thermo scientific

PRODUCT INFORMATION

Long PCR Enzyme Mix

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#_

Lot _
Exp. _
Store at -20 °C

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For Research Use Only. Not for use in diagnostic procedures.

CERTIFICATE OF ANALYSIS

Long PCR Enzyme Mix is functionally tested in PCR amplification of 47.4 kb fragment from lambda DNA.

Quality authorized by:  Jurgita Zilinskiene

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COMPONENTS

Component	#K0181 100 U	#K0182 500 U
Long PCR Enzyme Mix, 5 U/µL	20 µL (100 U)	100 µL (500 U)
10X Long PCR Buffer with 15 mM $MgCl_2$	0.6 mL	2 × 1.25 mL
10X Long PCR Buffer	0.6 mL	2 × 1.25 mL
25 mM $MgCl_2$ Solution	0.6 mL	2 × 1.25 mL
Dimethylsulfoxide (DMSO)	0.3 mL	1 mL
Water, nuclease-free	1.25 mL	2 × 1.25 mL

STORAGE

Store all components at -20 °C, except DMSO.
Store DMSO at room temperature.

DESCRIPTION

Long PCR Enzyme Mix is a unique blend of *Taq* DNA Polymerase and a thermostable DNA polymerase with proofreading activity. The two enzymes synergistically generate long PCR products with greater yield and three times higher fidelity than with *Taq* DNA Polymerase alone. The ratio of enzymes in the Long PCR Enzyme Mix is optimized for generation of very long amplicons: up to 47 kb with viral DNA and up to 21 kb with genomic DNA templates. The specially formulated Long PCR Buffer protects DNA from depurination and nicking during long thermal cycling. The products generated with the Long PCR Enzyme Mix are mostly 3'-dA tailed.
Long PCR Enzyme Mix is also used for efficient amplification of GC-rich DNA regions.

APPLICATIONS

- Long range PCR.
- PCR from GC-rich or difficult templates.
- Generation of PCR product for TA cloning.
- RT-PCR.

PROTOCOL

Reaction set-up

Gently vortex and briefly centrifuge all solutions after thawing. Set up the PCR reaction on ice. Setting reaction up at room temperature may result in primer degradation by 3'→5' exonuclease activity of the enzyme mix.

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by adding water, buffer, dNTPs, and primers. Prepare enough master mix for the number of reactions and add one extra to compensate for pipetting errors. Aliquot the master mix into individual PCR tubes and add template DNA and enzyme mix.

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 50 µL reaction:

Components	**PCR <30 kb**	**PCR >30 kb and GC-rich PCR**
10X Long PCR buffer with 15 mM $MgCl_2$*	5 µL	5 µL
dNTP Mix, 2 mM each (#R0241)	5 µL (0.2 mM each)	5 µL (0.2 mM each)
Forward primer	0.3-1 µM	0.3-1 µM
Reverse primer	0.3-1 µM	0.3-1 µM
Template DNA	10 pg – 1 µg	1 ng – 1 µg
DMSO	–	2 µL (4%)
Long PCR Enzyme Mix	1.25-2.5 U	2.5 U
Water, nuclease-free	to 50 µL	to 50 µL

*If using 10X Long PCR Buffer (without $MgCl_2$), add following volumes of 25 mM $MgCl_2$ solution.

Final concentration of $MgCl_2$ in 50 µL reaction mix	1.0	1.25	1.5	1.75	2.0	2.5	3.0	4.0
Volume of 25 mM $MgCl_2$, µL	2.0	2.5	3.0	3.5	4.0	5.0	6.0	8.0

3. Gently vortex and briefly centrifuge to collect all drops.
4. If the thermal cycler is not equipped with a heated lid, overlay the reaction mixture with a half volume of mineral oil.
5. Place the samples in a cycler and immediately start PCR.

Reaction volumes can be scaled up or down as long as the final concentrations of the reaction components remain the same.

Recommended thermal cycling conditions

Two-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Initial Denaturation	94	1-3 min	1
Denaturation	94-96	20 s	10
Annealing/ Extension	68	45-60 s/kb*	
Denaturation	94	20 s	15-25
Annealing/ Extension	68	45-60 s/kb+ x* s/cycle	
Final Extension	68	10 min	1

Three-step cycling protocol

Step	Temperature, °C	Time	Number of cycles
Initial Denaturation	94	1-3 min	1
Denaturation	94-96	20 s	10
Annealing	Tm-5	30 s	
Extension	68	45-60 s/kb*	
Denaturation	94	20 s	15-25
Annealing	Tm-5	30 s	
Extension	68	45-60 s/kb+x* s/cycle	
Final Extension	68	10 min	1

* Calculation of extension and auto-extention times:

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

RECOMMENDATIONS FOR LONG PCR

Primer design

- PCR primers should be 27-36 nucleotides long.
- Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.
- Prefer one or two G or C at the 3'-end of the primer, but avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of nonspecific priming.
- Avoid primer self-complementarities, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization.
- Check for possible complementary sites between primers and template DNA.
- When designing degenerate primers, place at least 3 conservative nucleotides at the 3'-end.
- Differences in melting temperatures (Tm) of the two primers should not exceed 5 °C

Template DNA

Use 0.01-1 ng of plasmid and phage DNA, and 0.1-1 µg of genomic DNA in 50 µL of PCR. Higher amounts of template increase the risk of nonspecific PCR products. Lower amounts of template may reduce the accuracy of the amplification.
All routine DNA purification methods are suitable for template preparation e.g., Thermo Scientific GeneJET™ Genomic DNA Purification Kit (#K0721), GeneJET Plasmid Miniprep Kit (#K0502). Trace amounts of certain agents used for DNA purification, such as phenol, EDTA and proteinase K, may inhibit DNA polymerases. Ethanol precipitation and repeated washes of the DNA pellet with 70% ethanol normally remove trace contaminants from DNA samples. High quality and integrity of the template DNA is essential for reliable amplification of large DNA fragments. Extreme care must be taken during preparation and handling of template for long PCR. Nicked or damaged DNA can serve as a potential priming site resulting in high background. Avoid repeated freezing/thawing of template DNA.

Magnesium concentration

10X Long PCR Buffer with 15 mM $MgCl_2$ is optimal in most cases. If DNA samples contain EDTA or other chelators, increase $MgCl_2$ concentration in the reaction mixture accordingly. The 10X Long PCR buffer without $MgCl_2$ and 25 mM $MgCl_2$ solution are included for optimization, if necessary.

dNTP concentration

Final concentration of 0.2 mM for each dNTP is optimal in most cases.

Enzyme concentration

For PCR up to 20 kb, use 1-1.25 U of Long PCR Enzyme Mix per 50 µL reaction volume. For PCR of ≥20 kb, use up to 2.5 U per 50 µL.

DMSO

DMSO, a cosolvent, increases yields and improves reliability of the system for long PCR and PCR of complex targets. DMSO is used at a final concentration between 1 and 12%. For PCR fragments ≥30 kb, use 4% DMSO.

Cycling parameters

Initial denaturation step

It is essential to completely denature the template DNA at the beginning of PCR to ensure efficient utilization of the template during the first amplification cycle. If GC content of the template is 50% or less, an initial 1-3 min denaturation at 94 °C is sufficient. For GC-rich templates, this step may be prolonged to 5 min. Use shortest possible denaturation time. Exposure of DNA to high temperatures may cause depurination of single-stranded DNA, which leads to DNA truncation.

Denaturation step

Optimal denaturation time and temperature depend on PCR tubes and thermal cycler used. Thin-wall PCR tubes is the best choice. The optimal denaturation conditions for Applied Biosystems GeneAmp™ 9700 Thermocycler are 96 °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 cycling conditions have to be determined experimentally.

Insufficient denaturation time or temperature may cause either diffuse smearing after electrophoresis or poor amplification efficiency. Excess denaturation time or temperature may result in nonspecific PCR products.

Annealing/Extension step

In most cases a two-step cycling protocol (denaturation followed by annealing/extension) is preferred over three-step cycling protocol (denaturation followed by annealing followed by extension). The two step protocol saves time and reduces exposure of enzymes and template to long cycling conditions. The three-step cycling protocol is used when annealing temperature of the primers is lower than 65 °C.

For annealing/extension at 68 °C 1 min/kb is recommended for amplicons up to 6 kb. For longer amplicons annealing/extension time, starting from 11th cycle, has to be prolonged by approximately 5 seconds to compensate for enzyme loss during cycling. *See* recommendations in cycling protocol p.4.

Too low extension temperature reduces specificity of amplification. Insufficient extension time results in no amplification product or short nonspecific products. Excessive extension time causes smeared bands after electrophoresis.

Cycle number

Optimal number of cycles is in a range of 25-35 and depends on the amount and complexity of template DNA and also on amplicon length. Too few cycles may not generate sufficient amounts of PCR product, while too many cycles may produce a diffuse smear in electrophoresis gel. As a guideline, use 30-35 cycles for single copy targets in genomic DNA and 25-30 cycles for plasmid or phage DNA.

Final extension step

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

TROUBLESHOOTING

Problem	Possible cause and 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. **Poor template quality.** Evaluate 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. Also, try to increase enzyme amount in the reaction. **Difficult template.** If DNA is GC-rich or contains secondary structure, 1-12% dimethyl sulfoxide (DMSO) in the reaction mixture may help. Also, use higher enzyme concentration up to 2.5 U per 50 µL. **Denaturation temperature not optimal.** Optimize denaturation temperature by 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. **Insufficient number of cycles.** Increase the number of cycles in 3-5 increments. **Insufficient amount of template.** Use more template or increase number of cycles. **Suboptimal primer design.** Check sequence information. Re-design the primers according to recommendations on p.5. **Annealing temperature too high.** Decrease the annealing temperature in 2-4 °C increments. **Insufficient extension time.** Optimize by increasing the extension time in 1-minute increments. Make sure to include auto-extension per cycle starting from 11th cycle. **Insufficient magnesium concentration.** If DNA template contains EDTA or other chelators or RNA impurities, the $MgCl_2$ concentration should be increased in 0.2-0.3 mM increments.

(continued)

Problem	Possible cause and solution
Band smearing	**Excess template.** Reduce amount of template in reaction. **Too many cycles.** Reduce cycles in increments of 3-5. **Extention time too long.** Decrease elongation time in 1-2 min increments. **Denaturation time not optimal.** Optimize denaturation time by decreasing or increasing in 5 seconds increments. Too long denaturation time can lead to degradation of the template, especially for long target sequences. **Denaturation temperature too low.** Try increasing the denaturation temperature in 1 °C increments. **Excess magnesium concentration.** If dNTP concentration is lower than 0.2 mMof each dNTP, $MgCl_2$ concentration should be decreased accordingly. Make sure template DNA solution does not contain magnesium. **Excess amount of enzyme mix.** Be sure to add only 1.25-2.5 units of enzyme mix per 50 µL reaction and mix the reaction well after addition of the enzyme mix. **Insufficient extension time** Especially with longer templates, increase the elongation time in 1-2 min increments. **Poor template quality** Check template integrity by electrophoresis on agarose gel. If necessary, repurify the template using methods that minimize shearing and nicking. Isolate fresh template and resuspend it in TE buffer, pH 8 or sterile water.
Non-specific PCR 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. **Suboptimal primer design** Check sequence information. Re-design the primers according to recommendations on p.5. **Too many cycles** Reduce the number of cycles to eliminate nonspecific products. **Annealing temperature too low** Increase temperature in 2-3 °C increments. **Excess magnesium concentration** If dNTP concentration is lower than 0.2 mM, $MgCl_2$ concentration should be decreased accordingly. Make sure template DNA solution does not contain magnesium.

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