

KOD Hot Start Master Mix

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KOD Hot Start Master Mix	100 rxn	71842-3
	500 rxn	71842-4

About the Kits

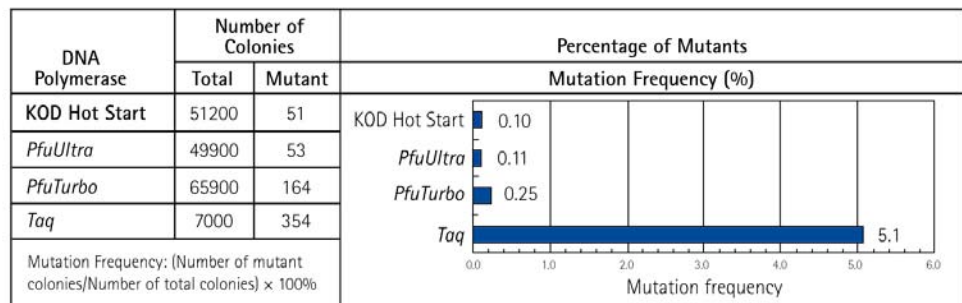
Description

KOD Hot Start Master Mix is a ready-to-use 2X mixture optimized for convenient high-fidelity PCR. The mix contains KOD Hot Start DNA Polymerase, two monoclonal antibodies, ultra pure deoxynucleotides, and reaction buffer with MgSO₄. The monoclonal antibodies inhibit the DNA polymerase and 3'→5' exonuclease activities at ambient temperatures (1). The Master Mix is ideal for use in high-throughput applications as it simplifies PCR set-up, offering time savings, consistency, and minimal risk of contamination. Simply add KOD Hot Start Master Mix to an equal volume of sample containing DNA template and primers. The final diluted reaction contains 1 U KOD Hot Start DNA Polymerase per 50 µl reaction. The smaller available size provides sufficient master mix for 100 (50 µl scale), or 250 (20 µl scale) reactions, while the larger size is adequate for 500 (50 µl scale) or 1250 (20 µl scale) reactions.

KOD Hot Start Master Mix amplifies genomic and phage/plasmid DNA targets up to 12 and 20 kbp, respectively. Amplification with KOD Hot Start Master Mix produces blunt-ended DNA products that are compatible for cloning with the Novagen Perfectly Blunt[®] and LIC Vector Kits.

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.

Components

- 2 × 1.25 ml or 10 × 0.125 ml KOD Hot Start Master Mix (0.04 U/μl)

Storage

Store at –20°C.

KOD Hot Start Master Mix Protocol

KOD Hot Start Master Mix is a unique PCR system. 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. Additional guidelines and troubleshooting sections provide details for optimizing reaction conditions.

Examples of amplification from phage DNA and plasmid DNA are shown in the Appendix on page 7.

Standard reaction setup

Standard reactions can be set up either in a 50 μl or a 20 μl format, as follows:

Component	Volume		Final Concentration
PCR Grade Water	X μl	A μl	
Sense (5') Primer (10 μM)	1.5 μl	0.6 μl	0.3 μM
Anti-Sense (3') Primer (10 μM)	1.5 μl	0.6 μl	0.3 μM
Template DNA ^a	Y μl	B μl	
KOD Hot Start Master Mix (0.04 U/μl)	25 μl	10 μl	0.02 U/μl
Total reaction volume	50 μl	20 μl	

^a See Template DNA section on page 4.

Cycling conditions

Temperature and time

The following recommended cycling conditions allow for primer extension, which will occur during temperature ramping between primer annealing and extension 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/kbp	70°C for 15 s/kbp	70°C for 20 s/kbp	70°C for 25 s/kbp
Repeat steps 2–4	20–40 cycles. For more information see "Cycle number" below			

s/kbp = seconds per kilobase pair

Cycle number

The number of cycles (Steps 2 through 4 in the table above) 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. We recommend 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 Master Mix exhibits strong 3'→5' exonuclease activity after thermal activation, primers should have at least 21 bases of their 3' end complementary to the target sequence. GC 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 are desirable 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.

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, optimal primer annealing temperature should be determined empirically.

There are several methods for determining the T_m of a primer. The nearest-neighbor method (4) using 50 mM monovalent salt is the preferred 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.

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 50-μl reaction.

Template DNA

The optimal amount of starting template may vary depending on template quality. In general, the suggested amount of template DNA for a 50 μl 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 can result in failed reactions since template denaturation is concentration-dependent. At high concentrations of DNA, denaturation is less efficient.

Plasmid templates

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

GC-rich templates

The addition of DMSO to 2–10% v/v final concentration may decrease template secondary structure and increase yield (5, 6). Final DMSO concentrations of less than 5% v/v have no effect on fidelity. 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 1/20th of the total reaction volume to reduce PCR inhibition.

Reaction components

High volumes of primer or template DNA, suspended in Tris-EDTA (TE) will chelate free Mg²⁺ in the reaction and may be detrimental to enzyme performance. Limit the volume of template DNA to 1/20th of the total reaction volume to reduce inhibition of the reaction due to EDTA.

Extension temperature and time

We recommend extension at 70°C since this results in a good balance of polymerization speed and accuracy. KOD Hot Start Master Mix exhibits optimal proofreading 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 ($>65^\circ\text{C}$) 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 to 10 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 suboptimal PCR.

Troubleshooting

Symptom	Possible cause	Solution
No PCR product	Extension time is too long	Lower extension time to 15 s/kbp
	Too much secondary structure in template DNA	Add DMSO to a final concentration of 5–10% v/v
	PCR primers are not long enough	Use primers longer than 21 bases
Low yield	Annealing temperature is too high	Lower annealing temperature in 3°C steps
	High GC content	Add DMSO to a final concentration of 5–10% v/v.
	Incomplete extension	Increase extension time 5 to 10 s/kbp
Smearing	Inefficient polymerization	Increase number of cycles
	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 5 s/kbp
Smearing above target size	Extension times too long	Reduce extension time 5 s/kbp
	Extension temperature too high	Reduce extension temperature 3 to 5°C
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.novagen.com/KOD for the latest 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., Kosteletzky, 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., Shcherbatko, A., Chavez-Noriega, L. E., Cohen, C.J., and Aiyar, J. (2005) <i>J. Biol. Chem.</i> 280 , 1257–1263.
Gene cloning using consensus shuffling	Miyazato, T., Toma, C., Nakasone, N., Yamamoto, K., and Iwanaga, M. (2003) <i>J. Med. Microbiol.</i> 52 , 283–288.
Multiplex cDNA-PCR	Binkowski, B. F., Richmond, K. E., Kaysen, J., Sussman, M. R., and Belshaw, P. J. (2005) <i>Nucleic Acids Res.</i> 33 , e55.
Multiplexed SNP genotyping	Sagara, N. and Katho, M. (2000) <i>Cancer Res.</i> 60 , 5959–5962.
Mutagenesis	Higasa, K. and Hayashi, K., (2002) <i>Nucleic Acids Res.</i> 30 , e11.
	Tabuchi, M., Tanaka, N., Nishida-Kitayama, H., Ohno, H., and Kishi, F. (2002) <i>Mol. Biol. Cell</i> 13 , 4371–5387.
PCR for PCR-Mass spectrometry based analysis	Matsumoto, N., Mitsuki, M., Tajima, K., Yokoyama, W. M., and Yamamoto, K. (2001) <i>J. Exp. Med.</i> 193 , 147–158.
PCR for sequence analysis	Benson, L. M., Null, A. P., and Muddiman, D. C. (2003) <i>J. Am. Soc. Mass. Spectrom.</i> 14 , 601–604.
Second strand cDNA synthesis	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.
	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.

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Appendix

Example amplifications

Amplification of 595 bp fragment (att region) from lambda DNA

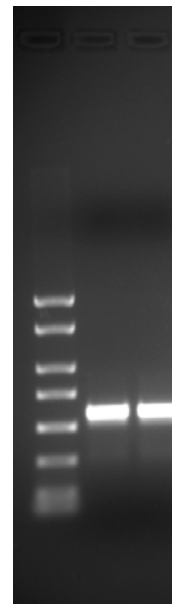
Reaction setup

Component	Volume		Final Concentration
PCR Grade Water	21 μ l	8.4 μ l	
Sense (5') Primer (10 μ M)	1.5 μ l	0.6 μ l	0.3 μ M
Anti-Sense (3') Primer (10 μ M)	1.5 μ l	0.6 μ l	0.3 μ M
Lambda DNA (10 ng/ μ l, diluted in TE)	1 μ l	0.4 μ l	0.2 ng/ μ l
KOD Hot Start Master Mix (0.04 U/ μ l)	25 μ l	10 μ l	0.02 U/ μ l
Total reaction volume	50 μl	20 μl	

Cycling conditions

Step	Temperature and time
1. Polymerase activation	95°C for 2 min
2. Denature	95°C for 20 s
3. Annealing/ Extension	70°C for 9 s
Repeat steps 2–4	25 cycles
5. Hold	4°C

1 2 3



1.0% TAE agarose gel

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

Lane 2 5 μ l of 50 μ l PCR Reaction

Lane 3 5 μ l of 20 μ l PCR Reaction

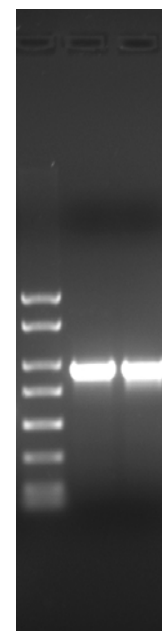
Amplification of 919 bp ORF from plasmid DNA**Reaction setup**

Component	Volume		Final Concentration
PCR Grade Water	21 μ l	8.4 μ l	
Sense (5') Primer (10 μ M)	1.5 μ l	0.6 μ l	0.3 μ M
Anti-Sense (3') Primer (10 μ M)	1.5 μ l	0.6 μ l	0.3 μ M
Plasmid DNA (10 ng/ μ l, diluted in TE)	1 μ l	0.4 μ l	0.2 ng/ μ l
KOD Hot Start Master Mix (0.04 U/ μ l)	25 μ l	10 μ l	0.02 U/ μ l
Total reaction volume	50 μl	20 μl	

Cycling conditions

Step	Temperature and time
1. Polymerase activation	95°C for 2 min
2. Denature	95°C for 20 s
3. Annealing	62°C for 10 s
4. Extension	70°C for 14 s
Repeat steps 2–4	25 cycles
5. Hold	4°C

1 2 3



1.0% TAE agarose gel

Lane 1 PCR Markers, 50–2000 bp
(Cat. No. 69278-3)Lane 2 5 μ l of 50 μ l PCR ReactionLane 3 5 μ l of 20 μ l PCR Reaction