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MasterAmp™ PCR Optimization Kits
MasterAmp™ 2X PCR PreMixes

Cat. Nos. MOS001, MO7201, and MO7205A→L

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1. Introduction

MasterAmp™ PCR Optimization Kits (without ammonium sulfate: for MasterAmp *Taq* and MasterAmp AmpliTherm™ DNA Polymerases) provide a complete buffer system for the optimization of individual template-primer pair combinations. The twelve MasterAmp 2X PCR PreMixes contain a buffered salt solution with all four deoxynucleotides, $MgCl_2$, and MasterAmp PCR Enhancer (with betaine).[+] Individual MasterAmp 2X PCR PreMixes are available separately for repeated amplification of a specific template-primer combination.

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.[1-5] Betaine, a natural osmoprotectant, lowers the melting temperature of G+C rich regions to a temperature more similar to A+T rich regions.[6] This results in destabilization of double-stranded DNA which limits polymerase pausing,[3] thereby increasing the yield of full-length product. In addition, betaine also may enhance PCR by protecting DNA polymerases from thermal denaturation.[7] 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.[1-4]

Also available are the MasterAmp PCR Optimization Kit containing ammonium sulfate and MasterAmp 2X PCR PreMixes containing ammonium sulfate, which are recommended for use with MasterAmp *Tfl* and MasterAmp *Tth* DNA Polymerases, and with proofreading DNA polymerases (such as *Pfu* or *Pwo*) that contain 3′→5′ exonuclease activity.

2. Product Specifications

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

Note: *Some of the PreMixes may not freeze completely.*

Contaminating Activity Assays: MasterAmp PCR Optimization Kits and individual MasterAmp 2X PCR PreMixes are free of detectable DNase and RNase activities as judged by agarose gel electrophoresis following over-digestion assays.

Quality Control: MasterAmp PCR Optimization Kits and individual MasterAmp 2X PCR PreMixes are function-tested in PCR reactions using AmpliTherm DNA Polymerase, and lambda phage *cII* gene and human *Apo*E gene segments as target templates.

MasterAmp 2X PCR PreMixes: The MasterAmp 2X PCR PreMixes contain 100 mM Tris-HCl (pH 8.3), 100 mM KCl, and 400 µM each dNTP. The concentrations of $MgCl_2$ (3-7 mM) and MasterAmp PCR Enhancer (0-8X) vary with the individual mixes.

3. Related Products

The following products are also available:

- MasterAmp™ PCR Optimization Kits with ammonium sulfate
- MasterAmp™ AmpliTherm™, *Taq*, *Tth*, and *Tfl* DNA Polymerases
- MasterAmp™ RT-PCR Kits
- MasterPure™ Nucleic Acid Purification Kits
- MasterAmp™ Buccal Swab DNA Extraction Kits
- BuccalAmp™ DNA Extraction Kits
- dNTP Solutions

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, minimize 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 minimize 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.[8,9]
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.[10] Nevertheless, too much primer may lead to the formation of nonspecific products or primer dimers.
4. **Choice of Enzyme:** Not all templates will be synthesized with equal efficiency by the various DNA polymerases. If amplification is not achieved with one enzyme, and after attempts to optimize 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.[8] Additional variations such as use of a hot start[11] or touchdown/stepdown PCR[12,13] 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[8,10,14] (see General Considerations). Assembly of amplification reactions in a clean area and using positive displacement pipettors with aerosol-barrier tips will minimize the risk of contamination from extraneous DNA templates.

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.

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 MasterAmp 2X PCR PreMixes A-L (see Step 2 below):

x	µl	sterile water
0.2-0.5	µM	primer 1 (final concentration)
0.2-0.5	µM	primer 2 (final concentration)
x	µl	DNA Template (1-1000 ng, 10^4-10^6 molecules)
x	µl	thermostable DNA polymerase
25	µl	Total volume

2. On ice, aliquot 25 µl of each MasterAmp 2X PCR PreMix into an individual PCR tube.
3. Add 25 µl of the template-primer-enzyme solution to the PCR tubes and mix.
4. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
5. Program the thermal cycler following the recommendations provided by the manufacturer.

   A suggested program is outlined below.

   a) Initially denature the template at 92°C-95°C for 3-5 minutes.

   b) Perform a 2- or 3-step cycling program, for 20-50 cycles as required:

      Denature at 92°C-95°C for 0.5-1.0 minutes.

      Anneal the primers at a temperature 5°C below the T_m of the primers for 0.5 minute.

      Extend the annealed primers at 68°C-72°C for 1 minute for every kb of expected product.

c) A final extension may be performed at 68°C-72°C for 10-30 minutes to ensure full extension and to increase the likelihood of 3′-uncoded nucleotide addition (3′-terminal A overhang).

6. Place the tubes in the thermal cycler and begin cycling.
7. After amplification, the samples may be kept at 4°C overnight or frozen at –20°C.

6. Troubleshooting Amplification Reactions

Little or no amplification detected

1) **Lower annealing temperature.** Lower the annealing temperature in 2°C increments.
2) **Perform hot start.**[11] 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°C-95°C.
3) **Perform Touchdown (TD)/Stepdown (SD) PCR.**[8,12,13] 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°C-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.
4) **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 5 minutes. Alternatively, denature the template by heating at 72°C for 10 minutes in the presence of 50 mM NaOH before amplification.
5) **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or primer dimers.
6) **Increase number of cycles.** Perform additional cycles in increments of five.
7) **Vary reaction components.** Vary the amount of DNA polymerase and primers. Try using an alternative DNA polymerase.
8) **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).[8,13] Organic extraction followed by ethanol precipitation may remove some inhibitors of amplification.
9) **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).[8,13] 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.**[11] 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°C-95°C.
4) **Perform Touchdown (TD)/Stepdown (SD) PCR.**[8,12,13] 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°C-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) **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.
7) **Decrease number of cycles.** Decrease number of cycles in increments of five.

7. References

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