

KOD FLEX™ PCR Master Mix

KMX-101 200 reactions

Store at -20°C

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CAUTION

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

- KOD FLEX™ is a trademark of Toyobo Co., Ltd. in Japan.

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[1] Introduction

Description

KOD FLEX™ PCR Master Mix is a highly efficient and high-success-rate 2× PCR master mix. It incorporates a modified KOD DNA polymerase (UKOD), an elongation accelerator, and an unique buffer technology to enable high-efficiency, low-bias amplification of hard-to-amplify targets.

Efficient amplification is possible even for hard-to-amplify targets such as high GC/AT content regions, long DNA fragments, and those with low amplification specificity. Amplification is possible even from crude samples such as biological samples.

This master mix demonstrates approximately 11 times the fidelity of Taq DNA polymerase, making the resulting amplified products suitable for conventional sequencing analysis via cloning. Furthermore, it is characterized by reduced amplification bias related to sequence composition or amplicon size, making it suitable for library amplification in next-generation sequencing.

KOD FLEX™ PCR Master Mix contains two types of anti-KOD DNA polymerase antibodies that inhibit the polymerase and its 3'→5' exonuclease activity, thus allowing for Hot Start PCR³⁾. Furthermore, KOD FLEX™ PCR Master Mix generates blunt-end PCR products because of its 3'→5' exonuclease (proof-reading) activity.

Features

- High success-rate • High elongation efficiency

Original buffer composition enables high specificity. PCR efficiency is improved by adding an “Elongation Accelerator”. The extension rate can be reduced by decrements of up to 15 s/kb in singleplex PCR. (Note that to realize sufficient amplification, the extension rate of ~30–60 s/kb is recommended for amplifications of crude samples or for the multiplexed amplification of long targets.)

- Applicable for amplification from crude samples

KOD FLEX™ PCR Master Mix is effective for amplification from crude samples (e.g. biological samples, foodstuffs, soil extract, etc) because of its tolerance of the PCR inhibitors commonly present in crude samples.

- Homogeneous amplification (Low bias)

Homogeneous multiplex PCR of targets up to 10 kb can be achieved. Various regions of the genome or transcriptome can be amplified homogeneously even if these regions contain GC bias. The resulting amplicons are also suitable for next-generation sequencing analyses.

Effect of GC bias on amplification is minimized, enabling uniform amplification regardless of the target. This master mix is suitable for library amplification for next-generation sequencers of long reads as well as short reads due to low bias and high elongation.

- High fidelity

KOD FLEX™ PCR Master Mix exhibits approximately 11-fold higher fidelity than Taq DNA polymerase, so this master mix can accurately amplify long targets. The amplified products can be used for various purposes, including next-generation sequencing analysis as well as cloning.

- Primers containing uracil (U) or Inosine (I) can be used

KOD FLEX™ PCR Master Mix can use primers containing inosines (I) or uracil (U), whereas conventional high-fidelity PCR enzymes cannot. Bisulfite-treated targets and degenerate primers can be used.

[2] Components

KOD FLEX™ PCR Master Mix 1.0 mL × 5

*This master mix contains anti-KOD DNA polymerase antibodies that neutralize the polymerase and inhibit its 3'→5' exonuclease activities.

**This master mix contains dNTPs (dATP, dGTP, dCTP, dTTP).

[3] Primer Design

- Primers should be 22–35 bases with a melting temperature (T_m) over 60°C.
- Optimal GC content of the primers is 45–60%. The ideal GC content of the 5'- and 3'-ends of the primers is 60–70% and 45–50%, respectively.
- The priming efficiency of the primers can be promoted by anchoring the 3'-end of the primers with a G or C.
- Primers should be designed so as not to generate intramolecular secondary structures or primer dimers.
- Primers for long target amplification should be 25–35 bases with T_m over 65°C.
- Primers containing inosine (I) or uracil (U) can be used and are applicable for many purposes such as metagenomic analyses.
- 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: Na^+ concentration, 50 mM and oligonucleotide concentrations, 0.5 μ M.

[4] Template

a. Purified DNA or cDNA

The following table indicates the applicable samples and appropriate amounts for PCR (50 μ L reaction):

Sample type	Appropriate amount	Typical amount
Genomic DNA: Eukaryotic	5–200 ng	50 ng
Genomic DNA: Prokaryotic	0.1–100 ng	10 ng
Plasmid DNA	10 pg–50 ng	1 ng
cDNA	~200 ng (RNA equiv.) ^a	50 ng (RNA equiv.)

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

^aExcessive amounts of RNA tend to inhibit PCR. The amount of cDNA to be prepared per reaction should be less than 200 ng (RNA equiv.).

b. Tissues and cells

When adding biological samples directly to the PCR reaction solution, the following samples can be applied to 50 µL 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 µL)
Yeast	Picked small amount of cells from colonies	
Fungus	Picked small amount of cells from colonies	
Cultured cells	10 ¹ –10 ⁵ cells/2µL medium or PBS	As the concentration of extracted DNA is low, 35–40 cycles are needed.
Whole Blood	1–2 µL	
Nail	1 × 1 mm	
Hair root	1–2 cm	
Leaf	2 × 2 mm	
Milled rice	0.5 × 0.5 mm	On an agarose gel assay, a portion of amplicon may remain in the slots.
Mouse tail	1 × 1 mm	

*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 µL of 20 mg/mL proteinase K to 50 µL PCR products prior to the electrophoresis is effective to dissociate the aggregates.

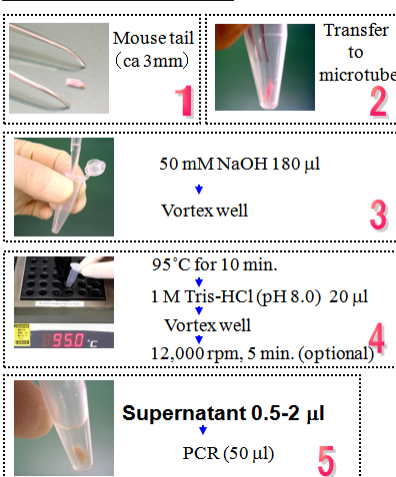
c. Lysate

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 μ L of 50mM NaOH and vortex.
3. Spin down.
4. Incubate at 95°C for 10 min. using thermal cycler.
5. Add 20 μ L of 1M Tris-HCl (pH 8.0) and vortex.
6. Spin down.

Supernatant 0.5–2 μ L → PCR (50 μ 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.

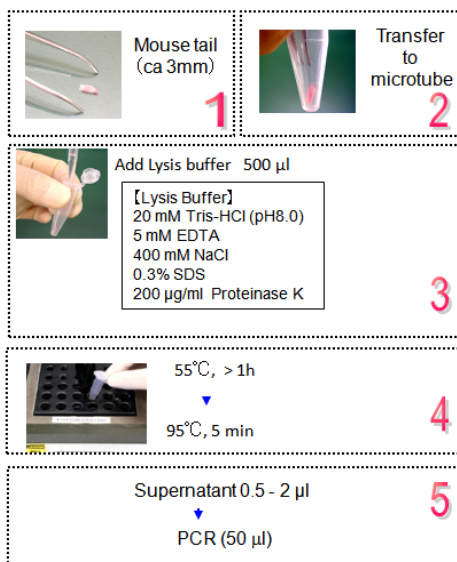
Mouse tail: 3 mm

Nail: 3 mm

Leaf: 3 × 3 mm

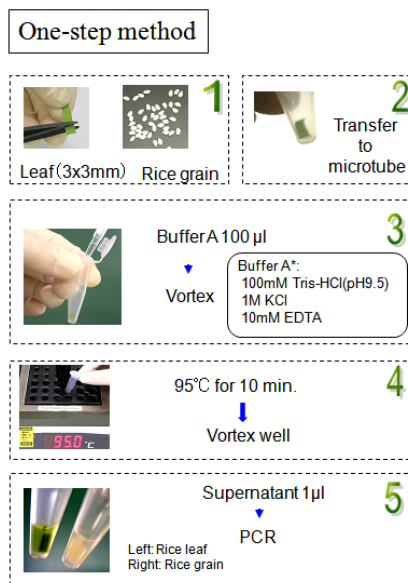
A rice grain

Proteinase K method



<One step method>

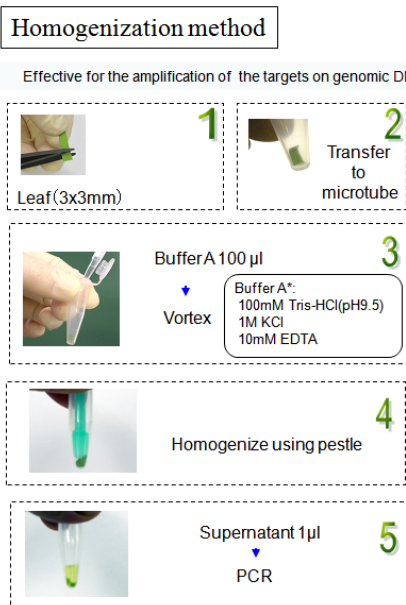
The following “One-step method” is recommended for the rapid preparation of a plant tissue lysate suitable for amplification with KOD FX Neo.



*BioTechniques, 19: 394 (1995)

<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.



151 Cloning of PCR products

- KOD FLEX™ PCR Master Mix generates blunt-end PCR products because of its 3'→5' exonuclease (proof-reading) activity. Therefore, the resulting PCR products can be cloned using blunt-end cloning methods.
- PCR products of KOD FLEX™ PCR Master Mix should be purified prior to restriction enzyme treatments in downstream cloning steps. The 3'→5' exonuclease activity of KOD DNA polymerase is present until 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 [9] Related products).

[6] Protocol

(1) Reaction set-up

The following protocol is designed for KOD FLEX™ PCR Master Mix.
Before preparing the mixture, the master mix should be completely thawed.

Component	Volume	Final Concentration
PCR-grade water	X μ L	
KOD FLEX™ PCR Master Mix(2 $\times$)	25 μ L	1 $\times$
10 pmol/ μ L Primer #1	1.5 μ L	0.3 μ M each
10 pmol/ μ L Primer #2	1.5 μ L	0.3 μ M each
Template DNA	Y μ L	{ Genomic DNA $\leq$ 200 ng/50 μL Plasmid DNA $\leq$ 50 ng/50 μL cDNA $\leq$ 200 ng (RNA equiv.)/50 μL Crude sample $\leq$ 5 μL/50 μL
Total reaction volume	50 μ L	

Notes:

- When non-specific or smeared bands are observed, the primer concentration should be decreased in decrements of up to 0.1 μ M (final).
- Optimal primer concentration is 0.3 μ M. In the case of long targets ($\geq$ 10 kb), reduced primers concentration (0.15 μ M) may give more effective amplification.
- In cases where degenerate primers are used, the primer concentrations can be increased up to 3.0 μ M (final) depending on the degree of degeneracy.

(2) Cycling conditions

The 3-step cycle can be applied in cases where the T_m of the primers are $\leq$ 65°C. A 2-step cycle can be applied in cases where the T_m of the primers are $>$ 65°C.

3-step cycle [In cases where primers' $T_m \leq 65^\circ\text{C}$]

Pre-denaturation:	94°C, 2 min	 30 cycles ^c
Denaturation:	98°C, 10 s	
Annealing:	T_m^a , 10 s	
Extension:	68°C, X s ^b	

2-step cycle [In cases where primers' $T_m > 65^\circ\text{C}$]

Pre-denaturation:	94°C, 2 min.	30 cycles ^b
Denaturation:	98°C, 10 s	
Extension:	68°C, X s ^c	

^aThe annealing temperature can be decreased in decrements of up to $T_m - 5^\circ\text{C}$ to enhance amplification yield.

^bThe PCR cycle number can be increased up to 40 cycles.

^cThe extension time should be determined according to the following table.

	Purified DNA (Genomic DNA, Plasmid DNA, cDNA)		Crude sample (Tissue, lysate),
	Target does not contain GC cluster*	Target contains GC cluster*	
Extension time	15–30 s/kb [#]	30 s/kb	60 s/kb

*GC cluster: a GC-rich region with GC content $\geq 70\%$.

[#]The extension time should be ≥ 30 s/kb in the case of a 2-step cycle or when using degenerate primers. In cases where low copy numbers of the template DNA are present or long targets over 10 kb require amplification, the yield can be increased by setting the extension time to 60 s/kb.

Notes for Multiplex PCR :

-The primer concentrations should be optimized using the following guidelines:

- Primer concentrations should be decreased in decrements of up to 0.1 μM if non-specific or smeared bands are observed.
- Amplification bias in multiplex PCR can be improved by increasing the concentration of primers that yield relatively weak bands, or by decreasing the concentration of primers that exhibit excessive amplification.
- Commercially available primer sets for multiple targets should be used according to the manufacturer's instructions.

-The annealing temperature should be determined based on the primer with the lowest T_m . The annealing temperature can be increased up to $T_m + 5^\circ\text{C}$ to reduce non-specific or smeared bands. To enhance amplification, it can be decreased in decrements up to $T_m - 5^\circ\text{C}$.

-The extension time should be determined according to the following table:

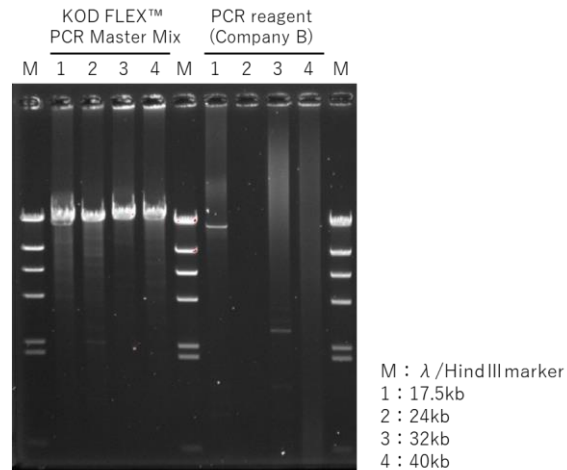
	Small targets (≤ 1 kb)		Large targets (1–10 kb)	
	Number of targets			
	≤ 10	> 10	≤ 10	> 10
<Purified DNA, cDNA> Extension times				
3-step cycle	15–30 s	30–60 s	30 s/kb*	30–60 s/kb*
2-step cycle	30 s	30–60 s	30 s/kb*	30–60 s/kb*
<Crude samples> Extension times				
3- and 2-step cycles	60 s	60 s	60 s/kb*	60 s/kb*

*The extension time should be determined based on the largest target.

(3) Application data

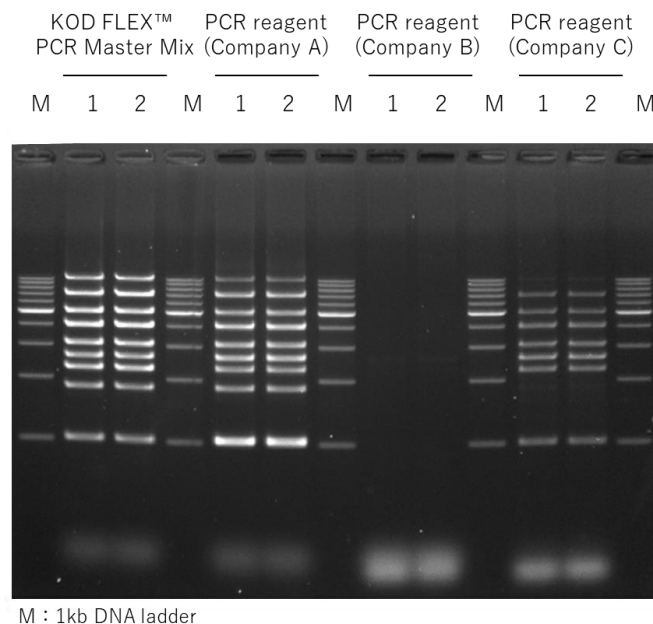
a. High elongation efficiency

Long targets were amplified using human genomic DNA as a template. Even extremely long targets of up to 40 kb were successfully amplified with KOD FLEX™ PCR Master Mix.



b. High efficiency • low bias

Multiplex PCR was performed using multiple targets (Chrome9 (1.0kb), MSH6 (1.8kb), BRCA2 (2.3kb), WT-1 (2.5kb), FANCE (3.0kb), RAD51D (4.0kb), KRAS (5.0kb), BRCA1 (7.0kb), DDB2 (10.0kb)) with various PCR reagents, and performance was compared (50μL reaction, N=2). As a result, good amplification with less bias was achieved when using KOD FLEX™ PCR Master Mix.



171 Troubleshooting

Symptom	Cause	Solution
No PCR product/low yield	Cycling conditions are not suitable.	Increase the extension time up to 60 s/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 procedure step-wise up to $T_m-5^{\circ}\text{C}$.
	Template DNA is of low quality and/or quantity.	In cases where degenerate primers are being used, the annealing temperature should be decreased step-wise up to $T_m-5^{\circ}\text{C}$.
		Increase the amount of template DNA.
		Change the DNA purification method.
		Use purified templates.
		Decrease the amount of cDNA to reduce inhibition by RNA contaminants.
		Degrade or eliminate RNA in the cDNA sample.
	Poor primer performance.	Decrease the primer concentration step-wise from 0.3 μM to 0.1 μM . (This solution may be effective for the amplification of targets over 10 kb.)
		Primer concentration should be increased up to 3.0 μM in cases where degenerate primers are being used.
		Use fresh primers.
		Redesign primers.
Smearing/Extra bands	Cycling conditions are not suitable.	Change from 3-step cycling to 2-step cycling.
		Decrease the number of cycles by 2–5 cycles.
	Too much template DNA.	Reduce the amount of template DNA.
	Poor primer performance.	Use fresh primers.
		Decrease the primer concentration step-wise from 0.3 μM to 0.1 μM .
	Low quality primers.	Redesign primers. (Longer primers may eliminate smearing or extra bands.)
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].

[8] 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)

[9] Related products

Product name	No. of Reactions	Code No.
TARget Clone™ -Plus-	10 reactions	TAK-201
10× A-attachment mix	25 reactions	TAK-301
Ligation high Ver.2	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], KOD FX Neo [Code No. KFX-201], KOD -Multi & Epi® [Code No. KME-101] or KOD FLEX™ PCR Master Mix [Code No. KMX-101]. The kit contains the pTA2 Vector, 2× Ligation Buffer, T4 DNA Ligase, and 10× A-attachment Mix.

The 10× A-attachment mix is a reagent comprising anti-KOD DNA polymerase antibody that specifically inhibits 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], KOD -Multi & Epi® [Code No. KME-101] or KOD FLEX™ PCR Master Mix [Code No. KMX-101] all possess blunt ends due to the 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 various T-vectors using ligation reagents such as Ligation high Ver.2 [Code No. LGK-201].

Principle of the 10× A-attachment mix:

