

KOD Hot Start DNA Polymerase

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emdchemicals.com

France
Freephone
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Germany
Freecall
0800 100 3496

Ireland
Toll Free
1800 409 445

United Kingdom
Freephone
0800 622 935

All other
European Countries
+44 115 943 0840

All Other Countries

Contact Your Local Distributor
www.merck4biosciences.com
bioscienceshelp@
emdchemicals.com

techservice@merckbio.eu

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About the Kits

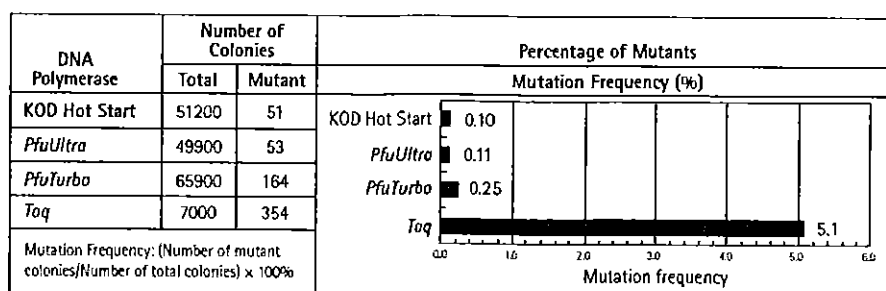
KOD Hot Start DNA Polymerase	20 U	71086-5
	200 U	71086-3
	1000 U	71086-4

Description

KOD Hot Start DNA Polymerase is a premixed complex of the high fidelity KOD DNA Polymerase and two monoclonal antibodies that inhibit the DNA polymerase and 3'→5' exonuclease activities at ambient temperatures (1). KOD Hot Start combines the high fidelity, fast extension speed, and outstanding processivity of KOD with the high specificity of an antibody-mediated hot start. Non-specific amplification is reduced because mispriming events during reaction set up and the initial temperature increase are avoided. In addition, primer degradation during setup at ambient temperature due to exonuclease activity is effectively inhibited. This enzyme quickly and accurately amplifies genomic and phage/plasmid DNA targets up to 12 and 20 kbp, respectively. GC-rich targets are also efficiently amplified. KOD Hot Start DNA Polymerase produces blunt-ended DNA products that are suitable for cloning with the Novagen Perfectly Blunt® and LIC Vector Kits and is compatible with site-directed mutagenesis protocols.

Unit definition: One unit is defined as the amount of enzyme that will catalyze the incorporation of 10 nmol of dNTP into acid insoluble form in 30 minutes at 75°C in a reaction containing 20 mM Tris-HCl (pH 7.5 at 25°C), 8 mM MgCl₂, 7.5 mM DTT, 50 µg/ml BSA, 150 µM each of dATP, dCTP, dGTP, dTTP (a mix of unlabeled and [³H]-dTTP) and 150 µg/ml activated calf thymus DNA.

Polymerase fidelity comparison



Mutation frequency comparison: KOD Hot Start, *PfuTurbo*®, *PfuUltra*®, and *Taq*

The fidelity of replication was measured as the mutation frequency in PCR products using a modified *rpsL*⁺ fidelity assay (2, 3).

Polymerase rate comparison

Enzyme	KOD DNA Polymerase	<i>Pfu</i> DNA Polymerase	<i>Taq</i> DNA Polymerase
Species	<i>Thermococcus kodakaraensis</i>	<i>Pyrococcus furiosus</i>	<i>Thermus aquaticus</i> YT-1
Elongation rate (bases/second)	106–138	25	61
Processivity* (nucleotide bases)	> 300	< 20	not determined

* Processivity is defined as the number of nucleotides that can be extended in one catalytic reaction by one DNA polymerase molecule.

USA and Canada
Tel (800) 628-8470
bioscienceshelp@
emdchemicals.com

Germany
Tel 0800 100 3496
techservice@merckbiosciences.de

United Kingdom and Ireland
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Ireland Toll Free 1800 409445
customer.service@merckbiosciences.co.uk

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Components

- 20 U or 200 U or 5 × 200 U KOD Hot Start DNA Polymerase (1 U/μl in 50 mM Tris-HCl, 1mM DTT, 0.1 mM EDTA, 50% glycerol, 0.001% Nonidet P-40, 0.001% Tween® 20, pH 8.0)
- 1.2 ml or 5 × 1.2 ml 10X PCR Buffer for KOD Hot Start DNA Polymerase
- 1 ml or 5 × 1 ml 25 mM MgSO₄
- 1 ml or 5 × 1 ml dNTPs (2 mM each)

Storage

Store all components at -20°C.

KOD Hot Start DNA Polymerase Protocol

KOD Hot Start DNA Polymerase and buffer are a unique PCR system. The following procedure is designed for use with the components provided in the KOD Hot Start DNA polymerase kit. Using reaction components or protocols designed for any other DNA polymerase may result in poor amplification. Reaction conditions listed below will provide satisfactory amplification for most primer/template combinations. Guidelines and troubleshooting sections provide details for optimizing reaction conditions.

Examples of amplification from human genomic DNA and plasmid DNA can be found in the Appendix on page 8.

Standard reaction setup

Component	Volume	Final Concentration
10X Buffer for KOD Hot Start DNA Polymerase	5 µl	1X
25 mM MgSO ₄ ^a	3 µl	1.5 mM
dNTPs (2 mM each)	5 µl	0.2 mM (each)
PCR Grade Water	X µl	
Sense (5') Primer (10 µM)	1.5 µl	0.3 µM
Anti-Sense (3') Primer (10 µM)	1.5 µl	0.3 µM
Template DNA ^b	Y µl	
KOD Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02U/µl
Total reaction volume	50 µl	

^a To optimize for targets greater than 2kb, final Mg²⁺ concentration may be adjusted to between 1.5 and 2.25 mM.

^b See Template DNA section on page 5.

Cycling conditions

Temperature and time

The following table allows for primer extension that occurs during temperature ramping between steps.

Step	Target size			
	< 500 bp	500–1000 bp	1000–3000 bp	> 3000 bp
1. Polymerase activation	95°C for 2 min	95°C for 2 min	95°C for 2 min	95°C for 2 min
2. Denature	95°C for 20 s	95°C for 20 s	95°C for 20 s	95°C for 20 s
3. Annealing	Lowest Primer T _m °C for 10 s			
4. Extension	70°C for 10 s/kb	70°C for 15 s/kb	70°C for 20 s/kb	70°C for 25 s/kb
Repeat steps 2–4	20–40 cycles. For more information see "Cycle number" below			

Cycle number

The number of cycles (steps 2 through 4 in the above table) required to generate a PCR product will depend on the source and amount of starting template in the reaction, as well as the efficiency of the PCR. In general, 20–40 cycles will be adequate for a wide range of templates. It is common to use fewer cycles when amplifying targets from plasmids (i.e., subcloning) where a high number of copies of template is easily attained, as this reduces the chance of amplifying a mutation. A higher number of cycles (e.g., 40) may be necessary when amplifying from genomic DNA since the target sequence will be in low abundance.

Additional Guidelines

Primers

Primer design is critical for successful PCR amplification. Because KOD Hot Start DNA polymerase exhibits strong 3'→5' exonuclease activity after thermo activations, primers should be at least 21 bases of 3' end complementary to the target sequence. G/C content of the primers should be 40–60%. Primer melting temperature (T_m) is defined as the temperature at which one half of the DNA duplex will dissociate to become single stranded. Some primer molecules will anneal as the temperature approaches the T_m of a primer, as a result PCR amplifications are usually successful over a range of annealing temperatures. Primer pairs with similar T_m values usually result in better amplifications because annealing and extension are better synchronized. If melting temperatures of a primer pair differ by more than 5°C, increasing the length of the lower- T_m primer will reduce the difference.

There are several methods for determining the T_m of a primer. The nearest-neighbor method (4) using 50 mM monovalent salt is one method for T_m prediction. Unlike other methods, the nearest-neighbor method takes into account the primer sequence and other variables such as salt and DNA concentration. The T_m can also be calculated with the % GC method (5). The most general method of calculating the T_m is based on the number of adenine (A), thymidine (T), guanidine (G) or cytosine (C) bases where $T_m(^{\circ}\text{C}) = 2(N_A + N_T) + 4(N_G + N_C)$.

Primer T_m values reported by manufacturers may vary by 5 to 10°C depending on the calculation method used. In addition, the exact T_m for a given primer in a reaction may be affected by DNA concentrations (primer and template), mono and divalent ion concentrations, dNTP concentration, presence of denaturants (e.g., DMSO), and nucleotide modifications. Therefore, an optimal primer annealing temperature should be determined empirically.

When receiving oligonucleotides from the manufacturer, prepare primer stocks at 100 pmol/μl (100 μM) in TE and store them at –20°C. To set up KOD reactions, dilute enough of each primer stock 10-fold (10 μM) to add 1.5 μl per reaction.

Template DNA

The optimal amount of starting template may vary depending on the template quality. In general the suggested amount of template DNA for amplification is 10 ng phage DNA, 10 ng plasmid DNA, 100 ng genomic DNA, or 2 μl of a reverse transcription reaction. Using too much template in the PCR reaction can result in failed reactions since template denaturation is concentration dependant. At high concentrations of DNA, denaturation is less efficient.

Plasmid templates

For subcloning, amplify from 10 ng of plasmid template and reduce the number of cycles to 20–25.

GC-rich templates

The addition of DMSO to 2–10% final concentration may decrease template secondary structure and increase yield. Final DMSO concentrations of less than 5% v/v have no effect on fidelity (6, 7). The effect of DMSO above 5% v/v on enzyme fidelity has not yet been determined.

Unpurified templates

Crude cell lysates, PCR products, plaques, and colonies can serve as template for PCR. Limit the volume of unpurified templates to reduce inhibition of the reaction.

Long target DNA

Amplification of targets longer than 3000 bp can be improved by increasing the concentration of MgSO_4 . Adjusting the final MgSO_4 concentration from 1.5 to 2.25 mM in 0.25 mM increments should be tried when suboptimal results are obtained for targets over 3000 bp. Also, the addition of DMSO to 2–10% v/v final concentration may reduce secondary structure of the template DNA and increase yield.

Reaction components

High volumes of primer and template DNA suspended in Tris-EDTA (TE) will chelate free Mg^{2+} in the reaction and may affect enzyme performance. If the combined volume of primer and template in TE in the reaction exceeds 5 μ l, then adjustment of the $MgSO_4$ concentration may be necessary. Increase the $MgSO_4$ in the reaction to compensate for the EDTA. Each molecule of EDTA will chelate one molecule of Mg^{2+} , so increasing the $MgSO_4$ by 0.25 μ l (0.125mM) for every 6.3 μ l of TE will compensate for the Mg^{2+} chelated by the EDTA.

Extension temperature and time

Extension at 70°C is recommended since a good balance of polymerization speed and accuracy is obtained. KOD Hot Start DNA Polymerase exhibits optimal proof reading activity at 68°C and optimal polymerization activity at 74°C. If using an extension temperature near 74°C, shortening the extension time 5 s/kbp may give better amplification. When using an extension temperature near 68°C, increasing the extension time by 5 s/kbp may give better results.

Two-step PCR

In two-step PCR, annealing and extension can be carried out at the same temperature. Primers for two-step cycling programs should be designed with high T_m values (> 65°C) to ensure proper annealing and extension at the same temperature. Initially try an annealing/extension temperature equal to the lowest T_m of the primer pair. Since polymerase speed is slower at 68°C, increase the annealing/extension time by 5 s/kbp during two-step cycling.

Optimization

When optimizing PCR reactions, it is best to change only one parameter at a time. The use of DMSO at 5% v/v final often improves a suboptimal PCR.

Troubleshooting

Symptom	Possible cause	Solution
No PCR product	Extension time is too long	Lower extension time to 15 s/kpb
	Too much secondary structure in template DNA	Add DMSO to a final concentration of 5–10% v/v
	PCR primers are not long enough Annealing temperature is too high	Use primers longer than 21 bases Lower annealing temperature in 3°C decrements
Low yield	High GC content	Add DMSO to a final concentration of 5–10% v/v
	Suboptimal PCR conditions	Increase final MgSO ₄ concentration in 0.25 mM increments
	Long target DNA	Increase final MgSO ₄ concentration in 0.25 mM increments
Smearing	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 5 s/kpb
	MgSO ₄ concentration too low	Increase final MgSO ₄ concentration 0.25 mM increments
Smearing above target size	Extension times too long	Reduce extension time 5 s/kpb
	MgSO ₄ concentration too high	Decrease final MgSO ₄ concentration in 0.25 mM increments
Primer dimers	Primers are complementary to each other	Design primers that are not self-complementary or complementary to each other
	Primer concentration is too high	Reduce primer concentration
	Annealing temperature too low	Raise annealing temperature

Application references

This section lists selected references for applications with KOD Hot Start DNA Polymerase. Please visit www.merck4biosciences.com for more information.

Application	Reference
Colony-direct PCR with Gram-positive bacteria	Tsuchizaki, N. and Hotta, K. (2003) <i>inNovations</i> 17, 9–11
Elongation PCR	Gao, X., Yo, P., Keith, A., Ragan, T. J., and Harris, T. K. (2003) <i>Nucleic Acids Res.</i> 31, e143
Gene cloning	Schilling, O., Spath, B., Kostelecky, B., Marchfelder, A., Meyer-Klaucke, W., and Vogel, A. (2005) <i>J. Biol. Chem.</i> 280, 17857–17862
	Williams, M. E., Burton, B., Urrutia, A., Shcherbakov, A., Chavez-Noriega, L. E., Cohen, C.J., and Aiyar, J. (2005) <i>J. Biol. Chem.</i> 280, 1257–1263
	Miyazato, T., Toma, C., Nakasone, N., Yamamoto, K., and Iwanaga, M. (2003) <i>J. Med. Microbiol.</i> 52, 283–288
Gene cloning using consensus shuffling	Binkowski, B. F., Richmond, K. E., Kaysen, J., Sussman, M. R., and Belshaw, P. J. (2005) <i>Nucleic Acids Res.</i> 33, e55
Multiplex cDNA-PCR	Sagara, N. and Katho, M. (2000) <i>Cancer Res.</i> 60, 5959–5962
Multiplexed SNP genotyping	Higasa, K. and Hayashi, K., (2002) <i>Nucleic Acids Res.</i> 30, e11
Mutagenesis	Tabuchi, M., Tanaka, N., Nishida-Kitayama, H., Ohno, H., and Kishi, F. (2002) <i>Mol. Biol. Cell</i> 13, 4371–5387.
	Matsumoto, N., Mitsuki, M., Tajima, K., Yokoyama, W. M., and Yamamoto, K. (2001) <i>J. Exp. Med.</i> 193, 147–158.
PCR for PCR-Mass spectrometry based analysis	Benson, L. M., Null, A. P., and Muddiman, D. C. (2003) <i>J. Am. Soc. Mass. Spectrom.</i> 14, 601–604.
PCR for sequence analysis	Okamoto, T., Yoshiyama, H., Nakazawa, T., Park, I. D., Chang, M. W., Yanai, H., Okita, K., and Shirai, M. (2002) <i>J. Antimicrob. Chemother.</i> 50, 849–856.
Second strand cDNA synthesis	Hirohashi, Y., Torigoe, T., Maeda, A., Nabeta, Y., Kamiguchi, K., Sato, T., Yoda, J., Ikeda, H., Hirata, K., Yamanaka, N., and Sato, N. (2002) <i>Clin. Cancer Res.</i> 8, 1731–1739.

USA and Canada
Tel (800) 628-8470
bioscienceshelp@emdchemicals.com

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Tel 0800 100 3496
techservice@merckbiosciences.de

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UK Freephone 0800 622935
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Appendix

Example amplifications

Amplification of 335 bp fragment (CFTR exon 11) from human genomic DNA

Reaction setup

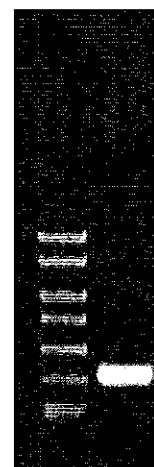
Component	Volume	Final Concentration
10X Buffer for KOD Hot Start DNA Polymerase	5 µl	1X
25 mM MgSO ₄	3 µl	1.5 mM
dNTPs (2 mM each)	5 µl	0.2 mM (each)
PCR Grade Water	32 µl	
Sense (5') Primer (10 µM)	1.5 µl	0.3 µM
Anti-Sense (3') Primer (10 µM)	1.5 µl	0.3 µM
Human Genomic DNA* (100 ng/µl)	1 µl	2 ng/µl
KOD Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02 U/µl
Total reaction volume	50 µl	

* Human Genomic DNA (Cat. No. 69237-3) diluted in TE to 100 ng/µl

Cycling conditions

Step	Temperature and time
1. Polymerase activation	95°C for 2 min
2. Denature	95°C for 20 s
3. Annealing	56°C for 10 s
4. Extension	70°C for 4 s
Repeat steps 2–4	30 cycles
5. Hold	4°C

1 2



1.2 % TAE agarose gel
 Lane 1 PCR Markers, 50 2000 bp
 (Cat. No. 69278-3)
 Lane 2 5 µl PCR Reaction

Amplification of 919 bp ORF from plasmid DNA

Reaction setup

Component	Volume	Final Concentration
10X Buffer for KOD Hot Start DNA Polymerase	5 µl	1X
25 mM MgSO ₄	3 µl	1.5 mM
dNTPs (2 mM each)	5 µl	0.2 mM (each)
PCR Grade Water	32 µl	
Sense (5') Primer (10 µM)	1.5 µl	0.3 µM
Anti-Sense (3') Primer (10 µM)	1.5 µl	0.3 µM
Plasmid DNA (10 ng/µl, diluted in TE)	1 µl	0.22 ng/µl
KOD Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02 U/µl
Total reaction volume	50 µl	

* Human Genomic DNA (Cat. No. 69237-3) diluted in TE to 100 ng/µl

Cycling conditions

Step	Temperature and time
1. Polymerase activation	95°C for 2 min
2. Denature	95°C for 20 s
3. Annealing	55°C for 10 s
4. Extension	70°C for 15 s
Repeat steps 2–4	25 cycles
5. Hold	4°C

1 2



1.4 % TAE agarose gel

Lane 1 PCR Markers, 50–2000 bp
(Cat. No. 69278-3)

Lane 2 5 µl PCR Reaction

