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Instruction manual KOD FX 1110 F0935K

KOD FX

KFX-101 200 U 200 reactions

Store at -20°C

Contents

CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precaution and utilize safety while using this kit.

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[1] Introduction

Description

KOD FX is based on DNA polymerase from the hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1[1) 2)]. KOD FX results in much greater PCR success based on efficiency and elongation capabilities than KOD -Plus- (Code No. KOD-201) or other Taq-based PCR enzymes. KOD FX enzyme solution contains two types of anti-KOD DNA polymerase antibodies that inhibit polymerase and 3'→5' exonuclease activities, thus allowing for Hot Start PCR[3)]. KOD FX generates blunt-end PCR products, due to 3'→5' exonuclease (proof-reading) activity.

Features

-This enzyme is effective for the amplification of GC-rich targets and crude samples.

-Hotstart technology using anti-KOD DNA polymerase antibodies enables highly efficient amplification.

-KOD FX enables the following amplifications (Maximum): 40kb from lambda phage DNA, 24kb from human genomic DNA, and 13.5kb from cDNA.

-The PCR error ratio of KOD FX is about 10 times less than that of Taq DNA polymerase.

Table 1. Comparison of the mutation frequency of each PCR enzyme.

	Total bases Sequenced	Number of mutated bases	Mutation frequency ($x10^{-5}$)
KOD FX	144,535	19	13.1
KOD -Plus-	145,753	5	3.4
Pfu DNA polymerase	113,080	12	10.6
Taq-base long-PCR enzyme	167,343	218	130.3
Taq DNA polymerase	102,708	145	141.2

Fidelity was measured as a mutation frequency by sequencing the PCR product. After cloning the PCR product (2.4kb of the human bata-globin region), about 96 clones were selected and sequenced.

[2] Components

This reagent includes the following components for 200 reactions;

KOD FX (1.0 U/µl) *	200 µl × 1
2× PCR Buffer for KOD FX**	1.7 ml × 3
2 mM dNTPs	1 ml × 2

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

** 2× PCR Buffer for KOD FX is a liquid (not congealed) when in storage at -20°C. Although it does congeal below -20°C, the quality is not affected.

[3] Quality Testing

Quality check is performed by amplification of the tPA gene (24kb).

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[4] Primer Design

-Primers should be 22-35 bases with a melting temperature (T_m) over 60°C. For amplification of a long target, 25-35 bases with high T_m values (≥ 65°C) are recommended. The primer Tm value for the 2-step or step-down cycles should be ≥ 73°C.

-For amplification of longer targets (≥ 10kb), primer oligonucleotides should be cartridge- or HPLC- purified. Primers purified by gel filtration tend to result in smeared PCR products.

[5] Cloning of PCR products

-KOD FX generates blunt-end PCR products, due to 3'→5' exonuclease (proof-reading) activity. Therefore, the product can be cloned according to a blunt-end cloning method.

-PCR products of KOD FX should be purified prior to restriction enzyme treatments. The 3'→5' exonuclease activity of KOD DNA polymerase remains after the PCR cycles.

[6] Protocol

1. Standard reaction setup

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

Component	Volume	Final Concentration
2x PCR buffer for KOD FX	25 µl	1×
2mM dNTPs*	10 µl	0.4 mM each
10pmol /µl Primer #1	1.5 µl	0.3 µM
10pmol /µl Primer #2	1.5 µl	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 Crude sample ≤0.5-4 µl (see [7])
PCR grade water	Y µl	
KOD FX (1.0U/µl)	1 µl	1.0 U / 50 µl
Total reaction volume	50 µl	

* Do not use dNTPs from other kits or companies.

Notes:

-For PCR reaction, thin-wall tubes are recommended. Reaction setup to a total reaction volume of 50 µl is also recommended.

-Addition of DMSO (final conc. 2-5%) might be effective for amplification of GC-rich target. No decreasing of the PCR fidelity by adding DMSO has been confirmed.

-Crude samples (e.g., cultured animal cell suspension) should be added up to 2 µl for each 50-µl reaction.

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2. Cycling conditions

The following cycling steps are recommended.

< 2-step cycle >		
Pre-denaturation :	94°C, 2 min.	
Denaturation :	98°C, 10 sec.	↰ 25-40 cycles
Extension :	68°C, 1 min. /kb	

Note: If the Tm value of the primer is under 73 °C, the 3-step cycle is recommended.

< 3-step cycle >		
Pre-denaturation :	94°C, 2 min.	
Denaturation :	98°C,10 sec.	↰ 25-40 cycles
Annealing :	Tm-[5-10]°C*, 30 sec.	
Extension :	68°C, 1 min. /kb	

*Tm value of the primer minus 5°C-10°C

< Step-down cycle >		
Pre-denaturation :	94°C, 2 min.	
Denaturation :	98°C, 10 sec.	↰ 5 cycles
Extension :	74°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	↰ 5 cycles
Extension :	72°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	↰ 5 cycles
Extension :	70°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	↰ 15-25 cycles
Extension :	68°C, 1 min. /kb	
Extension :	68°C, 7 min.	

Note : If the Tm value of the primer is under 73 °C, the 3-step cycle is recommended.

Notes:

-Extension time should be set at 1min. per 1kbp of target length. Although even 30 sec./ kb will give amplification in many cases, amplification efficiency or reliability may be decreased (See [8] Example 3).

-Because this enzyme possesses an extremely high amplification efficiency, 25-30 cycles usually give sufficient amplification. For a low-copy number target, or over 10 kb target length, 30-40 cycles are recommended.

-The step-down cycle condition is effective for trouble shooting a smear amplification in a long-distance PCR (>10kb).

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[7] Amplification from crude samples

KOD FX exhibits superior efficiency when amplifying targets from crude samples (*e.g.*, whole blood, mouse tail lysate, and plant lysate). The following table lists specimens that have been successfully amplified with KOD FX. Certain samples require pretreatment prior to PCR. The pretreatment methods are described below.

Pretreatment	Sample
Not required	Whole blood
Not required	Dried blood on filter paper
Not required	Cultured cells
Not required	Yeast (colony)
Not required	Bacteria (colony)
Not required	Fungus (colony)
Not required	Sperm
Not required	Plankton
Lysis	Mouse tail
Lysis	Plant (leaf, rice grain, etc)
Lysis	Hair root

(1) Amplification from not-treated samples

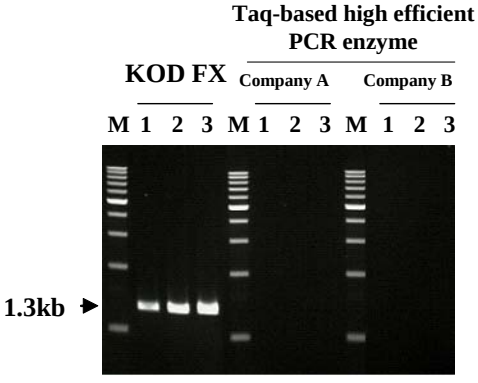
<Samples>

The following samples can be applied directly to 50 µl of reaction mixture.

-Whole blood: 1-4 µl
-Cultured cells: <2 × 10^4 cells [*e.g.*, 2 µl of cell suspension in RPMI/10% FCS (1 × 10^4 cells/µl)]
-Yeast, bacteria, and fungus: Small amount of the colony (enzymatic treatment is not required).

<Example>

Sample: whole blood (EDTA) [1: 1 µl; 2: 2 µl; 3: 4 µl]



Target: Human β-globin gene

Cycling conditions:

94°C, 2 min.
98°C, 10 sec.
68°C, 1.5 min.
30 cycles

Primer F: 5'-TTAGGCCTTAGCGGGCTTAGAC-3'
Primer R: 5'-CCAGGATTTTTGATGGGACACG-3'

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(2) Amplification from lysates

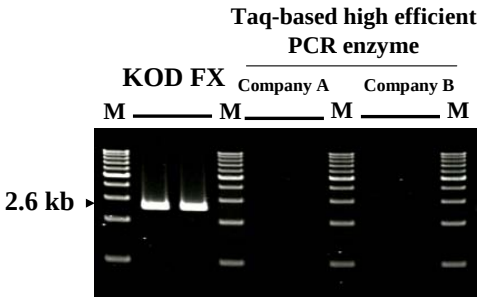
(a) Mouse tail

<Pretreatment>

The following "Alkaline lysis method" is recommended for rapid preparation of mouse tail lysate suitable for amplification with KOD FX.

<Example>

Sample: mouse tail lysates prepared by "Alkaline lysis method" (0.5 µl/50 µl reaction)



M: 1 kb Ladder Marker

Target: Mouse membrane glycoprotein (Thy-1) gene

Cycling conditions:

94°C, 2 min.

98°C, 10 sec. ↩ 30 cycles

68°C, 2.5 min.

Primer F :5'-CCACAGAATCCAAGTCGGAACTCTTG-3'
Primer R :5'-GTAGCAGTGGTGGTATTATACATGGTG-3'

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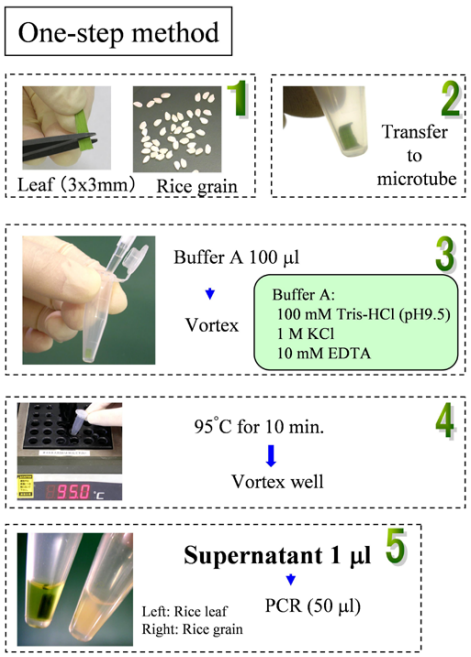
(b) Plant tissues

<Pretreatment>

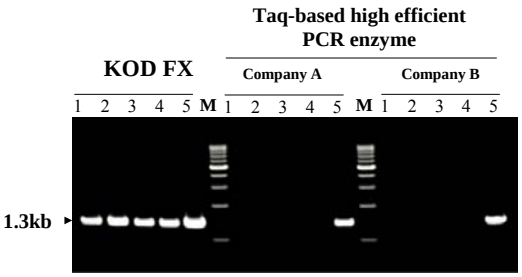
The following "One-step method" is recommended for a rapid preparation of the plant tissue lysate suitable for amplification with KOD FX.



*Buffer A is not supplied with this kit.

<Example>

Sample: Plant lysates prepared by "One-step method" (1 µl/50 µl reaction)



1: Leaf (Tomato)
2: Leaf (Tobacco)
3: Leaf (Rice)
4: Grain (Rice)
5: Purified rice leaf DNA

Target: rbcL gene 1.3 kb

Cycling conditions:

94°C, 2 min.
98°C, 10 sec.
68°C, 1.5 min.
30 cycles

Primer F1:5'-ATGTCACCACAAACAGAGACTAAAGC-3' (Tomato & Tobacco)
Primer R1 :5'-AAGCAGCAGCTAGTTCCGGGCTCCA-3' (Tomato & Tobacco)
Primer F2::'-ATGTCACCACAAACAGAAACTAAAGC-3' (Rice)
Primer R2 :5'-AAGCTGCGGCTAGTTCAGGACTCCA-3' (Rice)

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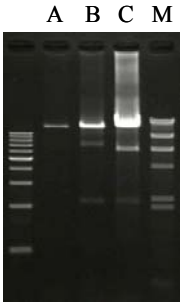
[8] Example

Example 1. Large PCR products (≥10 Kb) with various number of cycles.



Template: cDNA from Adult Skeletal Muscle total RNA
100 ng equiv. / 50 µl

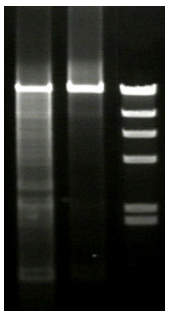
Target: Dystrophin [NM_004006] 13.5 kb

PCR cycling conditions:

94 ℃ 2 min.
98 ℃ 10 sec. ⌐ A:30 cycles B: 35 cycles C: 40 cycles
68 ℃ 14 min. ⌙

M : λ /*Hin*dIII digest

Example 2. Large PCR products (≥ 10 Kb) with various cycle conditions.



Template: Human Genomic DNA 200 ng / 50 µl

Target: tPA 24 kb

PCR cycling conditions:

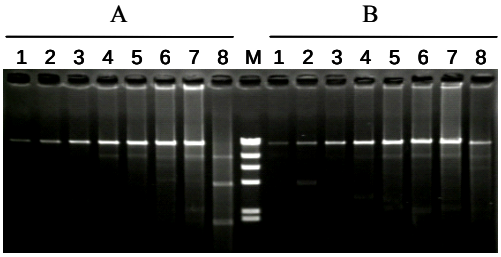
A

94 ℃ 2 min.
98 ℃ 10 sec. ⌐ 30 cycles
68 ℃ 24 min. ⌙

B

94 ℃ 2 min.
98 ℃ 10 sec. ⌐ 5 cycles
74 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
72 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
70 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 20 cycles
68 ℃ 24 min. ⌙
68 ℃ 7 min.

Example 3. Correlation between amount of the template DNA and extension time.



Target: tPA 24kb

Template: Human genomic DNA

1: 6 ng / 50 µl
2: 12 ng
3: 25 ng
4: 50 ng
5: 100 ng
6: 200 ng
7: 400 ng
8: 800 ng

PCR cycling conditions:

A

94 ℃ 2 min.
98 ℃ 10 sec. ⌐ 5 cycles
74 ℃ 12 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
72 ℃ 12 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
70 ℃ 12 min. ⌙
98 ℃ 10 sec. ⌐ 20 cycles
68 ℃ 12 min. ⌙
68 ℃ 7 min.

B

94 ℃ 2 min.
98 ℃ 10 sec. ⌐ 5 cycles
74 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
72 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 5 cycles
70 ℃ 24 min. ⌙
98 ℃ 10 sec. ⌐ 20 cycles
68 ℃ 24 min. ⌙
68 ℃ 7 min.

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[9] Trouble shooting

Symptom	Cause	Solution
No PCR product / low yield	Cycling condition is not suitable.	Use 3-step instead of 2-step cycling. Lower annealing temperature in 3°C decrements up to Tm-10°C.
		Increase the number of cycles by 2-5 cycles. ＜See [8] Example 1＞
	Primer is not good.	Check the quality of primers.
		Redesign primers.
	Template DNA is not good in quality and/or quantity.	Check the quality of template DNA.
		Increase the amount of template DNA ＜See [8] Example 3＞
	Too much sample	Excessive amounts of crude sample may inhibit amplification. Decrease the sample volume to 0.5 µl/50 µl reaction.
Smearing / Extra band	Cycling condition is not suitable.	Decrease the number of cycles by 2-5 cycles. ＜See [8] Example 1＞
		Use step-down cycling＜See [8] Example 2＞
	Primer is not good.	Check the quality of primers.
		Redesign primers.
	Too much template DNA	Reduce the amount of template DNA ＜See [8] Example 3＞
	Too much enzyme	Reduce Enzyme to 0.5-0.8U/ 50µl reaction

[10] References

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)

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[11] 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] 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].

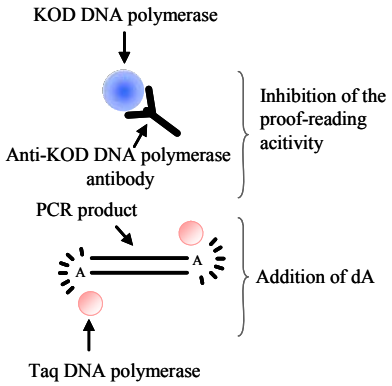

Fig. Principle of the 10 x A-attachment mix

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