LONG PCR ENZYME MIX

The Polymerase Chain Reaction (PCR) using a single DNA polymerase is generally limited to amplifications up to 3- 8 kb. Long PCR Enzyme Mix is an efficient and robust PCR system, designed for long PCR or for synthesis of PCR fragments with problematic sequences. Fermentas' Long PCR Enzyme Mix contains a unique blend of a high processive *Taq* DNA polymerase and second thermostable polymerase that exhibits 3' $\rightarrow$ 5' exonuclease (proofreading) activity.

The composition of Long PCR Buffer gives DNA strands greater protection against depurination and nicking during thermal cycling. Long PCR Enzyme Mix and Long PCR buffer amplify genomic and viral DNA targets in excess of 21 kb and 47 kb, respectively.

The fidelity of Long PCR Enzyme Mix is 3 times greater than that of *Taq* DNA polymerase alone.

Long PCR Mix offers significant advantages over conventional PCR using single, non- proofreading polymerases including: **increased range, increased fidelity, increased efficiency and greater yields.**

PCR products generated using Long PCR Enzyme Mix have mainly single dA overhangs at the 3'-end; cloning of PCR products by the T/A method is recommended. For blunt end cloning, the PCR product has to be polished with Klenow fragment or T4 DNA polymerase.

COMPONENTS OF THE KIT

Component	#K0181 40-50 rxns of 50 µl	#K0182 200-400 rxns of 50 µl
Long PCR Enzyme Mix, 5 u/µl Enzyme solution in buffer, containing 50% glycerol	20 µl	100 µl
10X Long PCR Buffer with $MgCl_2$ 10X Long PCR buffer with 15 mM $MgCl_2$	0.6 ml	2 x 1.25 ml
10X Long PCR Buffer 10X Long PCR buffer	0.6 ml	2 x 1.25 ml
$MgCl_2$ Solution 25 mM $MgCl_2$ solution	0.6 ml	2 x 1.25 ml
Dimethyl Sulfoxide (DMSO)* Dimethyl sulfoxide	0.3 ml	1 ml
Water, nuclease-free 0.22 µm membrane-filtered molecular biology grade water	1.25 ml	2 x 1.25 ml

*Store DMSO at room temperature. Frozen DMSO may be melted at approx. 30ºC without any loss of performance.

PCR GUIDELINES

Primer design

For long PCR, use primers up to 27-36 nucleotides in length, whose melting temperature is near 65°C- 70°C. The higher annealing temperature allows the use of an annealing-elongation step at 68°C. In most cases this enhances reaction specificity and increases PCR product yield.
The primer's GC content should be 40-60%. The C and G nucleotides should be distributed uniformly within the full length of the primer. More than three G or C nucleotides at the 3'-end of the primer should be avoided, as non-specific priming might be increased in such cases. In order to avoid primer-dimer and hairpin formation, the primer should not be self-complementary or complementary to any other primer in the reaction mixture. The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly. Check for all possible sites of complementarity between primers and the template DNA. If primers are degenerate, at least 3 conservative nucleotides must be located at the primer's 3'-end. For long fragment PCR, use primers at 0.3 - 1 µM concentration.

DMSO

Addition of DMSO as a cosolvent increases yields and improves reliability of the system with long PCR fragments and some complex PCR targets. DMSO should be maintained at a final concentration between 1 and 8%. For PCR fragments ≥30 kb, use 4% DMSO.

Template DNA

Usually, the amount of template DNA is in the range of 0.01-2.5 ng for plasmid or phage DNA and 0.1-1 µg for genomic DNA, in a total reaction volume of 50 µl. Higher amounts of template DNA usually increase the yield of non-specific PCR products. However if the fidelity of synthesis is crucial, the maximal allowable template DNA quantities should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification, although even, trace amounts of agents used in DNA purification procedures (phenol, EDTA, proteinase K, etc.) strongly inhibit DNA polymerases. Ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from the DNA sample. High quality and adequate length of the template are essential for reliable amplification of larger fragments. Extreme care must be taken in the preparation and handling of long DNA targets. Nicked or damaged DNA can serve as a potential priming site resulting in high background. Avoid repeated freeze-thawing to minimize damage of large DNA fragments.

Magnesium concentration

10X Long PCR Buffer with 15 mM $MgCl_2$ is optimized for most long PCR applications. If the DNA samples contain EDTA or other chelators, raise the $MgCl_2$ concentration in the reaction mixture proportionally. The set includes 10X Long PCR buffer without $MgCl_2$ and a 25 mM $MgCl_2$ solution for use when optimization is required.

dNTP concentration

0.2 mM for each dNTP is optimal for most PCR applications. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP and dTTP), as inaccuracy in the concentration of even a single dNTP increases the misincorporation level.

Enzyme concentration

For PCR fragments up to 20 kb, use 1-1.25 u of Long PCR Enzyme Mix per 50 µl reaction volume. For PCR fragments ≥20 kb, Long PCR Enzyme Mix should be increased to 2.5 u per 50 µl.

GENERAL PCR PROTOCOL

Preparation of Reaction Mixture

Always set up the PCR reaction on ice. Setting up at room temperature can result in primer degradation by the 3'→5' exonuclease proofreading activity of the enzyme mix.

Gently vortex and briefly centrifuge all solutions after thawing. To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers and template DNA solutions in a single tube, which can then be aliquoted into individual tubes. Long PCR Enzyme Mix should be added last. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

- Add, in a thin walled PCR tube, on ice:

for synthesis of PCR fragments up to 30 kb

Reagent	Quantity for 50 µl	Final concentration
Water, nuclease-free	Variable	–
10X Long PCR Buffer with $MgCl_2$*	5 µl	1X
2 mM dNTP mix	5 µl	0.2 mM of each
Primer I	Variable	0.3-1 µM
Primer II	Variable	0.3-1 µM
Template DNA: Plasmids or phages Genomic DNA	Variable	 0.01-2.5 ng/50 µl 0. 1-1 µg/50 µl
Long PCR Enzyme Mix	0.25-0.5 µl	1.25-2.5 u/50 µl

for synthesis of PCR fragments ≥30 kb

Reagent	Quantity for 50µl	Final concentration
Water, nuclease-free	Variable	–
10X Long PCR Buffer with $MgCl_2$*	5 µl	1X
2 mM dNTP mix	5 µl	0.2 mM of each
Primer I	Variable	0.3-1 µM
Primer II	Variable	0.3-1 µM
DMSO	2 µl	4%
Template DNA: Plasmids or phages Genomic DNA	Variable	 1-2.5 ng /50 µl 0.1-1 µg/50 µl
Long PCR Enzyme Mix	0.5 µl	2.5 u/50 µl

*If using 10X Long PCR Buffer (without $MgCl_2$) refer to the table below for the selection of 25 mM $MgCl_2$ solution volume:

Final concentration of $MgCl_2$ in 50 µl reaction mix, mM	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, µl	2	2.5	3	3.5	4	5	6	8

- Gently vortex the sample and briefly centrifuge to collect all drops from tube walls. If the thermal cycler is not equipped with a heated cover, overlay the sample with a half volume of mineral oil or add an appropriate amount of wax.
- Place samples in a thermocycler and immediately start PCR.

Cycling Conditions

Optimal reaction conditions vary according to the amplified fragment size, template, primers, reaction volume, PCR tubes and thermal cycler used.

❶ **Initial Denaturation Step**

The complete denaturation of the DNA template at the start of the PCR is of key importance. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and a poor yield of PCR product. The initial denaturation should be performed over an interval of 1-2 min at 94°C if the GC content is 50% or less. This interval should be extended up to 5 min for GC rich templates.

Use the shortest possible denaturation time. Exposure of DNA to high temperatures causes some depurination of single-stranded DNA during denaturation, which eventually leads to truncation. High temperatures also lead to gradual loss of enzyme activity. Minimizing denaturation time is particularly important in experiments with very large templates where total cycling time can exceed 12 hours.

❷ **Denaturation Step**

Denaturation time is very dependent on the PCR tubes and thermal cycler used. Thin-wall type PCR tubes are recomended. The optimal denaturation conditions for Applied Biosystems GeneAmp® 9700 Thermocycler are 94°C for 10 s., for Eppendorf

Mastercycler® - 95°C for 15 s, for Perkin Elmer 480 - 94°C for 20 s. When using other thermocyclers the cycle conditions have to be adjusted.

A too short denaturation time or too low denaturation temperature may cause either diffuse smearing upon electrophoresis or poor amplification efficiency. A too long denaturation time or too high denaturation temperature may result in a nonspecific product.

❸ **Annealing and Elongation Step**

For most applications, two-step cycles (denaturation followed by annealing - elongation) are preferable to instead three-step cycles (denaturation followed by annealing followed by elongation). Three-step cycles are necessary when the annealing temperature of the primers is less than 65°C.

To carry out the annealing - elongation at 68°C, the recommended time setting is 45 to 60 seconds per 1 kb. Annealing at too high temperature generates no amplification products, while too low temperature enhances nonspecific reactions. Abbreviated elongation time generates no amplification products or short nonspecific products. Prolonged elongation time causes diffusely smeared electrophoresis bands.

❹ **Numbers of Cycles**

Set the optimum cycle number around 25-35 cycles after considering the quantity or complexity of template DNA and the length of amplified DNA fragments. Too few cycles may not generate enough amplified product, while too many cycles may produce a diffuse smear upon electrophoresis. For single copy genomic targets, use 0.1-1 µg DNA and 30-35 cycles; for plasmid or phages- 0.01-2.5 ng DNA and 25-30 cycles.

❺ Final Elongation Step

After the last cycle, the samples are usually incubated at 68-72°C to fill in the protruding ends of newly synthesized PCR product.

Recommended cycling conditions

Segment	Temperature	Duration	Number of cycles
Initial denaturation	94°C	1- 2 min	1
Denaturation	94- 96°C	10- 20 s.	10
Annealing	Primer T_a*	30 sec.	
Elongation	68°C	45-60** s./kb PCR target	
Denaturation	94- 96°C	10-20 s.	15-25
Annealing	Primer T_a*	30sec.	
Elongation	68°C	45-60** s./kb PCR target + 10** s./cycle	
Final elongation	68°C	10 min	1

*Annealing temperature depend on the primer used.

**Table for recommended elongation time

PCR fragment length, kb	3	6	10	15	20	25	30	35	40	45
Elongation time, min	2	4	7	10	14	17	20	24	27	30
Auto-extension per cycle, s.	1	2	5	5	10	10	15	15	20	20

The quality of Long PCR Enzyme Mix can be tested using Long PCR Control Set (#K0201), containing λ DNA and primers for synthesis of 20 kb and 30 kb PCR fragments.

TROUBLESHOOTING

Problem	Potential Reason	Proposed Solution
Low or no PCR product	PCR component may be missing or degraded	Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent.
	Poor template quality	Check template integrity by electrophoresis on agarose gel. If necessary, repurify template using methods that minimize shearing and nicking. Isolate fresh template and resuspend it in TE buffer, pH 8.0 or sterile water.
	Target template is difficult	In most cases, inherently difficult targets are due to unusually high GC content and/or secondary structure. The addition of 1-8% dimethyl sulfoxide (DMSO) may help. Simultaneously increase enzyme mix concentration up to 2.5 u per 50 µl.
	Not enough enzyme	If inhibitors are present in the reaction mix (e.g. if the template DNA used is not highly purified), increase amount of Long PCR Enzyme Mix in 0.2 u increments.
	Denaturation temperature not optimal	Optimize denaturation temperature by decreasing or increasing in 1°C increments. Too high denaturation temperature can lead to degradation of the template, especially for long target sequences.
	Denaturation time not optimal	Optimize denaturation time by decreasing or increasing in 5 s. increments. Too long denaturation time can lead to degradation of the template, especially for long target sequences.

Problem	Potential Reason	Proposed Solution
Low or no PCR product	Not enough cycles	Increase the number of cycles in 3-5 increments.
	Not enough template	Target is scarce. Use more template or increase cycle number.
	Suboptimal primer	Confirm the accuracy of the sequence information. If the original primer(s) is less than 27 nt long, try using a longer primer. If the original primer(s) has a GC content of less than 40%, try to design a primer with a GC content of 40–60%.
	Annealing temperature too high	Decrease the annealing temperature in 2-4°C increments.
	Elongation time is too short	Increase the elongation time in 1-2 min increments. Remember to use 45-60 s. per kb of DNA.
	Magnesium concentration too low	If DNA template contains EDTA or other chelators or RNA impurities, the $MgCl_2$ concentration should be raised in 0.2-0.3 mM increments.
Band smearing	Too much template	Reduce amount of template in reaction.
	Too many cycles	Reduce cycles in increments of 3-5.
	Elongation time too long	Decrease elongation time in 1-2 min increments.
	Denaturation time not optimal	Optimize denaturation time by decreasing or increasing in 5 s. increments. Too long denaturation time can lead to degradation of the template, especially for long target sequences.

Problem	Potential Reason	Proposed Solution
Band smearing	Denaturation temperature too low	Try increasing the denaturation temperature in 1°C increments.
	Magnesium concentration too high	Do not inadvertently add additional magnesium. When decreasing the dNTP concentration, $MgCl_2$ concentration should be proportionally decreased. Be sure the buffer containing template does not contain magnesium.
	Too much enzyme	Be sure to add only 1.25-2.5 units of enzyme mix per 50 µl reaction volume and mix the reaction well after addition of enzyme.
	Elongation time too short	Especially with longer templates, increase the elongation time in 1-min increments.
	Poor template quality	Check template integrity by electrophoresis on agarose gel. If necessary, repurify template using methods that minimize shearing and nicking. Isolate fresh template and resuspend it in TE buffer, pH 8 or sterile water.
Multiple Products	Too much template	When amplifying genomic DNA, the initial concentration of the template in the PCR reaction should not exceed 1 µg per 50 µl reaction volume.
	Primer design: suboptimal primer	Confirm the accuracy of the sequence information. If the original primer(s) is less than 22 nt long, try using a longer primer. If the original primer(s) has a GC content of less than 40%, try to design a primer with a GC content of 40–60%.
	Too many cycles	Reduce the number of cycles to eliminate nonspecific products.

Problem	Potential Reason	Proposed Solution
Multiple Products	Annealing temperature too low	Increase temperature in 2- 3°C increments.
	Magnesium concentration too high	Do not inadvertently add additional magnesium. When decreasing the dNTP concentration, $MgCl_2$ concentration should be decreased proportionally. Be sure the buffer containing the template does not contain magnesium.

QUALITY CONTROL

Each lot of Long PCR Enzyme Mix is tested in PCR for synthesis of 47.4 kb fragment from λ DNA.

APPENDIX

Conversions of Oligonucleotides

Molecular Weight

MW = 333 x N

Concentration of Oligonucleotides

C (µM or pmol/µl) = A_{260} / (0.01 x N)

C (ng/ml) = (A_{260} x MW) / (0.01 x N)

MW - molecular weight, Da

A_{260} - absorbance at 260 nm

N - number of bases

Trademarks

GeneAmp is a registered trademark of Applied Biosystems.
Eppendorf Mastercycler is a registered trademark of Eppendorf.

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SAFETY INFORMATION

Dimethyl Sulfoxide

Xi Irritant

Risk phrases

R36/38 Irritating to eyes and skin.

Safety phrases

S23 Do not breathe gas/fumes/vapour/spray.

S26 In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

S37 Wear suitable gloves.

S60 This material and its container must be disposed of as hazardous waste.