KOD Xtreme™ Hot Start DNA Polymerase

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About the Kits

KOD Xtreme™ Hot Start DNA Polymerase	200 U	71975-3

Description

The KOD Xtreme™ Hot Start DNA Polymerase kit is an optimized PCR system for the amplification of long or GC-rich DNA templates. The system includes an ultra high fidelity KOD DNA Polymerase complexed with two monoclonal antibodies to permit hot start thermocycling, along with specially formulated 2X Xtreme buffer. KOD Xtreme Hot Start DNA Polymerase quickly and accurately amplifies genomic and phage/plasmid DNA targets up to 24 and 40 kbp, respectively. KOD Xtreme Hot Start DNA Polymerase successfully amplifies challenging DNA templates with up to 90% GC content.

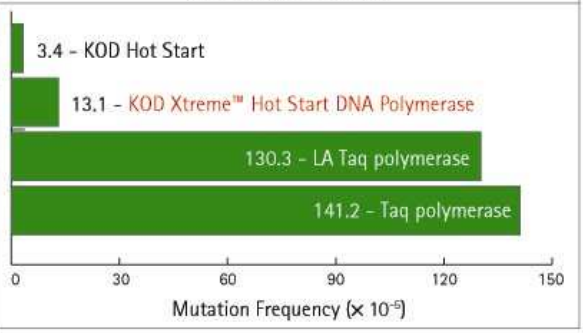
Each kit provides 200 U KOD Xtreme Hot Start DNA Polymerase, an optimized buffer and dNTPs sufficient for 200 amplification reactions. The polymerase produces blunt-ended DNA products compatible for cloning with the Novagen Perfectly Blunt® and LIC Vector Kits.

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 $MgCl_2$, 7.5 mM DTT, 50 µg/ml BSA, 150 µM each of dATP, dCTP, dGTP, dTTP (a mix of unlabeled and [^{3}H]-dTTP) and 150 µg/ml activated calf thymus DNA.

Polymerase fidelity data

DNA Polymerase	Number of Bases Sequenced	Number of Bases Mutated
KOD Hot Start	145,753	5
KOD Xtreme	144,535	19
LA Taq+	167,343	218
Taq	102,708	145

+ LA Taq is representative of polymerases mixes which are a blend of Taq DNA polymerase and a proofreading enzymes, such as AccuTaq™ LA DNA Polymerase, Advantage polymerases, Expand PCR systems, and TaKaRa LA Taq™ polymerase



Components

- 200 U KOD Xtreme 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)
- 3 x 1.7 ml 2X Xtreme Buffer
- 2 x 1 ml dNTPs (2 mM each)

Storage

Store all components in a constant-temperature freezer at –20°C.

KOD Hot Start Master Mix Protocol

KOD Xtreme™ Hot Start DNA Polymerase and Xtreme buffer are a unique PCR system. The following procedure is designed for use with the components provided in the KOD Xtreme 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. *Additional guidelines* and *Troubleshooting* sections provide details for optimizing reaction conditions.

Examples of amplification from human genomic DNA can be found in the *Appendix* on p 7.

KOD Xtreme standard reaction setup

Component	Volume	Final Concentration
2X Xtreme Buffer	25 µl	1X
dNTPs (2 mM each)	10 µl	0.4 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[a]	Y µl	
KOD Xtreme™ Hot Start DNA Polymerase (1 U/µl)	1 µl	0.02 U/µl
Total reaction volume	**50 µl**	

[a] See *Template DNA* section on p 5.

Cycling conditions

Temperature and time

The three-step cycling protocol below will result in successful amplification of most targets.

3-step cycling	
1. Polymerase activation	94°C for 2 min
2. Denature	98°C for 10 s[b]
3. Annealing	Lowest Primer Tm°C for 30 s
4. Extension	68°C for 1 min/kbp
Perform Steps 2–4 for20–40 cycles. For more information see "Cycle number" on p 4	

[b] For some applications, it may be desirable to limit the time of the denaturation step. For more information, see *Shortened denaturation step and DNA thermal damage* section on p 6.

If extra bands or smearing are observed with long targets (> 10 kbp), the following step-down cycling conditions may be helpful:

Step-down cycling	
1. Polymerase activation	94°C for 2 min
2. Denature	98°C for 10 s[b]
3. Annealing/Extension	74°C for 1 min/kbp
Perform Steps 2 and 3 for 5 cycles	
4. Denature	98°C for 10 s[b]
5. Annealing/Extension	72°C for 1 min/kbp
Perform Steps 4 and 5 for 5 cycles	
6. Denature	98°C for 10 s[b]
7. Annealing/Extension	70°C for 1 min/kbp
Perform Steps 6 and 7 for 6 cycles	
8. Denature	98°C for 10 s[b]
9. Annealing/Extensions	68°C for 1 min/kbp
Perform Steps 8 and 9 for 15–25 cycles. For more information see "Cycle number" below.	

[b] For some applications, it may be desirable to limit the time of the denaturation step. For more information, see *Shortened denaturation step and DNA thermal damage* section on p 6.

Cycle number

The number of cycles (steps 2–4 in the three-step cycling protocol on p 3) 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 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 Xtreme™ Hot Start DNA Polymerase exhibits strong 3'→5' exonuclease activity after thermal activation; primers should be at least 21 bases of 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, an 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 Xtreme™ 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 can result in failed reactions since template denaturation is concentration-dependent. 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 2X Xtreme Buffer supplied in the kit is optimized for a variety of templates. The addition of DMSO or other additives is generally not needed.

Unpurified templates

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

Reaction components

The 2X Xtreme Buffer contains 4mM $MgSO_4$. High volumes of primer or template DNA, suspended in Tris-EDTA (TE) will chelate free Mg^{2+} in the Xtreme buffer and may be detrimental to enzyme performance. Limit the volume of template DNA to 1/20th of the total reaction to reduce inhibition of the reaction due to EDTA or adjust the Mg^{2+}concentration accordingly.

Extension temperature and time

We recommend extension at 68°C since a good balance of polymerization speed and accuracy is obtained at this temperature. KOD Xtreme Hot Start DNA Polymerase exhibits optimal proofreading activity at 68°C and optimal polymerization activity at 74°C.

Two-step PCR

In two-step PCR, annealing and extension are carried out at the same temperature. Primers for two-step cycling programs should be designed with high T_m values (> 65°C) to allow proper annealing and extension at the same temperature.

Shortened denaturation step and DNA thermal damage

DNA thermal damage such as depurination or cytosine deamination to uracil occurs at high temperature, and can contribute greatly to errors in synthetic DNA molecules produced during PCR (5). Therefore, for some applications it may be necessary or desirable to limit the duration of denaturation steps. With many targets the denaturation step can be shortened to 5 s or 1 s and still achieve excellent yield of the desired PCR product (see the *Appendix* for example amplifications).

Optimization

When optimizing PCR reactions, it is best to change only one parameter at a time.

Troubleshooting

Symptom	Possible cause	Solution
No PCR product	Annealing temperature is too high	Lower annealing temperature in 3°C increments
	PCR primers are not long enough	Use primers longer than 21 bases
Low yield	Suboptimal PCR conditions	Increase number of cycles
Smearing	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 10 s/kbp
Smearing above target size	Extension times too long	Reduce extension time 10 s/kbp
Primer dimers	Primers are complementary to each other	Design primers that do not form hairpins (are not self complementary) or dimers (are not complementary to each other)
	Primer concentration is too high	Reduce primer concentration
	Annealing temperature too low	Raise annealing temperature
Unexpected mutation	Denaturation step is too long	Lower denaturation time to 5 s or 1 s.

References

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3. Fujii, S., Akiyama, M., Aoki, K., Sugaya, Y., Higuchi, K., Hiraoka, M., Miki, Y., Saitoh, N., Yoshiyama, K., Ihara, K., Seki, M., Ohtsubo, E., and Maki, H. (1999) *J. Mol. Biol.* **289**, 835–850.
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Appendix

Example amplifications

Amplification of 6977 bp of the GC-rich BAX coding region from human genomic DNA. The 6977 bp product exhibits 53% GC content. The first 529 bp are 71.8% GC and contain a segment that is 84.3% GC.

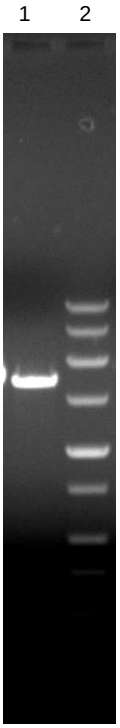
Reaction setup

Component	Volume	Final Concentration
2X Xtreme Buffer	25 µl	1X
dNTPs (2 mM each)	10 µl	0.4 mM (each)
PCR Grade Water	10 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* (5 ng/µl)	1 µl	0.1 ng/µl
KOD Xtreme™ 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 5 ng/µl

Cycling conditions

Step	Temperature and time
1. Polymerase activation	95°C for 2 min
2. Denature	98°C for 1 s
3. Anneal/Extend	74°C for 7 min
Perform Steps 2 and 3 for 5 cycles	
4. Denature	98°C for 1 s
5. Anneal/Extend	72°C for 7 min
Perform Steps 4 and 5 for 5 cycles	
6. Denature	98°C for 1 s
7. Anneal/Extend	70°C for 7 min
Perform Steps 6 and 7 for 5 cycles	
8. Denature	98°C for 1 s
9. Anneal/Extend	68°C for 7 min
Perform Steps 8 and 9 for 15 cycles	
10. Hold	4°C



0.4 % TAE agarose gel
Lane 1 5 µl amplification reaction
Lane 2 Perfect DNA™ Markers, 0.5-12kbp
(Cat. No. 69002-3)

Amplification of 8462 bp p53 region from human genomic DNA.

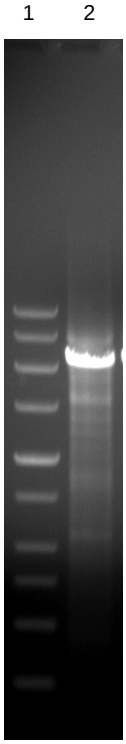
Reaction setup

Component	Volume	Final Concentration
2X Xtreme Buffer	25 µl	1X
dNTPs (2 mM each)	10 µl	0.4 mM (each)
PCR Grade Water	10 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 Xtreme™ 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	94°C for 2 min
2. Denature	98°C for 1 s
3. Annealing	60°C for 10 s
4. Extension	68°C for 8.5 min
Repeat steps 2–4	30 cycles total
5. Hold	4°C



0.4 % TAE agarose gel
Lane 1 Perfect DNA™ Markers, 0.5-12kbp (Cat. No. 69002-3)
Lane 2 5 µl amplification reaction