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MasterAmp™ Extra-Long PCR Kit
MasterAmp™ Extra-Long PCR 2X PreMixes
MasterAmp™ Extra-Long DNA Polymerase Mix

Cat. Nos. MHF9220, MHF925A→I, QU92125, QU92500, and QU9201K

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1. Introduction

The MasterAmp™ Extra-Long PCR Kit* is a complete system for rapid, consistent, and accurate amplification of long PCR products (>5-40 kb). This kit improves upon the Barnes technology[1] and permits efficient amplification of up to approximately 40 kb from lambda DNA, 28 kb from human DNA, and 30 kb from E. coli DNA. MasterAmp Extra-Long DNA Polymerase Mix combines MasterAmp Taq DNA Polymerase with a 3′→5′ proofreading enzyme to achieve PCR fidelity at least three times better than Taq DNA polymerase alone. The nine MasterAmp Extra-Long PCR 2X PreMixes contain a buffered salt solution with nucleotides, Mg^{2+}, and MasterAmp 10X PCR Enhancer (with betaine).+ The nine individual 2X PreMixes are available separately for repeat amplification of a specific template/primer pair combination. Since all other components are included in the PreMixes, only DNA polymerase, template, and primers must be added to the reaction.

The betaine component of the MasterAmp 10X PCR Enhancer substantially improves amplification yield and specificity for many target sequences, especially those containing high GC content or secondary structure.[2-7] In addition, betaine may enhance PCR by protecting DNA polymerases from thermal denaturation.[8]

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.

MasterAmp Extra-Long DNA Polymerase Mix Storage Buffer: MasterAmp Extra-Long 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.

Unit Definition: One unit of polymerase enzyme converts 10 nmol of dNTPs into acid-insoluble material in 30 minutes at 74°C under standard assay conditions.

MasterAmp Extra-Long PCR 2X PreMixes: The Extra-Long PCR 2X PreMixes contain Extra-Long PCR 2X Buffer and 400 µM of each dNTP. The concentrations of Mg^{2+} (3-7 mM) and MasterAmp 10X PCR Enhancer (with betaine) (0-6X) vary with the individual PreMixes.

*,+ See page 7 for patent and licensing information.

3. Kit Contents

Cat. #	Concentration	Quantity
MasterAmp™ Extra-Long DNA Polymerase Mix	2.5 U/µl	50 µl
Control Lambda DNA Template/Primer		30 µl

MasterAmp™ Extra-Long PCR 2X PreMixes 1-9 each at 300 µl
MHF9220 — 1 Kit

MasterAmp™ Extra-Long PCR 2X Premixes
(Sold separately, each at 5 ml)

PreMix 1 ..... Cat. # MHF925A
PreMix 2 ..... Cat. # MHF925B
PreMix 3 ..... Cat. # MHF925C
PreMix 4 ..... Cat. # MHF925D
PreMix 5 ..... Cat. # MHF925E
PreMix 6 ..... Cat. # MHF925F
PreMix 7 ..... Cat. # MHF925G
PreMix 8 ..... Cat. # MHF925H
PreMix 9 ..... Cat. # MHF925I

MasterAmp™ Extra-Long DNA Polymerase Mix
(Sold separately at 2.5 U/µl)

QU92125	125 Units
QU92500	500 Units
QU9201K	1000 Units

4. Related Products

The following products are also available:

- MasterPure™ Nucleic Acid Purification Kits
- dNTP Solutions

5. General Considerations

1. **Template:** The purity and integrity of the template DNA is very critical to the success of long PCR amplification. DNA prepared using standard isolation techniques is generally suitable 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. Assemble reactions in a clean area or use positive displacement pipettors with aerosol-barrier tips to minimize contamination risk from extraneous DNA templates. The optimal template amount for long PCR amplification may be 4-5 times greater than standard PCR. For example, 1 ng of lambda DNA, 100 ng of *E. coli* DNA or 100-500 ng of human DNA is typically used for long PCR, though this may vary depending on the source and quality of the template, as well as the length of the PCR product.[9]
2. **Primer Design:** We recommend designing primers 20-30 bases in length containing 40%-60% GC residues with melting temperatures of 62°C-70°C. Optimal primer pair annealing temperatures should be within 2-5 degrees of each other. Take care to design primers that do not form hairpin loop structures or are self-complementary.

The 5′ primer end may contain bases not complementary to the template; however, the 3′ primer end must be complementary to the template. Primers of 30-35 bases in length are also commonly used for long PCR to achieve high specificity.

3. **Reaction Components:** Mg^{2+} concentration is particularly critical for amplification of a specific target. Excess Mg^{2+} results in accumulation of nonspecific amplification products, while insufficient Mg^{2+} results in reduced yield of the desired PCR product. The primer-to-template ratio is important for controlling the amplification specificity and efficiency; primer excess ensures the denatured template molecules bind to the primers instead the complementary DNA strand.[10] Nevertheless, too much primer may lead to nonspecific product formation or primer dimers. 200 µM (final concentration) of each dNTP (already included in the 2X Long PCR PreMixes), is sufficient for most PCR amplification. However, if desirable, additional dNTPs may be added to the reaction (dNTP stock solutions are available separately).
4. **Cycling Parameters:** Many parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing, and extension, as well as ramp speed, and cycle number. Moderate denaturation temperatures and short incubation periods will help maintain the integrity of long DNA templates. Programming an increase in extension time automatically for later cycles may also improve the yields of long amplification. 20-30 cycles of amplification is typically sufficient, since amplification beyond ~10^{12} molecules may result in nonspecific products.[8] Additional variations such as use of a "hot start"[11] or touchdown/stepdown PCR[12,13] may also dramatically improve specificity and yield.
5. **DNA Polymerase:** Amplification of sequences longer than 5 kb may be difficult with Taq DNA polymerase alone. Therefore, inclusion of a small amount of 3′→5′ proofreading DNA polymerase is commonly recommended for amplifications longer than 5 kb. Addition of larger DNA polymerase amounts than for standard PCR may also be necessary to achieve longer amplification.
6. **Cosolvents:** Cosolvents are typically used to improve PCR amplification. Glycerol, gelatin[14], DMSO[15], and betaine[16] can improve long PCR amplification. Varying concentrations of MasterAmp 10X PCR Enhancer (with betaine) are already included in the MasterAmp Extra-Long PCR 2X PreMixes for fast and convenient long PCR optimization.

6. Suggested PCR Protocol

Assemble amplification reactions in a clean area and use positive displacement pipettors with aerosol-barrier tips to minimize contamination risk from extraneous DNA templates.

7. Assembly of Amplification Reactions

The volumes listed below are for one amplification reaction containing a total reaction volume of 50 µl. Assemble an appropriate amount of mix corresponding to the total number of reactions, and prepare nine tubes for each DNA template. (Preparation of an extra volume of mix may be useful to offset pipetting losses.) Assemble the reactions at room temperature.

Note: *We have found "hot start" to be unnecessary for most amplifications when using this kit.*

1. Thaw and thoroughly mix all of the reagents before dispensing. Combine the following:

0.2-1	µM	Forward Primer (final concentration)
0.2-1	µM	Reverse Primer (final concentration)
x	µl	DNA template (4-5 times more DNA than standard PCR may be required)
x	µl	sterile water to a reaction volume of 24 µl
1	µl	MasterAmp Extra-Long DNA Polymerase Mix
25	µl	Total reaction volume

2. Aliquot 25 µl of each MasterAmp Extra-Long PCR 2X PreMix into an individual PCR tube.
3. Add 25 µl of the template-primer-enzyme solution to the PCR tubes and mix well.
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-98°C for 1-2 minutes.

   b) Perform a 2- or 3-step cycling program, for 20-30 cycles as required:

      2-step cycling program:

      Denature at 92°C-98°C for 0.5-1 minute.

      Anneal and Extend at 68°C-72°C for 1 minute per kb of expected product.

      3-step cycling program:

      Denature at 92°C-98°C for 0.5-1 minute.

      Anneal the primers at a temperature 2°C-5°C below the T_m of the primers for 0.5-1 minute.

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

   c) Alternatively, extend for 1 minute per kb for the first 10-15 cycles, then lengthen the extension time 10 to 20 seconds for each of the next 10-15 cycles.
6. After amplification, the samples may be kept at 4°C overnight or frozen at –20°C.

8. Amplification of the Control DNA Template

The control PCR amplification is designed to amplify a 20-kb region of lambda DNA in MasterAmp Extra-Long PCR 2X PreMixes 4 and 6. Users may also observe weak amplification with Extra-Long PCR 2X PreMixes 1, 8, and 9.

1. Thaw and thoroughly mix all of the reagents before dispensing. Combine the following at room temperature:

21	µl	sterile water
3	µl	control template & primers (1 ng DNA and 1 µM each primer, final concentration)
1	µl	MasterAmp Extra-Long DNA Polymerase Mix
25	µl	Total reaction volume

2. Aliquot 25 µl of each MasterAmp Extra-Long PCR 2X PreMix into an individual PCR tube.
3. Add 25 µl of the template-primer-enzyme solution to the PCR tubes and mix well.
4. Add mineral oil and centrifuge briefly if using a thermal cycler without a heated lid.
5. Denature the template at 94°C for 1 minute.
6. Perform 20 cycles as follows:
   Denature at 98°C for 20 seconds.
   Anneal the primers at 56°C for 1 minute.
   Extend the annealed primers at 68°C for 20 minutes.
7. After amplification, the samples may be kept at 4°C overnight or frozen at –20°C.
8. Analyze the amplification products by agarose gel electrophoresis.

9. Troubleshooting Amplification Reactions

If Little or No Desired PCR Product is Detected:

1. **Check template quantity and quality.** Increase the amount of template DNA. Check template DNA quality by agarose gel electrophoresis. Organic extraction followed by ethanol precipitation may remove some inhibitors of amplification.
2. **Check DNA polymerase concentration.** Increase the DNA polymerase concentration in increments of 0.5 units per 100-µl reaction.
3. **Lower annealing temperature.** Lower the annealing temperature in 2°C increments.
4. **Perform hot start.**[11] The assembly of amplification reactions at temperatures above the reaction annealing temperature improves both the yield and specificity. Assemble the reactions without the primers or DNA polymerase; subsequently place the reactions in a thermal cycler heated to >80°C, then add the appropriate amount of each primer or DNA polymerase 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 that is preheated to 92°C-98°C.
5. **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; then lower the annealing temperature by 1°C-4°C every other cycle to approximately 10°C below the calculated T_m. A hot start must be performed if using a TD or SD cycling protocol.

6. **Check the initial denaturation time or temperature.** Lengthen or shorten denaturation time in increments of five seconds. Increase or decrease denaturation temperature in increments of 1°C.
7. **Increase extension time.** Increase the extension time, generally one minute for every kilobase of product.
8. **Increase number of cycles.** Perform additional cycles in increments of five.
9. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or anneal to one another.

If Multiple Product Bands or Smear is Detected:

1. **Decrease concentration of reaction components.** Decrease the amount of DNA template, DNA polymerase, or primers in the reaction mix.
2. **Increase annealing temperature.** Increase the annealing temperature in 2°C increments.
3. **Perform hot start.**[11] The assembly of amplification reactions at temperatures above the reaction annealing temperature improves both the yield and specificity. Assemble the reactions without the primers or DNA polymerase; subsequently place the reactions in a thermal cycler heated to >80°C, then add the appropriate amount of each primer or DNA polymerase 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 that is preheated to 92°C-98°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; then lower the annealing temperature by 1°C-4°C every other cycle to approximately 10°C below the calculated T_m. A hot start must be performed if using a TD or SD cycling protocol.
5. **Decrease number of cycles.** Decrease number of cycles in increments of five.
6. **Decrease extension time.** Reduce the extension time in increments of 1 minute.
7. **Check primers for degradation.** Check primer integrity by electrophoresis on a denaturing polyacrylamide gel.
8. **Redesign primers.** Design primers that have higher annealing temperatures and do not form hairpin loops or anneal to one another.

10. References

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