentration of template DNA by agarose gel electrophoresis or fluorimetry. Use 10^4-10^6 molecules of template for each reaction (e.g., up to ng amounts for cloned templates or µg amounts for genomic DNA).[7,12] Decrease the amount of magnesium, enzyme, and primer added to the reaction.
2) **Increase annealing temperature.** Increase the annealing temperature in 2 °C increments.
3) **Perform hot start.** [10] The final assembly of amplification reactions at temperatures above the reaction annealing temperature improves both the yield and specificity. Assemble the reactions without Mg^{2+}; subsequently place the reactions in a thermal cycler heated to >80 °C, then add the appropriate amount of $MgCl_2$ and begin the cycling protocol. Alternatively, a modified hot start may be performed in which reactions assembled on ice are added directly to a thermal cycler pre-heated to 92-95 °C.

4) **Perform touchdown (TD)/stepdown (SD) PCR.** [7,11,12] TD or SD PCR consists of a series of cycles that are performed at decreasing annealing temperatures. This protocol favors amplification of the target at temperatures greater than or equal to the optimum annealing temperature, while enhancing target yield in later cycles at annealing temperatures below the T_m of the reaction. Perform the initial series of cycles at an annealing temperature a few degrees above the calculated T_m of the primers; annealing temperature is lowered by 1-4 °C every other cycle to ~10 °C below the calculated T_m. A hot start must be performed if using a TD or SD cycling protocol.
5) **Alter the concentration of MasterAmp PCR Enhancer in the reaction.** While 3X MasterAmp PCR Enhancer works well for many templates, concentrations of 1X, 2X, or 4X may work better.
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.
8) **Decrease number of cycles.** Decrease number of cycles in increments of five.

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9. Further support

If you require any further support, please do not hesitate to contact our Technical Support Team: techsupport@lgcgroup.com.

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