



# KOD FX Neo

KFX-201 200 U 200 reactions

**Store at -20°C**

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## CAUTION

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

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## [1] Introduction

### Description

KOD FX Neo is based on the DNA polymerase from the hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1<sup>1)</sup> 2). KOD FX Neo provides greater efficiency and elongation capabilities than conventional PCR enzymes. In particular, KOD FX Neo shows greater amplification success from crude specimens.

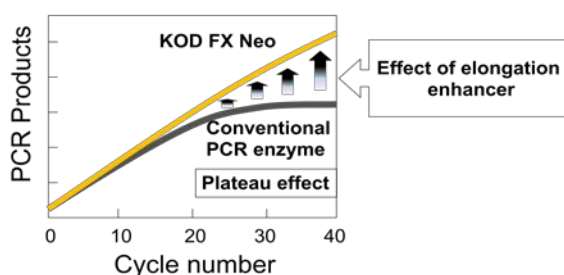
KOD FX Neo is the improved version of the previous KOD FX (Code No. KFX-101). This product contains a unique “elongation enhancer” that suppresses the “plateau effect”, enabling greater elongation rates and capabilities.

The KOD FX Neo enzyme solution contains two types of anti-KOD DNA polymerase antibodies that inhibit the polymerase and 3'→5' exonuclease activities, thus allowing for Hot Start PCR<sup>3)</sup>. KOD FX Neo generates blunt-end PCR products because of its 3'→5' exonuclease (proof-reading) activity.

### Features

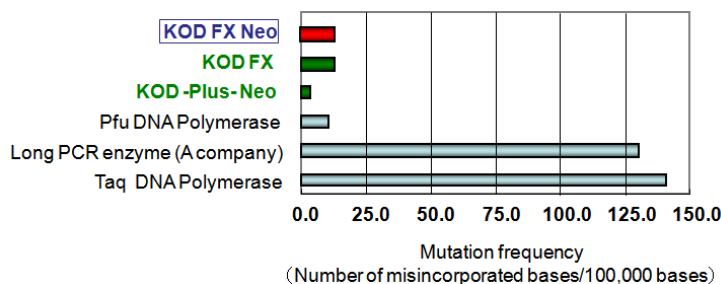
-This enzyme is effective for amplification from crude samples (e.g. biological samples, foodstuffs, soil extract, etc). Various samples or lysates can be used directly as templates.

-“Elongation enhancer” enables greater amplification efficiency and elongation capability (up to 40 kb from human genomic DNA) compared to conventional PCR. This enzyme is useful for amplifying difficult targets, such as high G/C, A/T, and/or long targets.



-Various microorganisms (e.g. yeast, fungus, gram-positive bacteria) can be directly used as templates for PCR.

-The PCR error ratio of KOD FX Neo is about 10 times less than that of Taq DNA polymerase and is equal to the previous version (KOD FX).



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

Fidelity was measured as the mutation frequency by sequencing the PCR product. After cloning the PCR product (5.7 kb of the human  $\beta$ -globin region), about 96 clones were selected and sequenced.

## [2] Components

KOD FX Neo (1.0 U/ $\mu$ L) *	200 $\mu$ L $\times$ 1
2 $\times$ PCR Buffer for KOD FX Neo**	1.7 mL $\times$ 3
2 mM dNTPs	1 mL $\times$ 2

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

\*\* 2 $\times$  PCR Buffer for KOD FX Neo is a liquid (not frozen) when stored at -20°C. Although it does freeze below -20°C, the quality is not affected.

## [3] Quality Testing

Quality testing is performed by amplification of the human  $\beta$ -globin gene (32 kb).

## [4] Primer Design

- Primers should be 22–35 bases with a melting temperature ( $T_m$ ) over 63°C.
- Optimal GC content of primers is 45–60%. The ideal GC contents of the 5' half and the 3' half are 60–70% and 45–50%, respectively.
- The priming efficiency of primers can be promoted by anchoring the 3' end of primers with G or C.
- Primers should be designed so as not to generate intermolecular secondary structures or primer dimers.
- Primers for long target amplification should be 25–35 bases with  $T_m$  over 65°C.
- Primers containing inosine cannot be used.
- The  $T_m$  of primers should be calculated using the Nearest Neighbor method. The  $T_m$  values in this manual were calculated using this method with the following parameters.  
 $\text{Na}^+$  concentration: 50 mM  
Oligonucleotide concentration: 0.5  $\mu$ M

## [5] Cloning of PCR products

- KOD FX Neo generates blunt-end PCR products because of its 3'  $\rightarrow$  5' exonuclease (proof-reading) activity. Therefore, PCR products can be cloned using blunt-end cloning methods.
- PCR products of KOD FX Neo should be purified prior to restriction enzyme treatments in cloning steps. The 3'  $\rightarrow$  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 [11] Related product).

## [6] Protocol

### 1. Standard reaction setup

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

Component	Volume	Final Concentration
PCR grade water	X $\mu$ L	1 $\times$
2x PCR buffer for KOD FX Neo	25 $\mu$ L	1 $\times$
2mM dNTPs*	10 $\mu$ L	0.4 mM each
10pmol / $\mu$ L Primer #1	1.5 $\mu$ L	0.15–0.3 $\mu$ M
10pmol $\mu$ L Primer #2	1.5 $\mu$ L	0.15–0.3 $\mu$ M
Template DNA	Y $\mu$ L	<div> Genomic DNA <math>\leq</math>200 ng / 50 <math>\mu</math>L  Plasmid DNA <math>\leq</math>50 ng / 50 <math>\mu</math>L  cDNA <math>\leq</math>200 ng (RNA equiv.) / 50 <math>\mu</math>L  Crude sample <math>\leq</math>0.5–2 <math>\mu</math>L/50 <math>\mu</math>L (see [ 7 ]) </div>
KOD FX Neo (1.0U/ $\mu$ L)	1 $\mu$ L	1.0 U / 50 $\mu$ L
Total reaction volume	50 $\mu$ L	

\* Do not use dNTPs from other kits or companies.

Notes:

-Optimal primer concentration is 0.3  $\mu$ M. In the case of long targets ( $\geq$ 10 kb), a reduced primer concentration (0.15  $\mu$ M) may give more effective amplification.

-For PCR reactions, thin-wall tubes are recommended. Reaction setup to a total reaction volume of 50  $\mu$ L is also recommended.

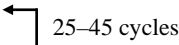
-Crude samples (e.g., cultured animal cell suspension) should be added up to 2  $\mu$ L for each 50- $\mu$ L reaction.

### 2. Cycling conditions

Two-step cycle conditions can be easily used for amplification using KOD FX Neo with primers  $\geq$ 20 mer and  $T_m \geq 68^\circ\text{C}$  [recommended cycle]. If the  $T_m$  value of the primer is under  $68^\circ\text{C}$ , the 3-step cycle condition is effective. For trouble shooting of poor amplification (no band, smear etc) or long target amplification ( $>$ 10 kb), the step-down cycle is recommended. The extension time should be set at 1 min./kb when crude samples are used as templates.

#### <Recommended cycle>

< 2-step cycle >	For purified DNA /cDNA	For crude samples
Pre-denaturation :	94 $^\circ\text{C}$ , 2 min.	94 $^\circ\text{C}$ , 2 min.
Denaturation :	98 $^\circ\text{C}$ , 10 sec.	98 $^\circ\text{C}$ , 10 sec.
Extension :	68 $^\circ\text{C}$ , 30 sec. /kb	68 $^\circ\text{C}$ , 1 min. /kb



### <Other cycles>

< 3-step cycle >	For purified DNA /cDNA	For crude samples	
Pre-denaturation :	94°C, 2 min.	94°C, 2 min.	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	
Annealing	T <sub>m</sub> °C*, 30 sec.	T <sub>m</sub> °C, 30 sec.	25–45 cycles
Extension :	68°C, 30 sec. /kb	68°C, 1 min. /kb	

\*T<sub>m</sub> value of the primer.

< Step-down cycle >	For purified DNA /cDNA	For crude samples	
Pre-denaturation :	94°C, 2 min.	94°C, 2 min.	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	74°C, 30 sec. /kb	74°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	72°C, 30 sec. /kb	72°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	70°C, 30 sec. /kb	70°C, 1 min. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	15–30 cycles
Extension :	68°C, 30 sec. /kb	68°C, 1 min. /kb	
Extension :	68°C, 7 min.	68°C, 7 min.	

### Notes:

- For amplification from crude samples, the extension time should be 1 min./kb.
- For amplification from a low copy DNA or for longer targets (> 10 kb), longer extension times may enhance the efficiency.
- Poor amplification may be improved by changing the denaturation step to 94°C, 15 sec.

## [7] Templates

### a. Purified DNA or cDNA

Appropriate template amounts for 50 µL reaction are summarized in the following table.

		Approved range	Recommended
Genomic DNA	Eukaryotic DNA	5–200 ng	50 ng
	Prokaryotic DNA	0.1–100 ng	10 ng
Plasmid DNA		10 pg–50 ng	1 ng
cDNA		< 200 ng (RNA equiv.)	50 ng (RNA equiv.)
Lambda phage DNA		0.01–10 ng	1 ng

-Contaminating RNA in cDNA or genomic DNA inhibits the PCR reaction by chelating Mg<sup>2+</sup>. 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.

## b. Tissues and cells

When adding biological samples directly to the PCR reaction solution, the following samples can be applied to 50  $\mu$ L reaction.

Sample	Appropriate template amount	Remarks
<i>E. coli</i>	Picked small amount of cells from colonies	When reproducibility is not good, suspended cells in TE buffer should be added (2–5 $\mu$ L)
Yeast	Picked small amount of cells from colonies	
Fungus	Picked small amount of cells from colonies	
Cultured cells	$10^1$ – $10^5$ cells/2 $\mu$ L medium or PBS	
Whole Blood	1–2 $\mu$ L	
Nail	1 $\times$ 1 mm	As the concentration of extracted DNA is low, 35–40 cycles are needed.
Hair root	1–2 cm	
Leaf	2 $\times$ 2 mm	
Milled rice	0.5 $\times$ 0.5 mm	
Mouse tail	1 $\times$ 1 mm	On an agarose gel assay, a portion of amplicon may remain in the slots.

\*In the case of the direct amplification from animal tissues, such as mouse tail, a portion of the amplification product may remain in the gel slot on an agarose gel assay. Adding 10  $\mu$ L of 20 mg/mL proteinase K to 50  $\mu$ L PCR products prior to the electrophoresis is effective to dissociate the aggregates.

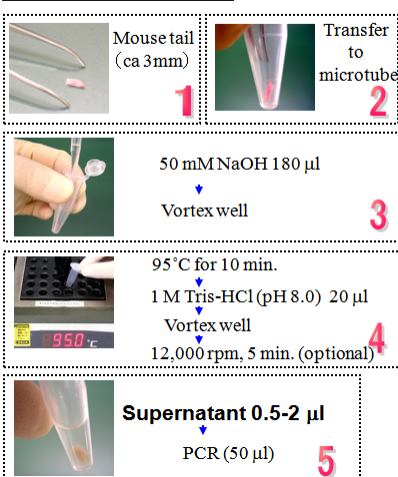
## c. Lysate

To make the lysate for PCR, the following methods are recommended. The lysates can be stored at 4°C for several weeks. For long term storage, the lysates should be stored at -20°C.

### <Alkaline lysis method>

The following “Alkaline lysis method” is recommended for rapid preparation of mouse tails or nail lysates suitable for amplification with KOD FX Neo.

#### Alkaline lysis method



\*Mouse tail cannot be dissolved completely.

### [96-well PCR plate protocol by alkaline lysis method]

The following protocol is useful for the preparation of lysates from a large number of mouse tail samples.

1. Transfer mouse tails (ca 3mm) to a 96-well PCR plate.
2. Add 180  $\mu$ L of 50mM NaOH and vortex.
3. Spin down.
4. Incubate at 95°C for 10 min. using thermal cycler.
5. Add 20  $\mu$ L of 1M Tris-HCl (pH 8.0) and vortex.
6. Spin down.

Supernatant 0.5–2  $\mu$ L → PCR (50  $\mu$ L)

### <Proteinase K method>

The following “Proteinase K method” is recommended for an efficient preparation of mouse tails or nail lysates suitable for amplification with KOD FX Neo. This protocol can also be applied to the following samples.

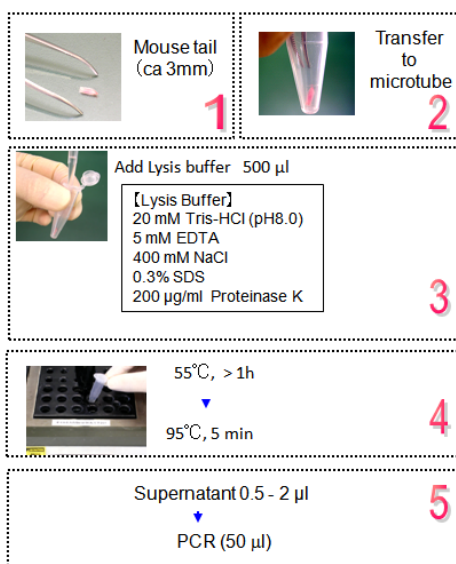
Mouse tail: 3 mm

Nail: 3 mm

Leaf: 3 × 3 mm

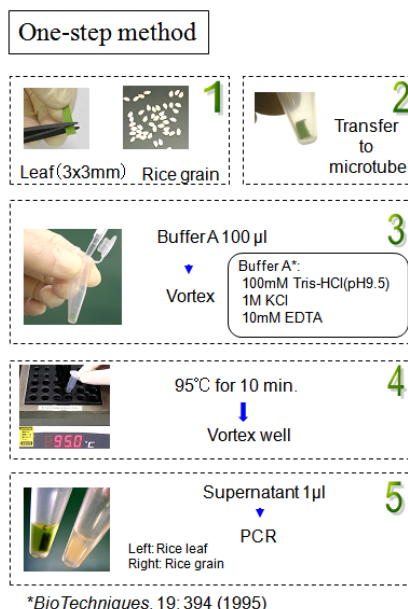
A rice grain

#### Proteinase K method



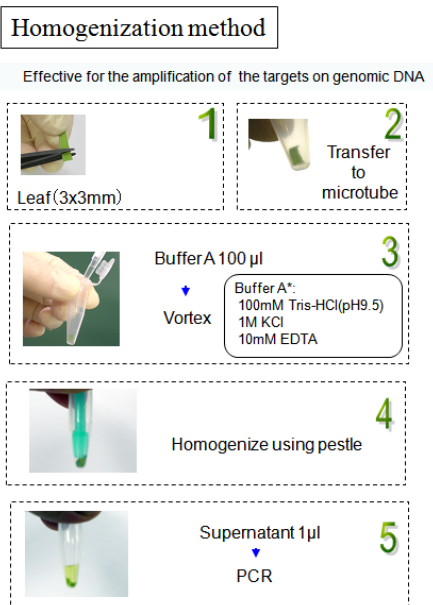
### <One step method>

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



### <Homogenization method>

The following “Homogenization method” is recommended for an effective preparation of a plant tissue lysate suitable for amplification with KOD FX Neo. This method is effective for the amplification of genomic DNA targets.





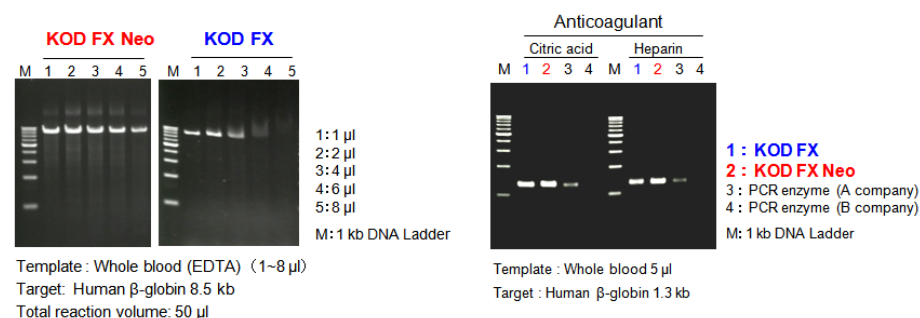
## [8] Examples

### Example 1. Amplification from crude samples

#### a. Whole blood samples

The human  $\beta$ -globin gene (8.5 kb) was amplified using KOD FX Neo and KOD FX (previous version) with various amounts of blood (EDTA) specimens as templates. PCR was performed using 50  $\mu$ L reaction solution contains 1–8  $\mu$ L whole blood samples according to the standard protocol with 30 cycles. As a result, KOD FX Neo successfully amplified the targets using 1–8  $\mu$ L whole blood specimens.

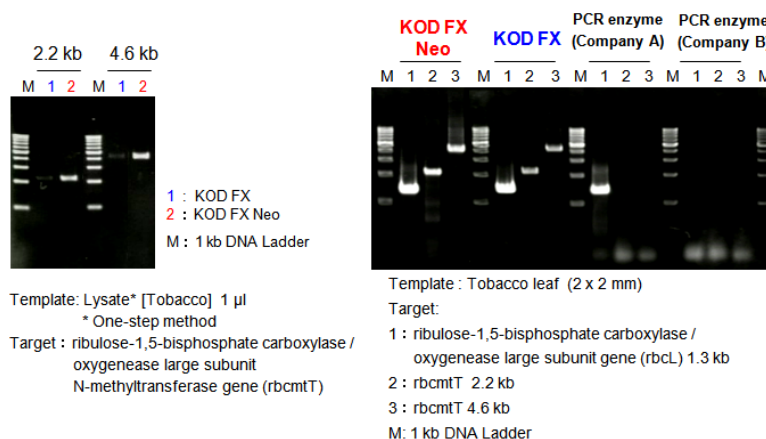
Next, the human  $\beta$ -globin gene (1.3 kb) was amplified using various PCR enzymes with whole blood specimens containing citric acid and heparin as anticoagulants. KOD FX Neo amplified the targets efficiently from both whole blood samples.



#### b. Leaf

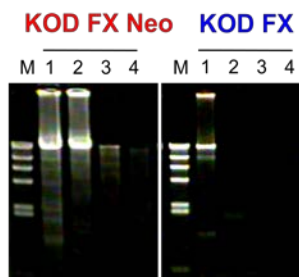
Two targets (2.2 and 4.6 kb) were amplified using lysates from tobacco leaves. Each PCR reaction was performed according to the recommended conditions with 35 cycles. KOD FX Neo showed greater amplification from lysates prepared by the “one-step method”.

Various targets were then directly amplified using small pieces of tobacco leaves (2  $\times$  2 mm). KOD FX Neo successfully amplified DNA using these templates.



## Example 2. Elongation capability

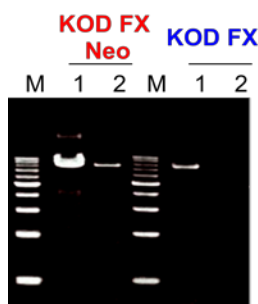
The efficiency for long targets was compared between KOD FX Neo and KOD FX (previous version) using human genomic DNA as templates. The amplification was performed according to the standard protocol with a step-down cycle. KOD FX Neo successfully amplified targets of up to 40 kb.



Template : Human genomic DNA 200 ng  
 1 : Human tissue-type plasminogen activator (tPA) 24 kb  
 2 : Human  $\beta$ -globin 32 kb  
 3 : Human  $\beta$ -globin 40 kb  
 4 : Human  $\beta$ -globin 50 kb  
 M :  $\lambda$ HindIII digest

## Example 3. Amplification efficiency and sensitivity

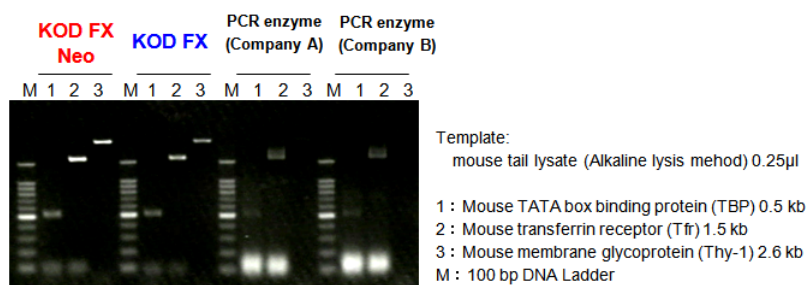
The human  $\beta$ -globin gene (8.5 kb) was amplified using the standard protocol with 40 cycles. KOD FX Neo showed higher sensitivity than KOD FX (previous version). KOD FX Neo can suppress the “plateau effect” on PCR after 20 cycles due to the “elongation enhancer”. Therefore, KOD FX Neo shows greater sensitivity than conventional reagents in the range of 30–45 cycles during PCR.



Template: Human DNA  
 Target : Human  $\beta$ -globin 8.5 kb  
 1 : 0.1 ng  
 2 : 0.01 ng  
 M : 1 kb DNA Ladder

#### Example 4. Amplification from mouse tail lysates

The amplification efficiency was then compared between various PCR enzymes using mouse tail lysates as templates. KOD FX Neo showed greater amplification efficiency than the other enzymes.

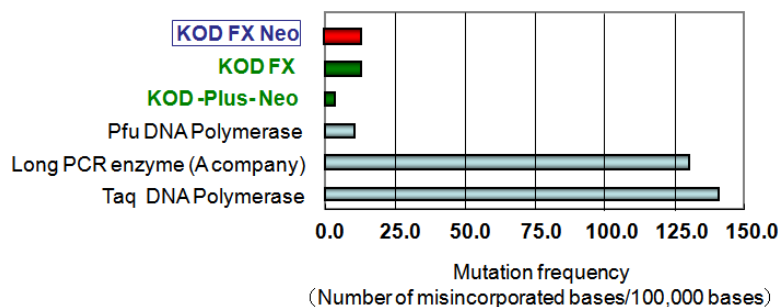


#### Example 5. PCR error ratio

The error ratio of various PCR enzymes were compared by determining the sequences of the amplicons from human  $\beta$ -globin gene. The amplicons were cloned into the vector using TArget clone™ -Plus- (Code No. TAK-201) and the sequences were determined.

The result indicates that the error ratio of KOD FX Neo is equal to that of KOD FX (previous version) and approximately 10 times lower than rTaq DNA polymerase.

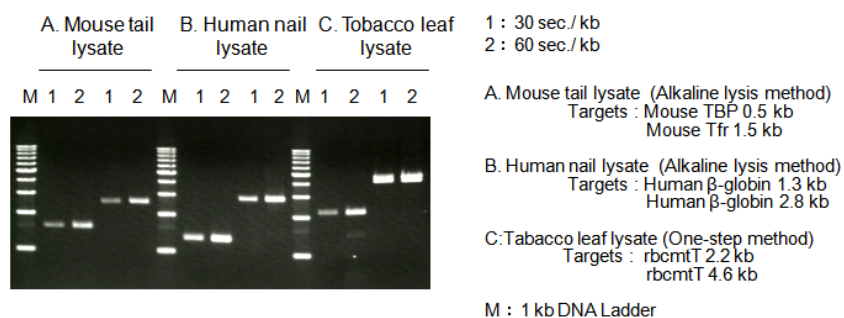
**Table. Comparison of the mutation frequency of each PCR enzyme.**



Fidelity was measured as a mutation frequency by sequencing the PCR product. After cloning, the PCR product (5.7 kb of the human  $\beta$ -globin region), about 96 clones were selected and sequenced.

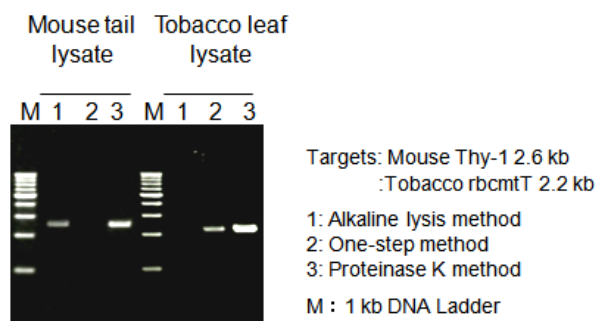
### Example 6 Extension time for crude samples

Various targets were amplified using crude lysates with two cycling conditions (extension time: 30 sec./kb or 60 sec./kb). As a result, longer extension conditions gave greater amplification.



### Example 7 Preparation methods of crude lysates

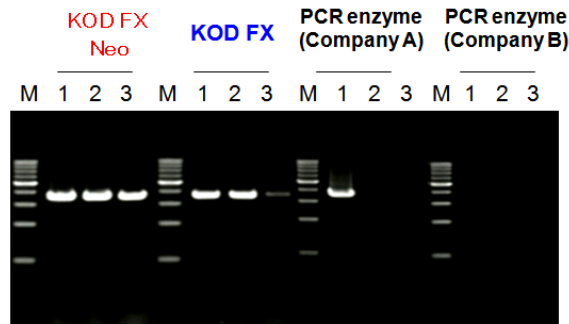
Amplification efficiency was compared using various lysates from mouse tail and tobacco leaf lysates. For mouse tail lysates, the alkaline lysis method and proteinase K method gave superior results. For plant lysates, the one-step and proteinase k methods showed superior results.



### Application data: Amplification using soil specimens.

Inhibitory effect of humic acid was compared between various PCR enzymes. Humic acid is the major organic constituent of soil or compost. It is produced by biodegradation of dead organic matters. The humic acid can be hardly separated from DNA on purification and inhibits PCR.

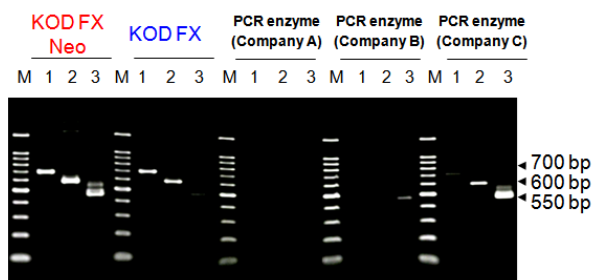
The results indicate that KOD FX Neo can amplify targets under high concentrations of humic acid.



M: 1kb Ladder Marker      Template: 10ng Human genomic DNA  
 1: Humic acid\* 0 µl      Target: Human  $\beta$ -globin gene 3.6kb  
 2: Humic acid\* 2 µl  
 3: Humic acid\* 4 µl

\*The solution of humic acid (OD280=1) was used as a inhibitor.

rDNA was amplified from roughly prepared extracts from 100 mg compost specimens using the alkaline method [see p5] using various PCR enzymes. In this experiment, three primer sets for prokaryote, *Bacillus* sp. and high G/C gram-negative bacteria were used. The results indicate that KOD FX Neo is the only PCR reagent can amplify all targets.



1 Prokaryote derived rDNA (700 bp)  
 2 *Bacillus* sp. derived rDNA (600 bp)  
 3 High G/C gram-positive bacteria derived rDNA (550 bp)

1 Fwd ATTAGATACCCTDGTAGTCC  
 Rev TACCTTGTTACGACTT  
 2 Fwd AGGGTCATTGGAACTGGG  
 Rev CGTGTTGTAGCCAGGTCATA  
 3 Fwd GGCCTTCGGGTTGTAAACC  
 Rev CTTTGAGTTTGTAGCCTTGCGGC

## [9] Troubleshooting

Symptom	Cause	Solution
No PCR product / low yield	Cycling condition is not suitable.	Increase the extension time up to 60 sec./kb.
		Increase the number of cycles by 2–5 cycles.
		Use a 3-step cycle instead of a 2-step cycle. Lower annealing temperature in the 3-step cycling decrements up to T <sub>m</sub> -5–10°C.
		Use a step-down cycle. (This solution is effective for long targets over 10 kb)
	Template DNA is not good in quality and /or quantity.	Increase the amount of template DNA.
		Decrease the amount of template DNA to reduce the contaminated PCR inhibitors.
		Use purified templates.
		Decrease the amount of cDNA to reduce the inhibition by contaminated RNA.
		Degrade or eliminate RNA in the DNA sample.
	Primer is not good.	Decrease the primer concentration from 0.3 mM to 0.15 μM. (This solution may be effective for the amplification of targets over 10 kb)
		Use fresh primers.
		Redesign primers.
	Enzyme concentration is low.	Increase enzyme concentration to 1.5–2.0 U/ 50 μL .
Smearing / Extra band	Cycling conditions are not suitable.	Change from 3-step cycling to 2-step cycling.
		Change from 2-step cycling to step-down cycling.
		Decrease the number of cycles by 2–5 cycles.
	Too much template DNA.	Reduce the amount of template DNA.
	Quality of primers is not sufficient.	Use fresh primers.
		Redesign primers. (Longer primers may eliminate smearing or extra bands)
	Too much enzyme	Reduce Enzyme to 0.5–0.8U/ 50 μL reaction
Poor TA cloning efficiency	PCR products have blunt-ends.	Clone the PCR products according to general blunt-end cloning guide lines. Use TArget Clone™ -Plus- (Code No. TAK-201) [see related products]

## [10] References

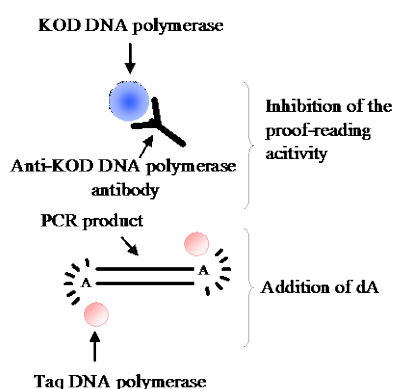
- 1) Takagi M, Nishioka M, Kakihara H, Kitabayashi M, Inoue H, Kawakami B, Oka M, and Imanaka T., *Appl Environ Microbiol.*, 63: 4504-10 (1997)
- 2) Hashimoto H, Nishioka M, Fujiwara S, Takagi M, Imanaka T, Inoue T and Kai Y, *J Mol Biol.*, 306: 469-77 (2001)
- 3) Mizuguchi H, Nakatsuji M, Fujiwara S, Takagi M and Imanaka T, *J Biochem.*, 126: 762-8 (1999)

## [11] Related products

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

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

10× 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], KOD -Plus- Neo [Code No. KOD-401], KOD FX [Code No. KFX-101] and KOD FX Neo [Code No. KFX-201], possess blunt ends due to 3'→5' exonuclease activity of the KOD DNA polymerase. The 10× 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].



**Figure. Principle of the 10× A-attachment mix**