KOD One™ PCR Master Mix

KOD One™ PCR Master Mix -Blue-

KMM-101 1 mL x 5
KMM-201 1 mL x 5

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 precautions and safety while using this kit. All trademarks, trade names, or company names referenced herein are used for identification only and are the property of their respective owners.

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[1] Introduction

Description

KOD One™ PCR master Mix and KOD One™ PCR Master Mix -Blue- are 2 x PCR master mixes based on genetically modified KOD DNA polymerase (UKOD). KOD One™ series enables fast PCR, which has an extension time of 5 sec./ kb by applying UKOD and a new Elongation Accelerator. In addition, these master mixes provide greater efficiency and elongation capabilities than conventional PCR enzymes. In particular, these show greater amplification success from crude specimens. Furthermore, these master mixes can be applied to amplify from templates containing uracils (dU) or using primers containing inosines (dI) and uracils (dU).

KOD One™ series contains two types of anti-KOD DNA polymerase antibodies that inhibit the polymerase and 3'→5' exonuclease activities, thus allowing for Hot Start PCR. These master mixes generate blunt-end PCR products because of 3'→5' exonuclease (proof-reading) activity of KOD DNA polymerase.

Features

- Fast

KOD One™ series can amplify the targets using the following very short conditions:

≤ 1 kb:	1 sec.
1~ 10 kb:	5 sec./ kb
10 kb~:	10 sec./ kb

The cycling conditions can be set flexibly when various targets having different sizes are amplified.

- Easy to Use

KOD One™ series contains all reaction components except primers and templates and provide high reproducibility by reducing operations. In addition, KOD One™ PCR Master Mix -Blue- includes a loading dye (BPB) to allow direct loading onto agarose gels.

- High Fidelity

KOD One™ series exhibits approximately 80-fold higher fidelity than Taq DNA polymerase. These mixes can be used for various purposes where this would be an advantage (e.g., in the preparation of long target amplicons for sequencing).

- High Efficiency

KOD One™ series 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.

- Primers or Templates Containing Inosines (dI) or Uracils (dU) Can Be Used

KOD One™ series can use primers or templates containing inosines (dI) or uracils (dU), whereas conventional high-fidelity PCR enzymes cannot.

- Low bias (Compatible for NGS library amplification)

KOD One™ series enables uniform amplification even for GC-rich and AT-rich targets. Library amplification bias affects coverage in next-generation sequencing. With its low bias and high-speed amplification capabilities, KOD One™ series is well-suited for amplifying both short-read and long-read libraries.

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[2] Components

KOD One™ series includes the following components for 200 reactions, 50 µl total reaction volume.

<KMM-101>
KOD One™ PCR master Mix 1 mL x 5

<KMM-201>
KOD One™ PCR master Mix -Blue- 1 mL x 5

Note:
The reagents can be stored at 4°C for a month. For longer storage, the reagents should be kept at -20°C.

[3] Quality Testing

Quality testing is performed by amplification of a 10 kb target using fast PCR, which has an extension time of 50 sec.
Technical information (data) contained in this instructional manual are for description representation and application of the product and does not constitute any guarantee.

[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 Tm over 65°C.

-Primers containing inosine (dI) or uracil (dU) can be used and are applicable for many purposes such as metagenomics analyses. The design of primers can be facilitated by use of specific software that allow replacement of thymine (dT) with uracil (dU) or inosine (dI).
The following online tool is recommended for amplification after bisulfate.
MethPrimer, http://www.urogene.org/methprimer/index.html

-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

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[5] Protocol

1. Standard reaction setup

Before preparing the mixture, all components should be completely thawed, except for the enzyme solution.

Component	Volume	Final Concentration
PCR grade water	X μL	
KOD One™ PCR Master Mix	25 μL	1×
10 pmol /μL Primer #1	1.5 μL	0.3 μM
10 pmol /μL Primer #2	1.5 μL	0.3 μM
Template DNA	Y μL	Genomic DNA ≤200 ng / 50 μL Plasmid DNA ≤50 ng / 50 μL cDNA ≤750 ng （RNA equiv.) / 50 μL Crude sample ≤5 μl/50 μL
Total reaction volume	50 μL	

Notes:

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

-When PCR yield is low, increased primers concentration (0.5 μM) may give more effective amplification.

-For PCR reactions, thin-walled tubes are recommended.

2. Cycling conditions

The following cycle is recommended.

3-step cycle	Amplicon size ≤ 1 kb	Amplicon size 1 ~10 kb	Amplicon size ≥ 10 kb
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	98°C, 10 sec.
Annealing :	(Tm -5) °C, 5 sec.	(Tm -5) °C, 5 sec.	(Tm -5) °C, 5 sec.
Extension :	68°C, 1 sec.	68°C, 5 sec. /kb	68°C, 10 sec. /kb

25–45 cycles

Notes:

-Longer extension time may enhance efficiency. For amplification from a low copy DNA or crude sample, the extension time should be 10 sec. /kb.

-The extension temperature should be adjusted in accordance with the Tm of primers. If the Tm value of the primer is over 73°C, the extension temperature should be set at 68°C.

-Poor amplification may be improved by changing the denaturation step to 94°C, 15 sec.

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-To fully inactivate the Hot Start antibody, incorporating a pre-denaturation step (e.g., 94°C, 2 min.) at the beginning of the cycle may improve amplification efficiency in some cases.

-To prevent incomplete double-strand formation, it is acceptable to add a final extension step (e.g., 68°C, 10 sec./kb) at the end of the cycle.

<Other cycles>

When non-specific or smeared bands are observed, the following cycles is recommended.

2-step cycle	Amplicon size < 10 kb	Amplicon size ≥ 10 kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	25–45 cycles
Extension :	68°C, 5 sec. /kb	68°C, 10 sec. /kb	

Step-down cycle	Amplicon size < 10 kb	Amplicon size ≥ 10 kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	74°C, 5 sec. /kb	74°C, 10 sec. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	72°C, 5 sec. /kb	72°C, 10 sec. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	5 cycles
Extension :	70°C, 5 sec. /kb	70°C, 10 sec. /kb	
Denaturation :	98°C, 10 sec.	98°C, 10 sec.	15–30 cycles
Extension :	68°C, 5 sec. /kb	68°C, 10 sec. /kb	

[6] Template

a. Purified DNA or cDNA

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

		Approved range	Recommended
Genomic DNA	Eukaryotic DNA	1–200 ng	50 ng
	Prokaryotic DNA	0.1–200 ng	10 ng
Plasmid DNA		1 pg–50 ng	10 ng
cDNA		< 750 ng (RNA equiv.)	50 ng (RNA equiv.)
Lambda phage DNA		0.01–10 ng	1 ng

-Contaminating RNA in cDNA inhibits the PCR reaction. PCR should be performed using template DNA containing <750 ng of RNA in a 50 µL reaction.

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

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b. Tissues and cells

When adding biological samples directly to the PCR reaction solution, the following samples can be applied to the 50 µL reaction.

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

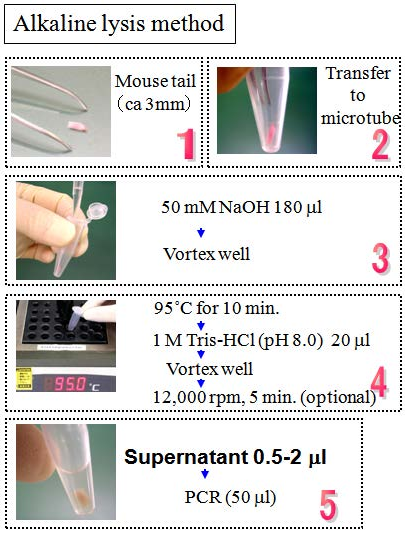
*In the case of the direct amplification from animal tissues, such as mouse tail, a portion of the amplification product may remain in the gel slot on an agarose gel assay. Adding 10 µL of 20 mg/mL proteinase K to 50 µL PCR products prior to the electrophoresis is effective for dissociating the aggregates.

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.



*Mouse tail cannot be dissolved completely.

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[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.

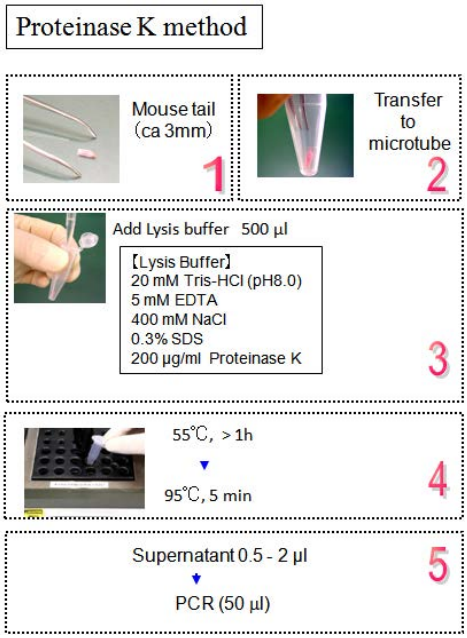
1. Transfer mouse tails (ca 3mm) to a 96-well PCR plate.
2. Add 180 µL of 50 mM NaOH and vortex.
3. Spin down.
4. Incubate at 95°C for 10 min using a thermal cycler.
5. Add 20 µL of 1M Tris-HCl (pH 8.0) and vortex.
6. Spin down.

Supernatant 0.5–2 µL → PCR (50 µL)

<Proteinase K method>

The following "Proteinase K method" is recommended for efficient preparation of mouse tails or nail lysates suitable for amplification. This protocol can also be applied to the following samples.

Mouse tail: 3 mm
Nail: 3 mm
Leaf: 3 × 3 mm
A rice grain



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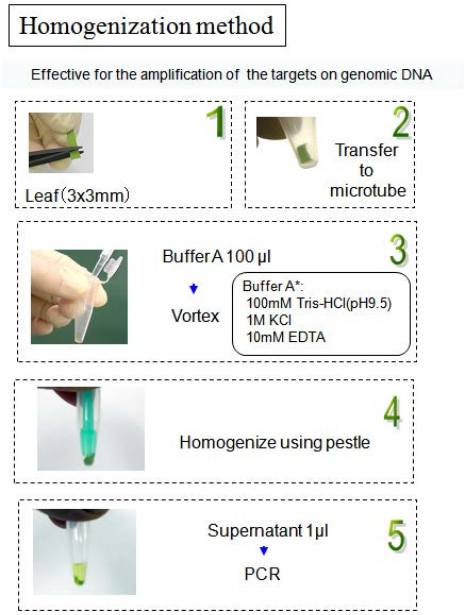
<One step method>

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

*BioTechniques, 19: 394 (1995)

<Homogenization method>

The following "Homogenization method" is recommended for effective preparation of a plant tissue lysate suitable for amplification. This method is effective for amplification of genomic DNA targets.



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[7] PCR products Cleanup

-PCR products cleanup may be required to remove enzyme, dNTPs, and reaction buffer prior to sensitive downstream applications. When purifying PCR products of KOD One™ PCR Master Mix / KOD One™ PCR Master Mix -Blue- using magnetic beads, abnormal pelleting of the beads may be observed. The following treatment before purification can be used to improve bead cleanup if this occurs.

<Proteinase K treatment>
Add 1 µL 10-20mg/mL Proteinase K to 50 µL of PCR product, mix well and incubate at room temperature for at least 1 min.

<Tween 20 treatment>
Add 1 µL 10% Tween 20 to 50 µL of PCR product.

[8] Cloning of PCR products

-KOD One™ PCR Master Mix / KOD One™ PCR Master Mix -Blue- generate blunt-end PCR products because of 3'→5' exonuclease (proof-reading) activity. Therefore, PCR products can be cloned using blunt-end cloning methods.

-PCR products of KOD One™ PCR Master Mix / KOD One™ PCR Master Mix -Blue- should be purified prior to restriction enzyme treatments in cloning steps. 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).

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[9] Examples

Example 1. Fast PCR

Various targets were amplified with KOD One™ PCR Master Mix and KOD One™ PCR Master Mix -Blue- using the fast cycling conditions. KOD One™ series successfully amplified all targets.

<Reaction Mix>

PCR grade water	21 µL
KOD One™ PCR Master Mix	25 µL
10 µM Primers	1.5 µL
10 ng/ µL human genomic DNA	1 µL
Total Volume	50 µL

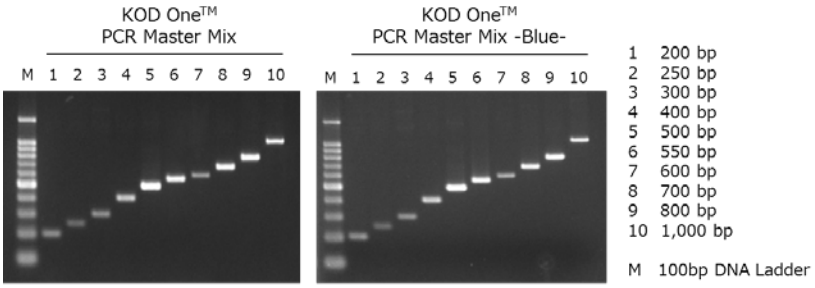
<PCR cycle>

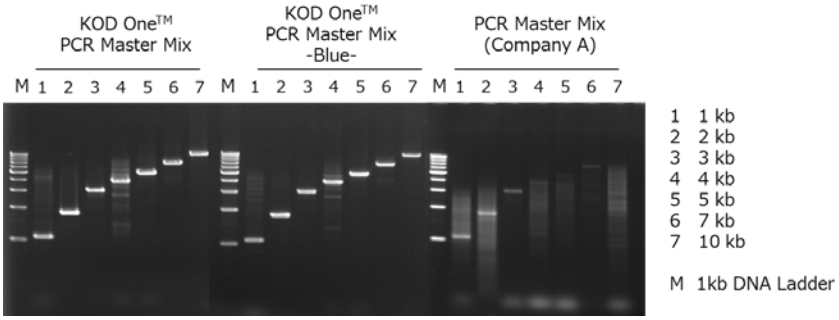
Amplicon size ≤ 1 kb

98℃ 10 sec.
60℃ 5 sec.
68℃ 1 sec.
] ×30 cycles

Amplicon size 1 ~10 kb

98℃ 10 sec.
60℃ 5 sec.
68℃ 5 sec.
] ×30 cycles





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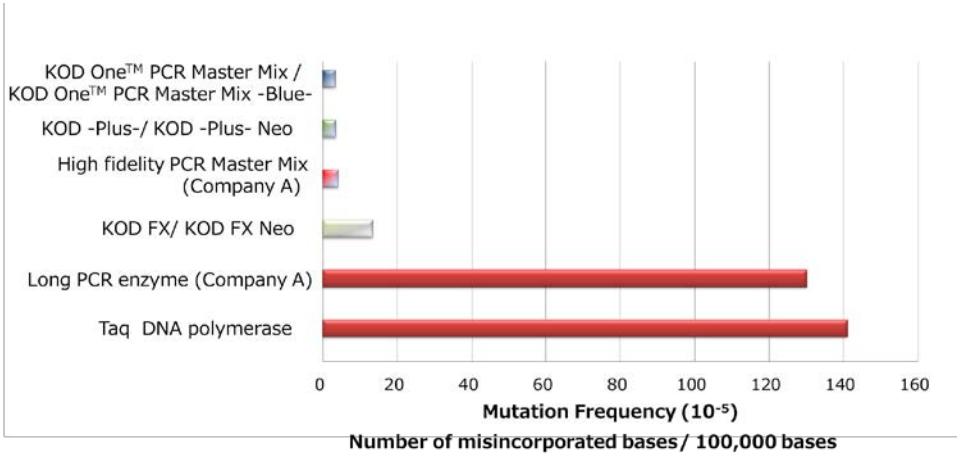
Example 2. PCR error ratio

The error ratio of various PCR enzymes were compared by determining the sequences of the amplicons from human β-globin gene. The amplicons were cloned into the vector using TArget clone™ -Plus- [Code No. TAK-201] and the sequences were determined. KOD One™ PCR Master Mix and KOD One™ PCR Master Mix -Blue- showed excellent fidelity and the mutation frequency was approximately 80 times lower than that of rTaq DNA polymerase.



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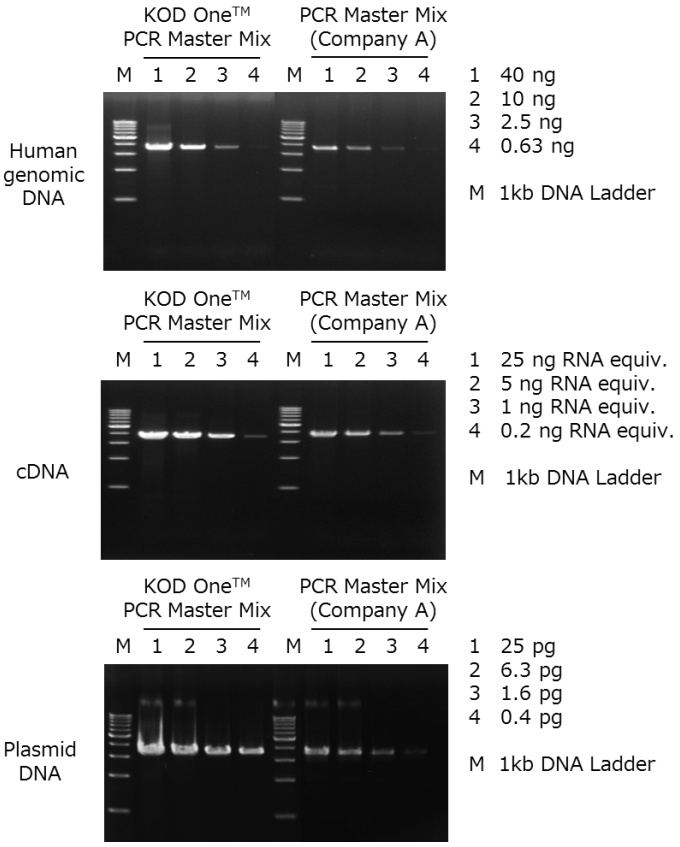
Example 3. Amplification efficiency and sensitivity

The 3.5 kb fragments were amplified from human genomic DNA, cDNA and plasmid DNA. Each PCR reaction was performed according to the recommended conditions. KOD One™ PCR Master Mix showed higher sensitivity using 5 sec./ kb extension time.

<Reaction Mix>		<PCR cycle>
PCR grade water	21 µL	98℃ 10 sec.
KOD One™ PCR Master Mix	25 µL	60℃ 5 sec.] ×30 cycles
10 µM Primers	1.5 µL	68℃ 18 sec.
Each template DNA	1 µL	
Total Volume	50 µL	



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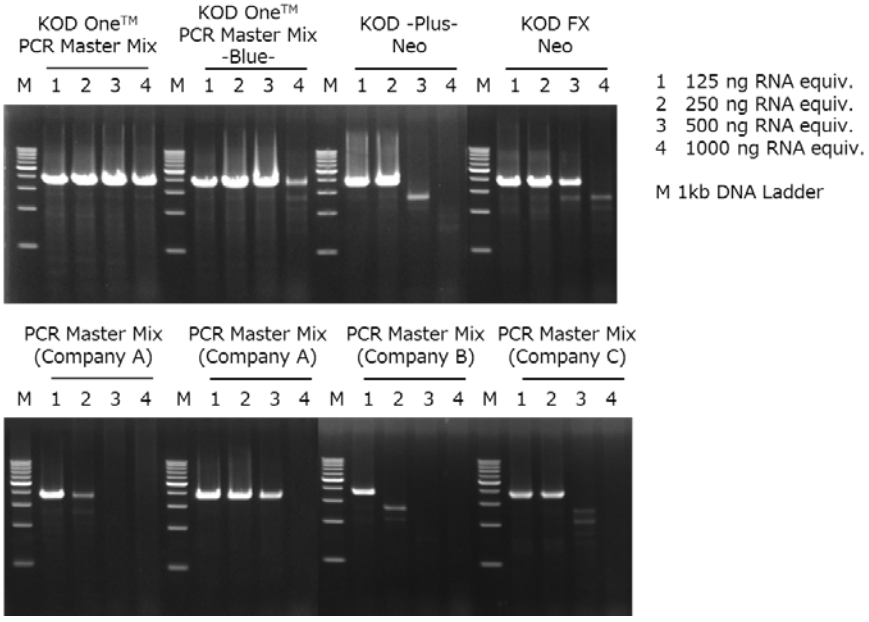
Example 4. Amplification from cDNA

The inhibitory effect of RNA in cDNA was compared using various PCR enzymes. KOD One™ Master Mix was not susceptible to RNA inhibition, and it was able to amplify targets under high concentrations of cDNA.

<Reaction Mix>		<PCR cycle>
PCR grade water	17 µL	98℃ 10 sec.
KOD One™ PCR Master Mix	25 µL	60℃ 5 sec. ×30 cycles
10 µM Primers	1.5 µL	68℃ 18 sec.
Each cDNA	5 µL	
Total Volume	50 µL	



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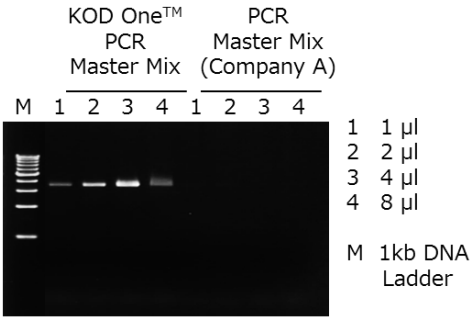
Example 5. Amplification from crude samples

Amplification from whole blood and that from mouse lysate were compared. KOD One™ PCR Master Mix amplified the targets efficiently.

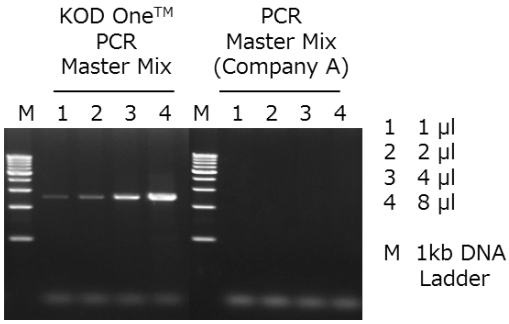
<Reaction Mix>	
PCR grade water	22 - X µL
KOD OneTM PCR Master Mix	25 µL
10 µM Primers	1.5 µL
Each template	X µL
Total Volume	50 µL

<PCR cycle>
98℃ 10 sec.
60℃ 5 sec.
68℃ 5 sec./ kb
×30 cycles

Amplification from whole blood



Amplification from mouse lysate



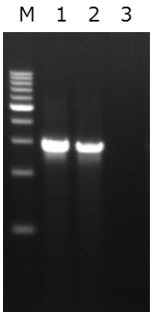
Example 6. Amplification using degenerate primers containing inosine.

The 2.8 kb fragments were amplified using degenerate primers containing inosine. KOD One™ PCR Master Mix was able to amplified, whereas conventional high-fidelity PCR enzymes were not.

<Reaction Mix>	
PCR grade water	21 µL
KOD One™ PCR Master Mix	25 µL
10 µM Primers*	1.5 µL
50 ng/µL E.coli genomic DNA	1 µL
Total Volume	50 µL

<PCR cycle>
98℃ 10 sec.
60℃ 5 sec.
68℃ 15 sec.
×30 cycles

<Primer sequence>
Fwd: ATGGTICARATHCCICARAAY
Rev: RTGIGCYTGRTCCCARTTYTC



1 KOD One™ PCR Master Mix
2 KOD One™ PCR Master Mix -Blue-
3 KOD -Plus- Neo

M 1kb DNA Ladder

*In cases where degenerate primers are used, the primer concentrations should be increased depending on the degree of degeneracy.

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Example 7. Amplification of NGS library

NGS library of *Thermus thermophilus* genome (GC content 70%) was amplified using KOD One™ PCR Master Mix and other companies' product, and the amplified libraries were sequenced on Illumina MiSeq. This is the result of examining the GC bias of amplified libraries. The Normalized Coverages (○) close to 1 indicate low bias. KOD One™ PCR Master Mix showed lower GC bias than PCR Reagent of company B. And KOD One™ PCR Master Mix shows a high coverage rate. It can be used for sequence analysis with a small number of reads.

The optimal number of cycles may be 1-3 cycles higher or lower depending on the sample type and size distribution of the input DNA. For details of cycle numbers, please refer to the following table.

<Reaction Mix>

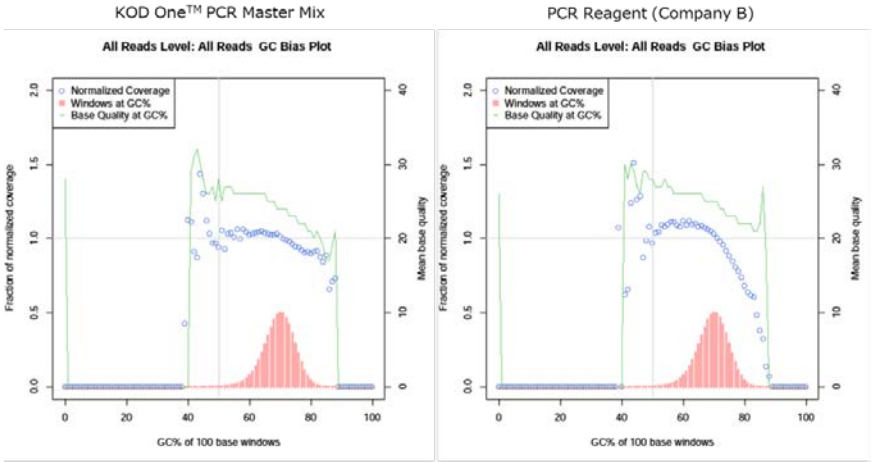
PCR grade water	14 µL
KOD One™ PCR Master Mix	25 µL
10 µM Primers*	5 µL
1 ng/µL Library for Illumina	1 µL
Total Volume	50 µL

<PCR cycle>

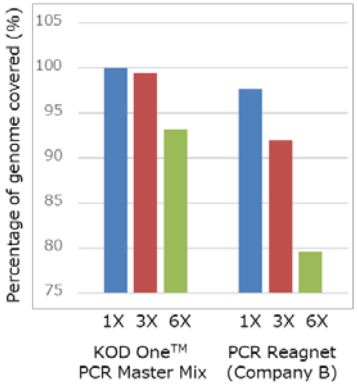
98℃ 10 sec.
60℃ 5 sec.] ×14 cycles
68℃ 1 sec.

<Primer sequence for TruSeq adapter>
Fwd: AATGATACGGCGACCACCGAGATC
Rev: CAAGCAGAAGACGGCATACGAG

Comparison of GC bias



Comparison of coverage



Recommended cycle numbers

Input DNA	Cycle numbers
1 µg	0-1
500 ng	1-2
100 ng	4-5
50 ng	5-6
10 ng	8-10
1 ng	13-15
0.25 ng	16-18

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[10] Troubleshooting

Symptom	Cause	Solution
No PCR product / low yield	Cycling condition is unsuitable.	Increase the extension time up to 10 ~30 sec./ kb.
		Increase the number of cycles by 2–5 cycles.
		Lower annealing temperature in the 3-step cycle decrements up to Tm-7–10°C.
	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.
		Degrade or eliminate RNA in the DNA sample.
	Primer is not good.	Decrease the primer concentration from 0.3 μM to 0.15 μM. This solution may be especially effective for the amplification of targets over 10 kb.
		Increase the primer concentration from 0.3 μM to 0.5 μM. This solution may be especially effective for amplification from low copies template.
		Use fresh primers.
		Redesign primers.
Smearing / Extra band	Cycling conditions are unsuitable.	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 (25-35 mer) 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 Follow blunt-end cloning guidelines. Use TArget Clone™ -Plus- [Code No. TAK-201] [see [10] Related products].

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[11] Related products

Product name	Package	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 products containing KOD DNA polymerase. (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 One™ PCR Master Mix [Code No. KMM-101, KMM-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 using KOD DNA polymerase 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].

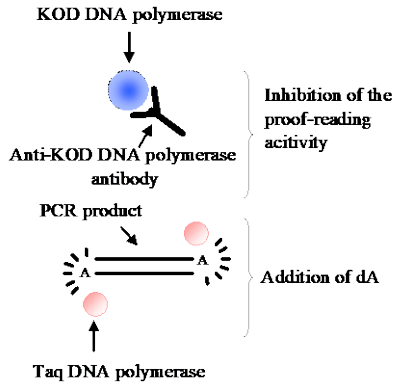

Figure. Principle of the 10× A-attachment mix

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