KOD -Plus- Neo

KOD-401 200 U 200 reactions

Store at -20°C

Contents

CAUTION
All reagents in this kit are intended for research purposes. Do not use for diagnostic or clinical purposes. Please observe general laboratory practices and safety precautions while using this kit.

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[1] Introduction

Description

KOD -Plus- Neo is based on a DNA polymerase from the hyperthermophilic Archaeon *Thermococcus kodakarensis* KOD1[1) 2)]. This polymerase exhibits excellent PCR fidelity because of its efficient 3'→5' exonuclease activity (proof reading activity). This product contains a unique "elongation enhancer" that suppresses the "plateau effect" produced by conventional PCR. Therefore, this reagent exhibits greater amplification efficiency and elongation capability compared to the previous version of KOD -Plus- (Code No. KOD-201).

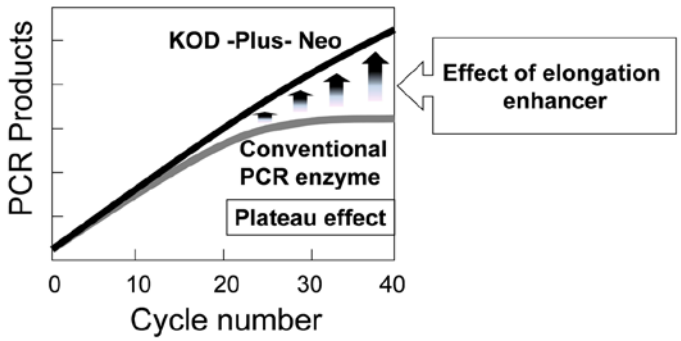
Moreover, this enzyme requires only 30 sec/kb for the PCR extension step. This facilitates the long range PCR.

This enzyme contains two anti-KOD DNA polymerase antibodies that inhibit polymerase and 3'→5' exonuclease activity, thus allowing for Hot Start PCR[3)]. This polymerase generates blunt-end PCR products due to 3'→5' exonuclease (proof-reading) activity.

Features

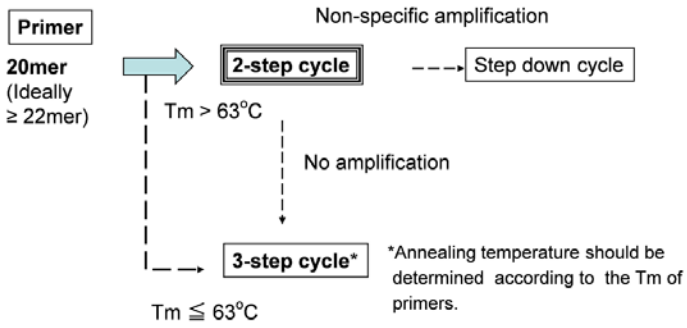
-KOD -Plus- Neo exhibits 80-fold greater PCR fidelity than Taq DNA polymerase.

-"Elongation enhancer" enables greater amplification efficiency and elongation capability (up to 24 kb from human genomic DNA) compared to conventional PCR.



-Requires only 30 sec/kb for the PCR extension step.

-2-step cycle conditions can be used for amplification using ≥ 20 mer primers (melting temperatures, Tm >63°C).



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*The Nearest Neighbor method is recommended to calculate the Tm of primers. The Tm values in this manual were calculated using this method with the following parameters.
Na^+ concentration: 50 mM
Oligonucleotide concentration: 0.5 μM

[2] Components

KOD -Plus- Neo (1.0 U/μL)*	200 μL x 1
10 x PCR Buffer for KOD -Plus- Neo	1 mL x 1
25 mM $MgSO_4$	1 mL x 1
2 mM dNTPs	1 mL x 1

*The enzyme solution contains anti-KOD DNA polymerase antibodies that neutralize polymerase and 3'→5' exonuclease activity.

[3] Quality Testing

Quality control was performed by amplifying the human β-globin gene (17.5 kb).

[4] Primer Design

-Primers should be 22–35 bases with Tm >63°C.

-Optimal GC content of primers is 45–60%. Ideal GC contents of the 5' half and the 3' half are 60–70% and 40–50%, respectively.

-Primers for long target amplification should be 25-35 bases with Tm >65°C.

-Primers containing inosine cannot be used.

-The Tm of primers should be calculated using the Nearest Neighbor method. The Tm values in this manual were calculated using this method with the following parameters.
Na^+ concentration: 50 mM
Oligonucleotide concentration: 0.5 μM

[5] Cloning of PCR products

-KOD -Plus- Neo generates blunt-end PCR products due to its 3'→5' exonuclease (proof-reading) activity. Therefore, PCR products can be cloned according to blunt-end cloning methods.

-PCR products of KOD -Plus- Neo should be purified prior to restriction enzyme treatments. The 3'→5' exonuclease activity of KOD DNA polymerase remains at the end of the PCR reaction.

-The dedicated TA cloning kit "TArget clone™ -Plus- (Code No. TAK-201)" is recommended for the cloning of blunt end PCR products produced by KOD DNA polymerase (see [10] Related product).

[6] Protocol

1. Standard reaction setup

The following procedure is designed for use with the components provided in this kit. Before mixture preparation, all components should be completely thawed, except for the enzyme solution.

Component	Volume	Final Concentration
10x Buffer for KOD -Plus- Neo	5 µL	1x
2 mM dNTPs*	5 µL	0.2 mM each
25 mM $MgSO_4$	3 µL	1.5 mM
10 pmol/µL Primer #1	0.75–1.5 µL	0.15–0.3 µM
10 pmol/µL Primer #2	0.75–1.5 µL	0.15–0.3 µM
Template DNA	X µL	Genomic DNA ≤ 200 ng/50 µL Plasmid DNA ≤ 50 ng/50 µL cDNA ≤ 200 ng (RNA equiv.)/50 µL
PCR grade water	Y µL	
KOD -Plus- Neo (1.0 U/µL)	1 µL	1.0 U/50 µL
Total reaction volume	50 µL	

*Do not use dNTPs from other kits or companies.

Notes:

-Optimal primer concentration is 0.3 µM. In the case of long target (≥10 kb), a reduced primer concentration (0.15 µM) may give more effective amplification.

-The addition of DMSO (final conc. 2–5%) is beneficial for the amplification of GC-rich targets. Decreased PCR fidelity does not occur in the presence of DMSO (See step 2, Cycling conditions).

-Contaminating RNA in cDNA or genomic DNA inhibits the PCR reaction by chelating Mg^{2+}. PCR should be performed using template DNA containing <200 ng RNA.

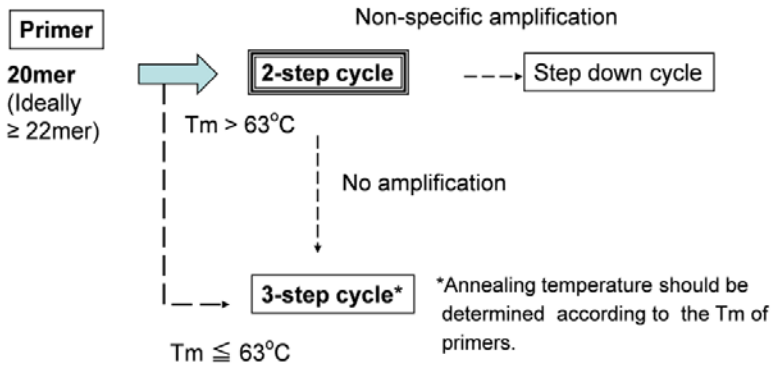
-Quality of template DNA should be checked by electrophoresis. The length and purity of template DNA affects amplification results.

-Templates containing uracil cannot be used for amplification.

-For PCR reactions, thin-wall tubes are recommended. A total reaction volume of 50 µL is also recommended.

2. Cycling conditions [Important]

Two-step cycle conditions with primers ≥20 mer, Tm >63°C are recommended for effective amplification using KOD -Plus- Neo.



A. 2-step cycle

If the Tm value of the primer is over 63°C, a 2-step cycle is recommended.

< 2-step cycle >	
Pre-denaturation:	94°C, 2 min.
Denaturation:	98°C, 10 sec.
Extension:	68°C, **30 sec./kb**

25-45 cycles

Notes:

-For amplification using low copy templates or amplification of long targets (>10 kb), longer extension times (up to 1min/kb) or higher Mg concentrations (up to 2 mM final concentration) may increase the yield.

-The addition of DMSO (final conc. 2–5%) may be beneficial for the amplification of GC-rich targets. The concentration of DMSO used should be determined according to the Tm of the primers.

<25 mer or Tm <68°C: up to 2%
≥25 mer or Tm ≥68°C: up to 5%

-In the case of amplification failure a 3-step cycle may be required.

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B. 3-step cycle

When the Tm of primers are less than 63°C, a 3-step cycle should be used.

< 3-step cycle >		
Pre-denaturation:	94°C, 2 min.	
Denaturation:	98°C, 10 sec.	↰ 25-45 cycles
Annealing:	[Tm]°C, 30 sec.	
Extension:	68°C, **30sec./kb**	

Notes:

-Amplification using low copy templates or amplification of long targets (>10 kb) may be improved by increasing the extension time (up to 1 min/kb) or Mg concentration (up to 2 mM final concentration).

-The addition of DMSO (final conc. 2–5%) might be beneficial for the amplification of GC-rich targets.

C. Step-down cycle

If non-specific amplification is observed with 2-step and 3-step cycle conditions (extra or smeared bands observed after electrophoresis of PCR product), then the step-down cycle may improve specificity.

< Step-down cycle >		
Pre-denaturation:	94°C, 2 min.	
Denaturation:	98°C, 10 sec.	↰ 5 cycles
Extension:	74°C , **30 sec./kb**	
Denaturation:	98°C, 10 sec.	↰ 5 cycles
Extension:	72°C , **30 sec./kb**	
Denaturation:	98°C, 10 sec.	↰ 5 cycles
Extension:	70°C , **30 sec./kb**	
Denaturation:	98°C, 10 sec.	↰ 15-30 cycles
Extension:	68°C , **30 sec./kb**	
Extension:	68°C , 7 min.	

Notes:

-Amplification from low copy templates or amplification of long targets (>10 kb), may be improved by increasing the extension time (up to 1 min/kb) or the Mg concentration (up to 2 mM final concentration).

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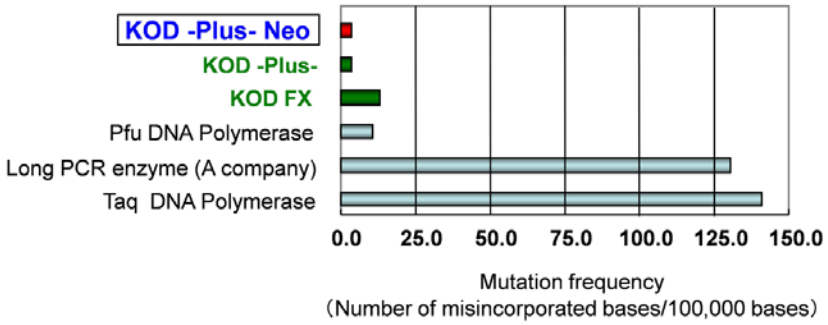
- The addition of DMSO (final conc. 2–5%) might be effective for the amplification of GC-rich targets. The DMSO concentration should be determined according to the Tm of primers.

<25 mer	or	Tm <68°C:	up to 2%
≥25 mer	or	Tm ≥68°C:	up to 5%

[7] Examples

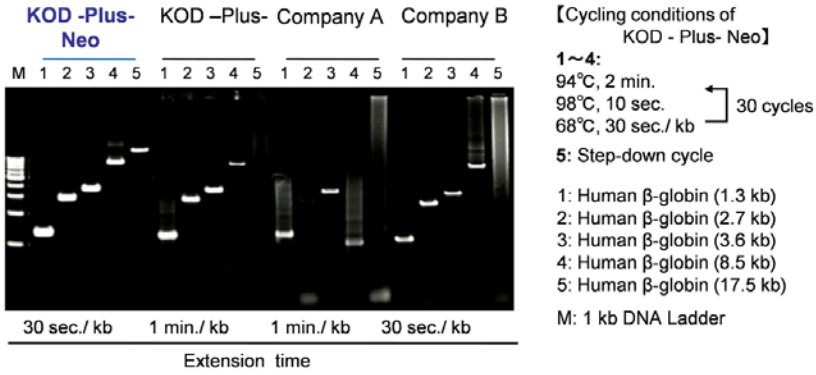
Performance data 1. PCR fidelity

Mutation frequency was measured by sequence analysis of human β-globin gene products amplified from human genomic DNA via TA cloning with TArget Clone™ -Plus-. KOD -Plus- Neo showed excellent fidelity and the mutation frequency was equal to that of the previous version of the enzyme (KOD -Plus-).



Performance data 2. Elongation capability

Targets of various sizes were amplified from human genomic DNA by several PCR enzymes according to the recommended conditions of each enzyme. KOD -Plus- Neo successfully amplified targets up to 17.5 kb.



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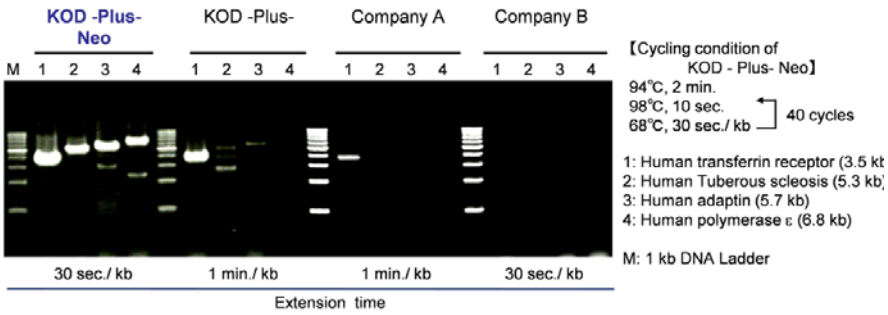
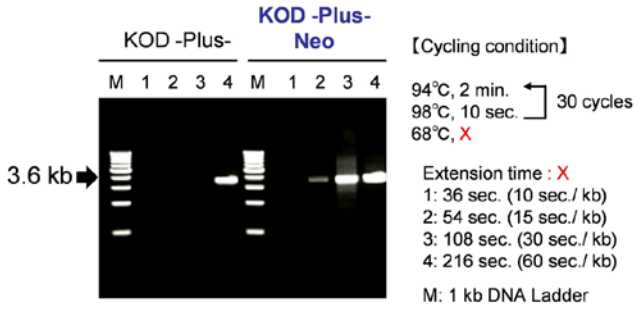
Performance data 3. Amplification from low copy templates

Four genes were amplified using 0.5 ng cDNA template (RNA equiv.). Templates were synthesized from total RNA of HeLa cells. KOD -Plus- Neo successfully amplified all genes.



Performance data 4. Elongation rate

The β-globin gene (3.6 kb) was amplified from human genomic DNA (50 ng) using various extension times. KOD -Plus- Neo can amplify a 3.6 kb target using an extension time of 30 sec/kb.



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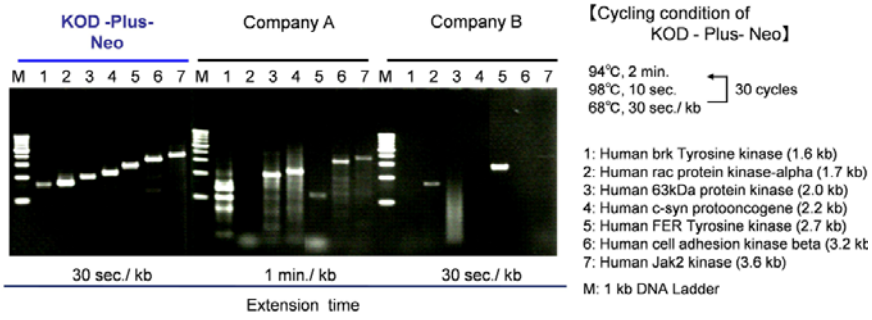
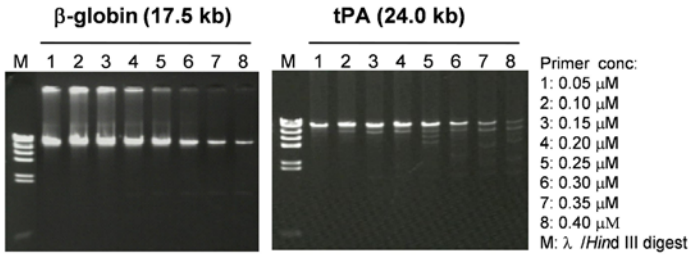
Application data 1. Amplification of various protein kinase targets

Various protein kinase open reading frames (ORFs) were amplified using cDNA synthesized from total RNA of HeLa cells. KOD -Plus- Neo successfully amplified all targets.



Application data 2. Amplification of long targets

Long targets were amplified from human genomic DNA using various concentrations of primers. Excessive amounts of primer inhibit the amplification. Therefore, long targets (>10 kb) should be amplified using lower primer concentrations of approximately 0.15 μM.



[Cycling condition]

β-globin (17.5 kb)
:Step-down cycle (Extension time: 30 sec. /kb)
tPA (24.0 kb)
:Step-down cycle (Extension time: 60 sec./kb)

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[8] Troubleshooting

Symptom	Cause	Solution
No PCR product/low yield	Cycling conditions are not suitable.	Using the 3-step cycle, lower annealing temperature incrementally to a maximum of Tm-5–10°C.
		Prolong the extension time to 1 min./kb.
		Increase the number of cycles by 2–5 cycles.
	Mg concentration is low.	Increase the Mg concentration up to 2 mM.
	High GC content of target sequence.	Add DMSO 2–5%. [See Cycling conditions]
	Quality and/or quantity of primers is not sufficient.	Decrease the primer concentration incrementally down to 0.15 μM.
		Use fresh primers.
		Redesign primers.
	Quality and/or quantity of template DNA is not sufficient.	Check the quality of template DNA. RNA inhibits amplification.
		Increase the amount of template DNA.
	Enzyme concentration is low.	Increase enzyme concentration up to 1.5–2.0 U/50 μL .
Smearing/extra band	Cycling conditions are not suitable.	Decrease the number of cycles by 2–5 cycles.
		Change from 3-step cycle to 2-step cycle.
		Change from 2-step cycle to Step-down cycle.
	Quality of primers is not sufficient.	Use fresh primers.
		Redesign primers.
	Too much template DNA.	Reduce the amount of template DNA.
	Too much Mg.	Reduce $MgSO_4$ incrementally down to 1.0 mM.
	Too much enzyme.	Reduce enzyme concentration to 0.5–0.8 U/50 μL .
Poor TA cloning efficiency.	PCR products have blunt-ends.	Clone the PCR products according to general blunt-end cloning guidelines. Use TArget clone™ -Plus- (Code No.TAK-201) [See Related products].

[9] Reference

1) Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, and Imanaka T., *Appl Environ Microbiol.*, 63: 4504-10 (1997)

2) Hashimoto H, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Inoue T and Kai Y, *J Mol Biol*., 306: 469-77 (2001)

3) Mizuguchi H, Nakatsuji M, Fujiwara S, Takagi M and Imanaka T, *J Biochem*., 126: 762-8 (1999)

4) 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, *J. Mol. Bio*l., 289: 835-850 (1999)

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[10] Related products

Product name	Package	Code No.
TArget Clone™ -Plus-	10 reactions	TAK-201
10x A-attachment mix	25 reactions	TAK-301
Ligation high Ver.2	750 μL (100 reactions)	LGK-201

TArget Clone™ -Plus- is a high efficient TA cloning kit. The kit can be applied to the TA cloning of blunt-end PCR products amplified using KOD -Plus- [Code No. KOD-201], KOD -Plus- Neo [Code No. KOD-401] or KOD FX [Code No. KFX-101]. The kit contains pTA2 Vector, 2x Ligation Buffer, T4 DNA Ligase and 10x A-attachment Mix.

10 x A-attachment mix is a reagent comprising anti-KOD DNA polymerase antibody specific to KOD 3'→5' exonuclease activity (proof-reading activity), as well as Taq DNA polymerase, which exhibits terminal transferase activity. PCR products from KOD -Plus- [Code No. KOD-201] and KOD FX [Code No. KFX-101] possess blunt ends due to 3'→5' exonuclease activity of the KOD DNA polymerase. The 10 x A-attachment mix allows for PCR products to acquire overhanging dA at the 3'-ends. Products with 3'-dA overhangs can be directly cloned into arbitrary T-vectors using ligation reagents, such as Ligation high Ver.2 [Code No. LGK-201].

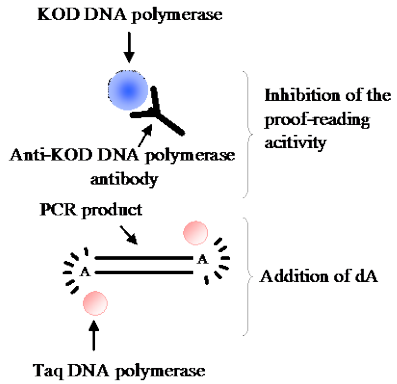

Fig. Principle of the 10 x A-attachment mix

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