TOYOBO
Beyond Horizons

KOD One™

PCR Master Mix
PCR Master Mix –Blue-

CASEBOOK

Contents

Storage temperature : −20℃**

	(Dye-free 2×PCR Master Mix) **KOD One™ PCR Master Mix**			(Dye-containing 2×PCR Master Mix) **KOD One™ PCR Master Mix -Blue-**		
Code No.	KMM-101	KMM-121	Bulk size	KMM-201	KMM-221	Bulk size
Packaging	5 tubes×1mL*	1 bottle × 50 mL	Please contact us	5 tubes × 1mL*	1 bottle × 50 mL	Please contact us

*For a 50 μL reaction system, it can be used 200 times.

**It can be stored at 2–8 ℃ if used within one month.

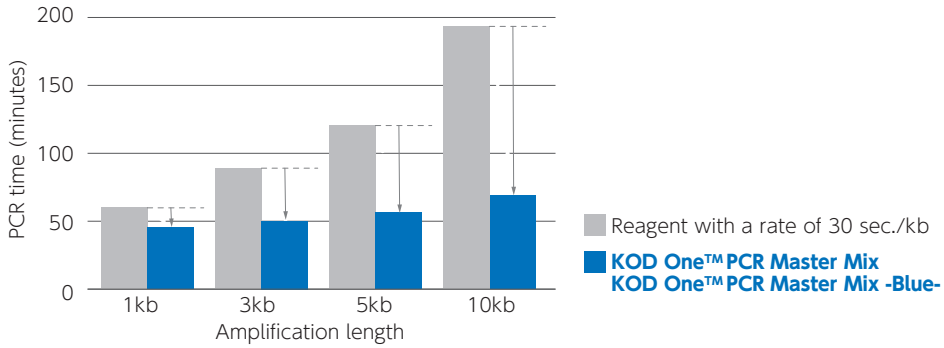
Product Overview and Features

We have achieved high-speed polymerase chain reaction (PCR) while maintaining high accuracy by adding an improved elongation accelerator to modified KOD DNA polymerase (UKOD), which possesses high accuracy and amplification efficiency. This product is available as a 2× master mix, and it can be simply used by mixing the template and primers. We also provide a version containing a loading dye. This allows for the conventional PCR process to be conducted more conveniently and in less time.

Dye-free 2×PCR Master Mix
KOD One™ PCR Master Mix

Dye-containing 2×PCR Master Mix
KOD One™ PCR Master Mix -Blue-

■ Achieves the fastest elongation rate of 1 sec./kb

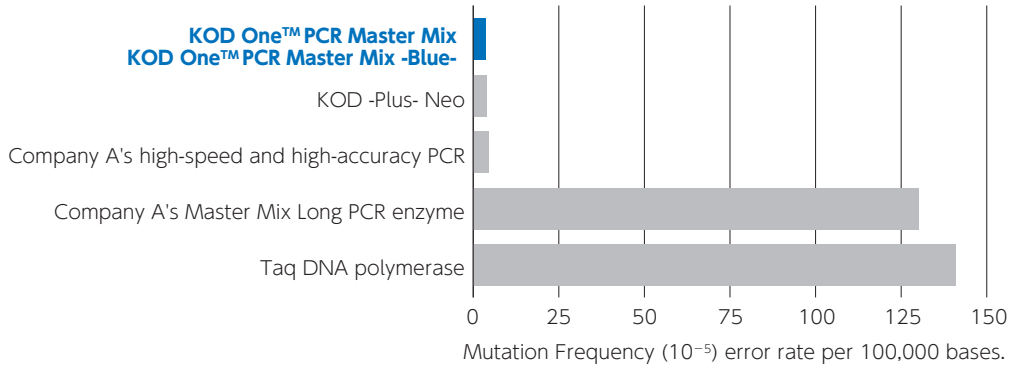


Applied Biosystems 9700

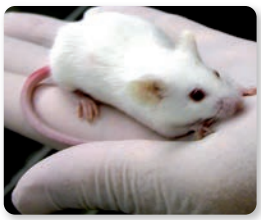
Amplicon size	Elongation time
-1kb	1sec.
1-10kb	5sec./kb
10kb-	10sec./kb

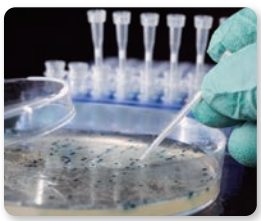
※We have confirmed the amplification of 40 kb using human genomic DNA as a template.

■ Highest level of accuracy in the KOD series



■ Efficient amplification from various samples without purification





■ Compatible with primers and templates containing inosine (dI) and uracil (dU)

You can request samples here.→

Basic reaction conditions

1）Preparation of Reaction mixture

The reagents should be completely thawed and thoroughly stirred before preparing the reaction solution. After mixing all of the reagents, the reaction solution should be thoroughly mixed by vortexing or other means and set in a thermal cycler.

Regent	Volume (μL)	Final Concentration
Sterile distilled water	X	
KOD One™ PCR Master Mix (2×)	25	1×
Primer (10μM each)	1.5	0.3μM*1 each
Template*2	Y	
Total Volume	50	

＊1：A primer concentration of 0.3 μM (final concentration) is recommended. However, when amplifying long chains of ≥ 10 kb, the primer concentration can be reduced to 0.15 μM (final concentration) to improve the amplification. If the detection sensitivity is poor, increasing the primer concentration to 0.5 or 1.0 μM (final concentration) may improve the detection sensitivity.

＊2：See below for the template addition amounts.

①When using purified template and cDNA (PCR Reaction mixture : 50 μL)

Template Example		Additive range	Standard addition amount
Genome DNA	Eukaryotic DNA	1-200ng	50ng
	prokaryotic DNA	0.1-200ng	10ng
Plasmid DNA		1pg-50ng	10ng
cDNA		1-750ng (RNA equivalent)	50ng (RNA equivalent)
λDNA		10pg-10ng	1ng

②Tissue sample, etc. (PCR Reaction mixture : 50 μL)

Please refer to the instruction manual and this example for the sample preparation methods.

Template Example	Standard addition amount	Template Example	Standard addition amount
Escherichia coli	Small amount on chip	Hair	1-2cm
Yeast	Small amount on chip	Plant leaf	2mm square
Filamentous Fungus	Small amount on chip	Rice polishing	about 1/5 of a grain of rice
Culture Cells	10^1-10^5cells	Mouse tail	Approx. 1mm
Blood (EDTA collection tube, citric acid collection tube)	1-2μL	Plant tissue lysate*	0.5-2μL
Nail	about 1/3 of a grain of rice	Animal tissue lysate	0.5-2μL

＊Please refer to Examples 7 and 8 for the preparation of animal and plant lysates.

2）Cycle conditions

3-step cycle			
Denaturation	98℃,	10sec.	25-45cycles
Annealing	(Tm-5)℃,	5sec.	
Extension	68℃,	1-10sec./kb	

The elongation time should be set as follows depending on the target amplicon size:

Less than 1kb : 1sec.
1-10kb : 5sec./kb
10kb- : 10sec./kb

One second may not be controllable depending on the PCR device. If the amount of amplification is small, the elongation time should be set to 5 sec./kb.

If the amount of amplification is small, such as when the copy number of the target is small or when amplifying from a crude sample, extending the elongation time to 10 to 30 sec./kb may improve the result.

If extra bands are observed, try the two-step, step-down cycle described in the instruction manual.

◆The instruction manual can be downloaded here →

Example 1

Amplification of 200 bp to 10 kb targets by fast PCR

Amplification was performed using human genomic DNA as a template in a fast PCR cycle with a short elongation time. Clear bands were confirmed at an elongation time of 1 sec. for targets of ≤1 kb and at elongation time of 5 sec./kb for targets of 1–10 kb.

Composition of Reaction mixture

	(μL)
Sterile distilled water	21
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
10ng/ μL human genomeDNA	1
Total Volume	50

PCR Cycle

Targets less than 1kb

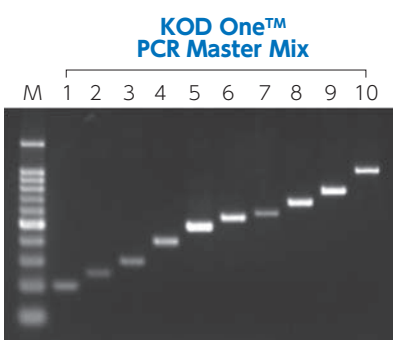
98℃, 10sec.
60℃, 5sec.
68℃, 1sec.
} 30cycles

1-10kb Target

98℃, 10sec.
60℃, 5sec.
68℃, 5sec./kb
} 30cycles

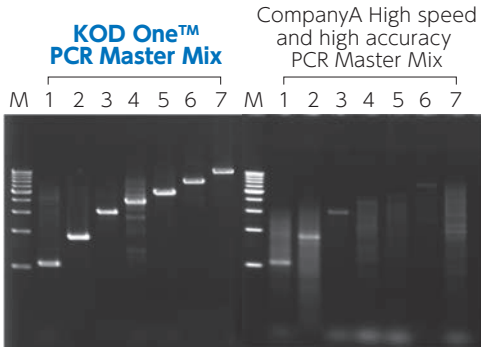
Result

[1 kb or less: Elongation time 1 sec.]



M: 100bp DNA Ladder
1: DDB2 (200bp)
2: FANCG (250bp)
3: HBg (300bp)
4: CDH1 (400bp)
5: Chrome9 (500bp)
6: ERCC4 (550bp)
7: HRAS (600bp)
8: PRF1 (700bp)
9: BRCA (800bp)
10: CDK4 (1,000bp)

[1-10kb: Elongation time 5sec./kb]



M: 1kb DNA Ladder
1: Chrome9 (1kb)
2: MSH6 (2kb)
3: FANCE (3kb)
4: RAD51D (4kb)
5: KRAS (5kb)
6: BRCA1 (7kb)
7: DDB2 (10kb)

Example 2

Amplification of 17.5-40 kb long targets by fast PCR

Amplification of long targets using human genomic DNA as a template was confirmed from 17.5 to 40 kb using KOD One™ PCR Master Mix.

Composition of Reaction mixture

	(μL) Lane 1-3	(μL) Lane 4-8
Sterile distilled water	7.8	3.8
KOD One™ PCR Master Mix	10	10
10pmol / μL Primer F	0.6	0.6
10pmol / μL Primer R	0.6	0.6
10ng/ μL human genome	1	5
Total Volume	20	20

Primer

Target 17.5kb Hbg
Forward Primer : TGCACCTGCTCTGTGATTATGACTATCCCACAGTC
Reverse Primer : ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA

Target 24kb tPA
Forward Primer : CCTTCACTGTCTGCCTAACTCCTTCGTGTGTTCC
Reverse Primer : TGTCTCCAGCACACAGCATGTTGTCGGTGAC

Target 32kb Hbg
Forward Primer : AGACTTCACATGCTGCTCTGTGCATCCGAGTG
Reverse Primer : ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA

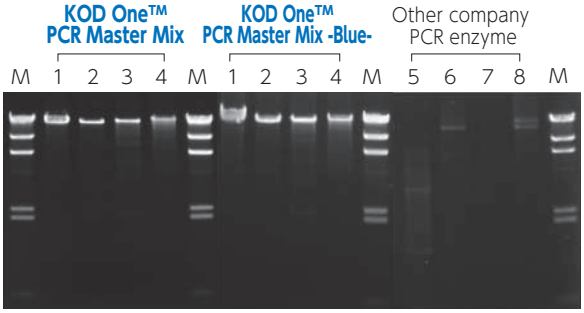
Target 40kb Hbg
Forward Primer : GTTCTCCTCTGCATATCTCCCCACCGCATCTCTTT
Reverse Primer : CCTTCAGTGCCCATGATGTAGGAAAGTGTGGCATA

PCR Cycle

98℃, 10sec.
60℃, 5sec.
68℃, 10sec./kb
} 30cycles

※PCR enzymes from other companies were used under the conditions recommended by the manufacturers.

Result



M: λ/*Hin*dⅢ

KOD One™ PCR Master Mix
1: 17.5kb
2: 24kb
3: 32kb
4: 40kb

Other company PCR enzyme (17.5 kb)
5: Company A fast and accurate PCR Master Mix
6: Company D Highly accurate PCR enzyme
7: Company B Highly accurate PCR enzyme
8: Company C highly accurate PCR enzyme

Example 3

Amplification of high-GC content regions above 70%

Amplification was performed targeting TGFb, ACE, CASP3, ATCB, and BRAF, including regions with high-GC content. Amplification was observed for all of the targets with KOD One™ PCR Master Mix, but not with the long PCR enzyme of company A.

Composition of Reaction mixture

	(μL)
Sterile distilled water	21
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
10ng/ μL human genome DNA	1
Total Volume	50

PCR Cycle

98℃, 10sec.
60℃, 5sec.
68℃, 50sec.
(5sec./kb)
30cycles

※The long PCR enzyme of company A was used under the conditions recommended by the manufacturer.

Primer

TGFb
Forward Primer : TCCACCTTGGTCAGTCTCCTATAAC
Reverse Primer : GCTGTGTGTACTCTGCTTGAACTTG

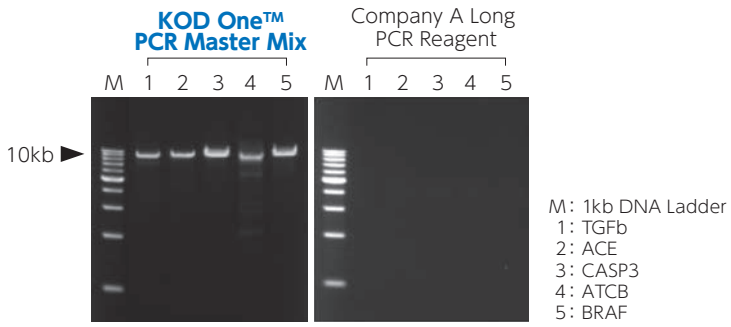
ACE
Forward Primer : CCTCAGGCTCTTCTCCTCTCTACTC
Reverse Primer : GGTCATATTCCTCCACAAACTTGC

CASP3
Forward Primer : CTGGCTTTGTCTCCCTTCTAGGTAC
Reverse Primer : TGACCTTCTCCACACTCTAGAAACC

ATCB
Forward Primer : AACACCACACTCTACCTCTCAAGC
Reverse Primer : TTTAATTACAGGTGGCTCATACGTG

BRAF
Forward Primer : CCTTTACCTGAGAGTAAGCATCAGC
Reverse Primer : AACAAAACTGGAACGATACTCCTTG

Result



Example 4

Amplification using primers containing inosine

We compared amplification with conventional products using degenerate primers containing inosine. Clear bands were only identified using KOD One™ PCR Master Mix (with and without dye).

Composition of Reaction mixture

	(μL)
Sterile distilled water	17
KOD One™ PCR Master Mix/-Blue-	25
100pmol / μL Primer F	1.5*
100pmol / μL Primer R	1.5*
50ng/ μL *E. coli* genome DNA	5
Total Volume	50

*When using degenerate primers, the molar concentration per primer type is lower for higher degeneracy. The sensitivity can be improved by increasing the primer concentration according to the level of degeneracy.

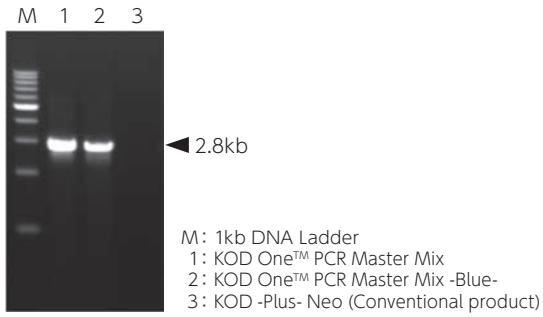
PCR Cycle

98℃, 10sec.
60℃, 5sec.
68℃, 15sec.
(5sec./kb)
30cycles

Primer

Forward Primer : ATGGTICARATHCCICARAAY
Reverse Primer : RTGIGCYTGRTCCCARTTYTC

Result



Example 5 Comparison of the detection sensitivity using plasmid DNA and cDNA templates

Plasmid DNA and cDNA were used to amplify a target of approximately 3.5 kb with an elongation time of 5 sec./kb, and the detection sensitivity was compared. The reactions were performed according to the recommended conditions for each PCR enzyme.
KOD One™ PCR Master Mix was able to amplify lower copy number regions compared with the reagents of Company A.

Composition of Reaction mixture

	(μL)
Sterile distilled water	21
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
Template (Plasmid DNA, cDNA)	1
Total Volume	50

PCR cycle

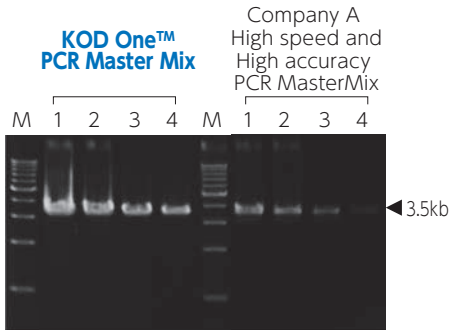
98℃, 10sec.
60℃, 5sec.
68℃, 18sec. } 30cycles
(5sec./kb)

※The PCR master mixes of company A were used in accordance with the manufacturer's recommended conditions.

Result

[Plasmid DNA]

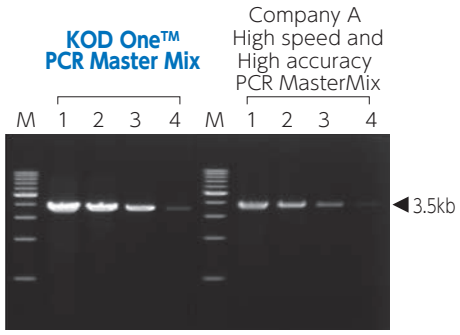
Template : plasmid DNA
Target : Polymerase Gene



M : 1kb DNA Ladder
1 : 25pg
2 : 6.3pg
3 : 1.6pg
4 : 0.4pg

[cDNA]

Template : HeLa cell total RNA-derived cDNA
Target : TFR



M : 1kb DNA Ladder
1 : 25ng (RNA equivalent)
2 : 5ng (RNA equivalent)
3 : 1ng (RNA equivalent)
4 : 0.7ng (RNA equivalent)

Example 6 Confirming the amount of reverse transcription reagent

Because RNA in the reverse transcription product (cDNA) is inhibitory to PCR, the addition of a large amount of cDNA leads to reaction inhibition. Therefore, to confirm the amount of cDNA that can be added, the added amount of cDNA was varied and the TFR gene was detected.
KOD One™ PCR Master Mix was not easily inhibited by RNA, and good amplification was achieved even under conditions in which a large amount of cDNA, which is difficult to amplify with conventional or other companies' products, was added.

Composition of Reaction mixture

	(μL)
Sterile distilled water	17
KOD One™ PCR Master Mix/-Blue-	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
cDNA	5
Total Volume	50

PCR cycle

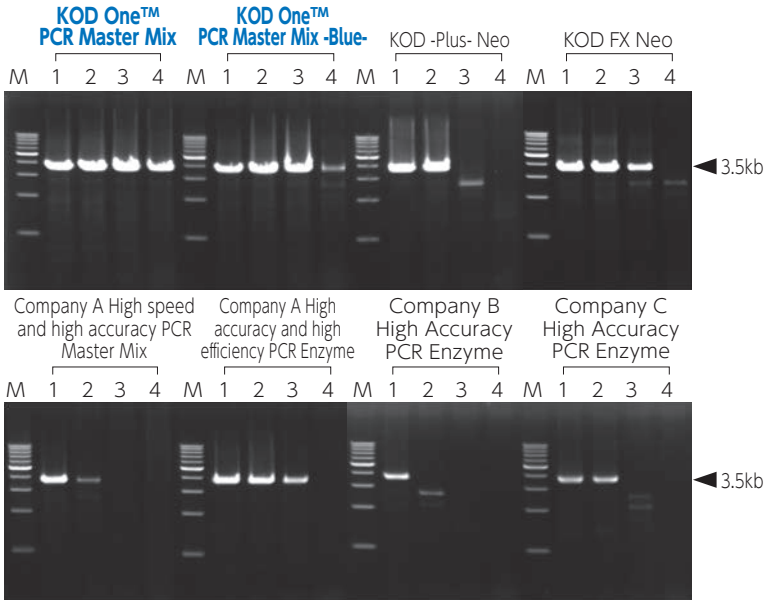
98℃, 10sec.
60℃, 5sec.
68℃, 18sec. } 30cycles
(5sec./kb)

※PCR enzymes other than KOD One™ PCR Master Mix are performed according to their recommended conditions.

Primer

Forward Primer : CCACCATCTCGGTCATCAGGATTGCCT
Reverse Primer : TACTCCTTAACGAGAAGACATCTCAAGAC

Result



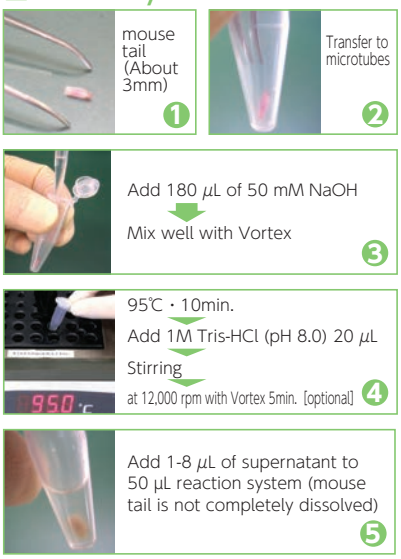
M : 1kb DNA Ladder
1 : 125ng (RNA equivalent)
2 : 250ng (RNA equivalent)
3 : 500ng (RNA equivalent)
4 : 1,000ng (RNA equivalent)

Template : HeLa cell total RNA-derived cDNA
Target : TFR

Example 7 Amplification using mouse tail lysate as a template

The mouse Thy-1 gene was amplified using mouse tail prepared by the alkaline lysis method. The reactions were performed at an elongation time of 5 sec./kb according to the recommended conditions for each PCR enzyme. Only KOD One™ PCR Master Mix showed a clear band.

■Alkaline lysis method



Composition of Reaction mixture

	(μL)
Sterile distilled water	Y
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
Lysate	X
Total Volume	50

PCR cycle

98℃, 10sec.
60℃, 5sec.
68℃, 13sec.
(5sec./kb)
30cycles

※Company A Fast and Accurate PCR Master Mix is performed according to its recommended conditions.

Primer

Forward Primer :
CCACAGAATCCAAGTCGGAACTCTTG
Reverse Primer :
GTAGCAGTGGTGGTATTATACATGGTG

Result

M : 1kb DNA Ladder
1 : Lysate 1μL
2 : Lysate 2μL
3 : Lysate 4μL
4 : Lysate 8μL

Example 8 Amplification using plant lysate as a template

rbcL gene amplification was compared between KOD One™ PCR Master Mix (with and without dye) and conventional products using tobacco leaf lysate prepared by the one-step method.

KOD FX Neo showed similar amplification to KOD One™ PCR Master Mix, but KOD-Plus-Neo showed no amplification with 1 μL of lysate, suggesting that the plant component inhibited the amplification.

This may be because of inhibition by the plant components.

■One step method

Bio Techniques, **19**: 394 (1995)

Composition of Reaction mixture

	(μL)
Sterile distilled water	Y
KOD One™ PCR Master Mix/-Blue-	10
10pmol / μL Primer F	0.6
10pmol / μL Primer R	0.6
Lysate	X
Total Volume	20

PCR cycle

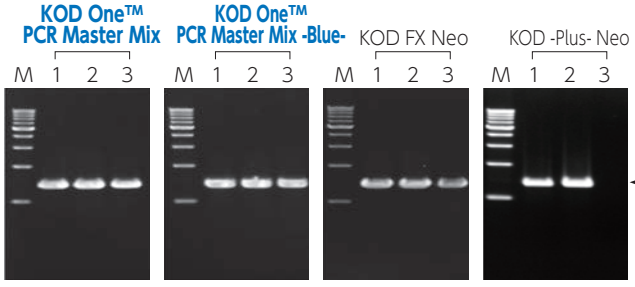
98℃, 10sec.
60℃, 5sec.
68℃, 7sec.
(5sec./kb)
30cycles

※Conventional PCR enzymes are performed under their recommended conditions.

Primer

Forward Primer : ATGTCACCACAAACAGAGACTAAAGC
Reverse Primer : AAGCAGCAGCTAGTTCCGGGCTCCA

Result



M : 1kb DNA Ladder
1 : Lysate 0.25μL
2 : Lysate 0.5μL
3 : Lysate 1.0μL

Example 9 Direct PCR using a blood sample

Unpurified blood was directly used to amplify the human β-globin gene. The reactions were performed at an elongation time of 5 sec./kb according to the recommended conditions for each PCR enzyme. Only KOD One™ PCR Master Mix showed a clear band.

Composition of Reaction mixture

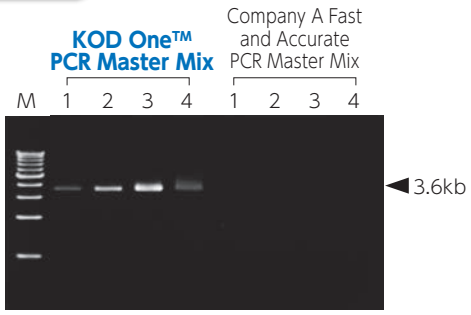
	(μL)
Sterile distilled water	Y
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
blood	X
Total Volume	50

PCR cycle

98℃, 10sec.
60℃, 5sec.
68℃, 18sec.
(5sec./kb)
30cycles

※Conpany A Fast and Accurate PCR Master Mix is performed according to its recommended conditions.

Result



M: 1kb DNA Ladder
1: blood 1μL
2: blood 2μL
3: blood 4μL
4: blood 8μL

Primer

Forward Primer : GGTGTTCCCTTGATGTAGCACA
Reverse Primer : ACATGTATTTGCATGGAAAACAACTC

Example 10 Evaluation of the PCR tolerance against humic acid

Humic acid is a reddish brown or blackish brown organic substance found in humus and soil, and it is known to inhibit PCR. Humic acid cannot be removed by the usual DNA purification process. Therefore, PCR using environmental and ecological samples as templates is considered to be very difficult. Therefore, we investigated the variation of the amplification of the HBg gene from the human genome with the humic acid content using KOD One™ PCR Master Mix and various PCR enzymes.

In the case of KOD One™ PCR Master Mix, amplification was observed regardless of the amount of humic acid, and the results showed high humic acid tolerance even under high-speed PCR conditions.

Composition of Reaction mixture

	(μL)
Sterile distilled water	Y
KOD One™ PCR Master Mix	10
10pmol / μL Primer F	0.6
10pmol / μL Primer R	0.6
OD_{280}=1 humic acid	X
4ng / μL human genom DNA	1
Total Volume	20

PCR cycle

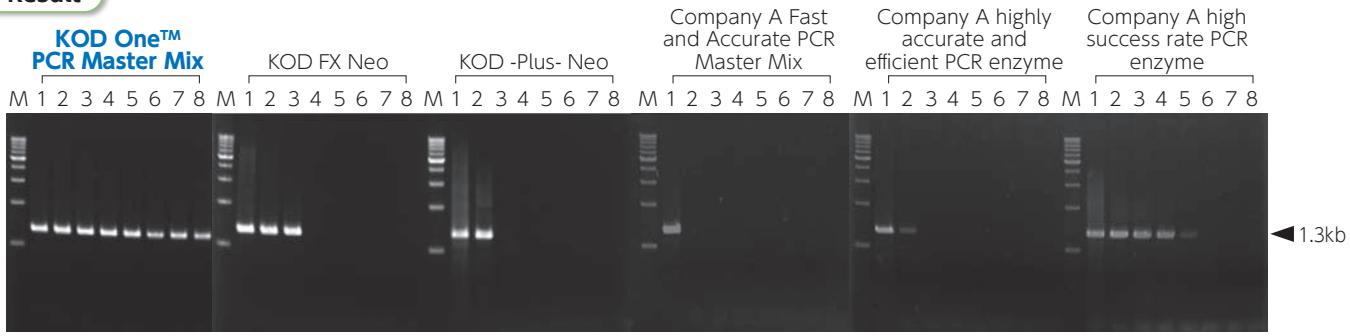
98℃, 10sec.
60℃, 5sec.
68℃, 7sec.
(5sec./kb)
30cycles

※PCR enzymes other than KOD One™ PCR Master Mix are performed under the recommended conditions.

Primer

Forward Primer :
TTAGGCCTTAGCGGGCTTAGAC
Reverse Primer :
CCAGGATTTTTGATGGGACACG

Result



M: 1kb DNA Ladder

Humic acid added (X)
1: 0μL
2: 1μL
3: 2μL
4: 3μL
5: 4μL
6: 5μL
7: 6μL
8: 7μL

Example 11 Direct PCR using filamentous fungi and yeast

Purpose of the Experiment

Extraction and amplification of DNA from filamentous fungi and yeasts without extraction and purification.

Experimental procedure

Sample Type

Aspergillus oryzae and *Saccharomyces cerevisiae* fungi

Sample Preparation Method

From the plate on which the filamentous fungi and yeast were grown, the fungi were removed with a toothpick and the following were used as templates:

1. 5 μL of the suspension in 50 μL of sterile water
2. 5 μL of the suspension in 20 μL of sterile water
3. as is

Collected *Aspergillus oryzae* bacteria

Target gene name and length

ITS1 region in 18S rRNA
Aspergillusoryzae: approx. 180 bp
Saccharomyces cerevisiae : approx. 450 bp

Primer

ITS1 Forward Primer : GTAACAAGGTYTCCGT
ITS1 Reverse Primer : CGTTCTTCATCGATG

※Owing to the short target region, shorter primers than the recommended length (22-35mer) were used in this experiment.

Composition of Reaction mixture

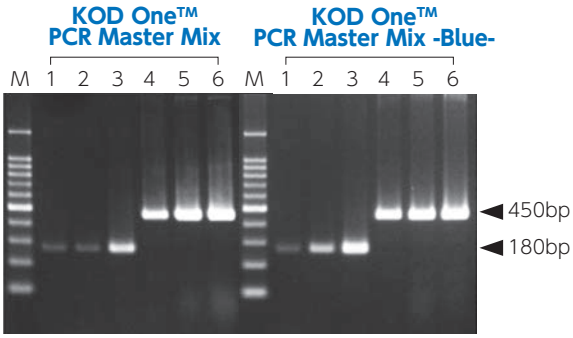
	(μL)
Sterile distilled water	3.8 or 8.8
KOD One™ PCR Master Mix	10
10μM Primer F	0.6
10μM Primer R	0.6
Bacteria suspension or bacteria	5 or 0
Total Volume	20

PCR cycle

98℃, 10sec.
45℃, 5sec.
68℃, 10sec.
30cycles

Results and Discussion

For *S. cerevisiae*, there was not much difference in the amount of amplification with the sample preparation method. However, for *A. oryzae*, the fungus was better amplified when directly suspended in the PCR reaction mixture. In addition, there was no difference in results between KOD One™ Master Mix and KOD One™ PCR Master Mix -Blue-.



M : 100bp DNA Ladder
Aspergillus oryzae
1 : Suspend 50 μL of the bacteria in sterile water and use 5 μL
2 : Suspend 20 μL of the bacteria in sterile water and use 5 μL
3 : DirectSaccharomyces cerevisiae
Saccharomyces cerevisiae
4 : Suspend 50 μL of the bacteria in sterile water and use 5 μL
5 : Suspend 20 μL of the bacteria in sterile water and use 5 μL
6 : Suspend the organisms in direct

＊Direct PCR from fungi may be inhibited by a large amount of the fungus, and amplification may not be successful. In addition, molds and other fungi have hard cells, so amplification may not be possible unless a certain amount of fungus is added. We recommend that the amount of fungus is considered in advance.

If the amplification does not work well considering the amount of the fungus, it may be possible to amplify the fungus by setting the elongation time to 10 sec./kb.

In addition, because KOD One™ PCR Master Mix does not have an initial denaturation step in the recommended PCR cycle, depending on the fungus species, the PCR efficiency may be improved by performing heat treatment of approximately 95 ℃ for 5 min. after suspension in sterile water when preparing the sample.

Example 12

Confirmation of the gene disruption in yeast

Data provided by Dr. Yasuhiro Araki, Department of Advanced Oral Biology, Oral Science Frontier Center, Graduate School of Dentistry, Osaka University

Purpose of the Experiment

Confirm that the target gene on the chromosome is disrupted by the marker gene.

Experimental procedure

Sample Type

Genomic DNA extracted from yeast

Sample Preparation Method

A colony the size of a match head was scraped off the tip of a yellow tip and suspended in a PCR tube containing 50 μL of 0.02 M NaOH. The yeast suspension was heat-treated in the PCR at 95 ℃ for 10 min.

Target gene name and length

PIB2/ 2,345bp　pib2::natNT2/ 1,783bp
GTR1/ 1,128bp　gtr1::kanMX4/ 1,679bp

Primer

PIB2
Forward Primer : CGGATGCTATGTCACGTTG
Reverse Primer : TCTTTACTGCTGTTGTGTCC

GTR1
Forward Primer : ACCCTAAATTGTGATTATGG
Reverse Primer : TTCTTCCTCTTATGTTTCTC

Composition of Reaction mixture

	(μL)
Sterile distilled water	3.9
KOD One™ PCR Master Mix -Blue-	5
10μM Primer F	0.3
10μM Primer R	0.3
Yeast suspension	0.5
Total Volume	10

Half of the PCR reaction solution (5 μL) was used for electrophoresis

PCR cycle

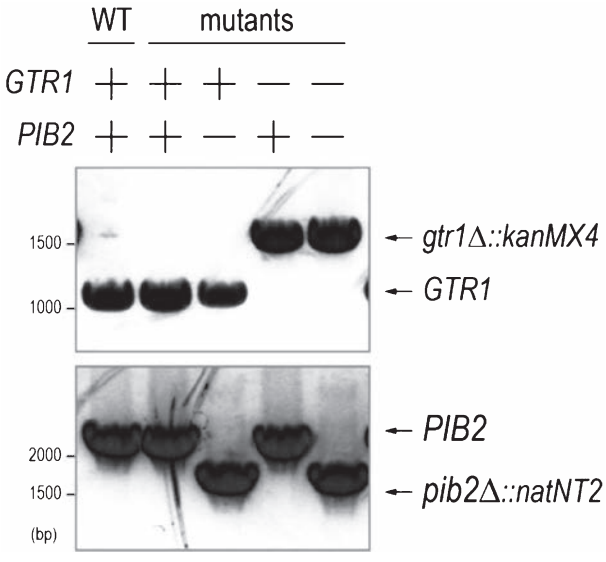
98℃, 10sec.
53℃, 5sec.
68℃, 10sec.
} 35cycles

Thermal cycler

Applied Biosystems GeneAmp® PCR System 9700

Results and Discussion

- The PCR time was significantly shortened.
- The amplification efficiency of the target gene improved.
- The all-in-one PCR reaction solution is easy to prepare.



Comments,opinions etc.

We usually have the opportunity to perform a large number of PCRs, which is a burden. However, this burden is greatly reduced by starting with KOD One™. We used to use several KOD products for different purposes, but we now only use KOD One™ considering the reaction speed and accuracy, which is advantageous from an economic point of view.

Example 13 PCR genotyping of *Aspergillus fumigatus flbC* knockout strains

Data provided by Mr. Dai Tanaka, Department of Infectious and Biodefense Sciences, Tohoku Medical and Pharmaceutical University, School of Pharmaceutical Sciences

Purpose of the Experiment

The PCR method is used to select *A. fumigatus flbC* recombinants.

Experimental procedure

Sample Type

Aspergillus fumigatus spore

Sample Preparation Method

A very small amount of *A. fumigatus* spores (on the tip or not) was suspended in 100 μL of TE buffer and microwaved (500 W, 2 min) to obtain the supernatant.

Target gene name and length

flbC (Afu2g13770)	1,500bp
ptrA	2,600bp

Primer

Forward Primer : TCTCATCTGCTCCCTTCATCT
Reverse Primer : ATTGTACAAGGGACGAGAGCC

Thermal cycler

T100 Thermal Cycler (Bio-Rad)

◉KOD One™ PCR Master Mix

Composition of Reaction mixture

	(μL)
Sterile distilled water	8.8
KOD One™ PCR Master Mix	10
10 μM Forward / Reverse Primer	Each 0.1
Microwave treatment Spore suspension	1
Total Volume	20

PCR cycle

98℃, 10sec. ┐
68℃, 20sec. ┘ 40cycles

◉KOD FX Neo

Composition of Reaction mixture

	(μL)
Sterile distilled water	7.4
2×PCR Buffer	10
10 μM Forward / Reverse Primer	Each 0.1
KOD FX Neo	0.4
Microwave treatment Spore suspension	1
Total Volume	20

PCR cycle

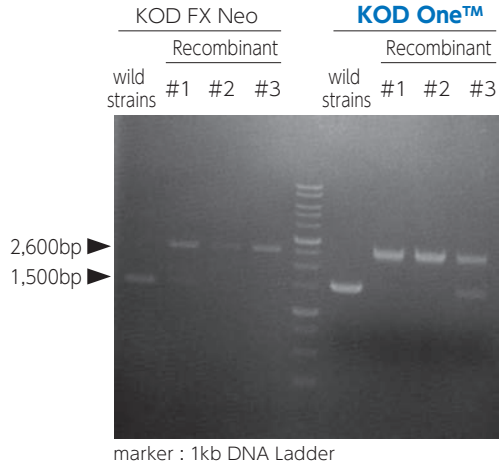
98℃, 10sec. ┐
68℃, 240sec. ┘ 40cycles

Results and Discussion

Aspergillus fumigatus A1159 strain was used as the parent strain, and recombinants (*flbC* gene deficient strains #1-3) were obtained by inserting DNA fragments containing homologous sequences upstream and downstream of the coding region of the *flbC* gene and the pyrithiamine resistance gene *ptrA*, respectively.

The solutions obtained by microwaving the spores of these recombinants and the parental strain were used as templates for colony PCR using KOD One™ and KOD FX Neo. The success or failure of genetic recombination and the performance of both enzymes were then compared by agarose electrophoresis.

The results showed that the PCR products were obtained without any problems in both transgenic and parental strains when either enzyme was used. In other words, both enzymes can be used to observe the success or failure of genetic recombination by the colony PCR method. The PCR took approximately 55 min. for KOD One™ and 200 min. for KOD FX Neo.



marker : 1kb DNA Ladder

These results indicate that KOD One™ is a "usable enzyme" that can shorten the time required for colony PCR while being resistant to crude samples.

Comments, opinions, etc.

A colleague told me that I should use KOD FX Neo for colony PCR of mold spores. I had been only using KOD FX Neo for genotyping of genetically modified molds. However, I was surprised to find that KOD One™ is as resistant to crude samples as KOD FX Neo, even though the time required for elongation is greatly reduced. I will continue to use KOD One™ in the future.

Example 14

Amplification of the high-GC mouse c-Maf cDNA full-length sequence

Data provided by Fukushima Medical University School of Medicine Radioisotope Research Facility Dr. Masayuki Sekiyoshi

Purpose of the Experiment

Amplify the mouse c-Maf cDNA full-length sequence for animal cell expression vector construction.

Experimental procedure

Sample Type

Total RNA extracted from mouse T cells

Sample Preparation Method

Total RNA was prepared by ISOGEN (Nippon Gene), and then cDNA synthesized by the PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio) was used as a template.

Target gene name and length

mouse c-Maf cDNA / 1.2 kb

Primer

Forward Primer : GGGATCCGATGGCTTCAGAACTGGCAA
length : 27mer Tm : 65.1℃
Reverse Primer : GGTCGACCATGAAAAATTCGGGAGAGG
length : 27mer Tm : 63.5℃

◉KOD One™ PCR Master Mix -Blue-, Company A High-Speed, High-Accuracy PCR Master Mixes Common

Composition of Reaction mixture

	(μL)
Each PCR Master Mix	3
10μM Primer F	1
10μM Primer R	1
cDNA	1
Total Volume	6

PCR cycle

94℃, 2min.
98℃, 10sec. ←┐
55℃, 5sec. ├ 30cycles
68℃, 15sec. ←┘

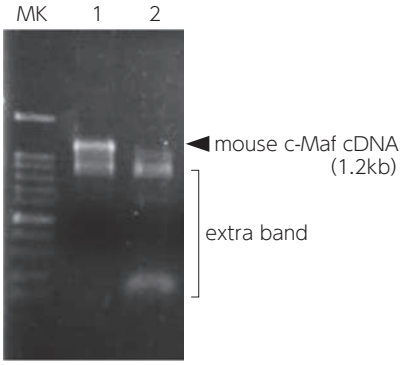
＊PCR is performed under the conditions of Company A Fast and Accurate PCR Master Mix.

Thermal cycler

Bioer Technology LifeECO ver 2.0

Results and Discussion

KOD One™ was able to amplify 1.2 kb of c-Maf cDNA with high GC content, while the fast and accurate PCR master mix of company A was able to amplify 1.2 kb of c-Maf cDNA with high GC content but produced a product lacking the high GC content region.



MK : 100bp DNA Ladder
1 : KOD One™ PCR Master Mix -Blue-
2 : Company A Fast and Accurate PCR Master Mix

Comments, opinions, etc.

The amplified sequence had a high GC content of 70%, and locally there were sequences with GC continuity exceeding 94%. The addition of dimethyl sulfoxide may improve the PCR reaction, but it is not clear whether stable results can always be obtained.

The fast and accurate PCR master mixes of company A also contain a highly accurate enzyme with high amplification capacity, but, as shown in the figure (lane 2), it amplifies as a short chain lacking the highly continuous GC sequence. In contrast, in KOD One™ PCR Master Mix -Blue- (lane 1), the full-length 1.2 kb amplification is detected as the main band. This main band was cloned and sequenced, and the nucleotide sequence was confirmed to be correct.

This indicates that KOD One™ PCR Master Mix -Blue- is also suitable for accurate amplification of highly continuous GC sequences.

Example 15

Transferring genes between expression vectors

Data provided by Mr. Kei Fujiwara, Laboratory of Life and Molecular Engineering, Faculty of Science and Technology, Keio University

Purpose of the Experiment

Transfer the separately cloned genes to different expression vectors.

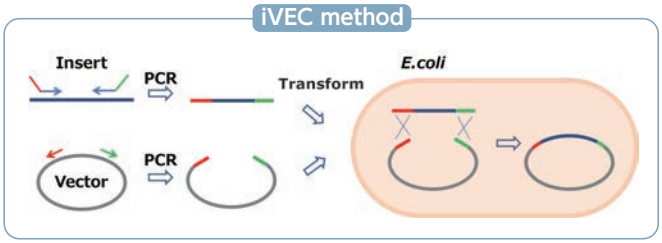
Experimental procedure

Sample

Plasmid DNA

Sample Preparation Method

DNA purified by the kit



Target gene name and length

Vector A (derived from pET15)	6,050bp
E. coli gene A-H	1,000-2,000bp

primer

For Vector	Forward Primer : 20nt	Tm : 55.4℃
	Reverse Primer : 18nt	Tm : 56.9℃
For gene	Forward Primer : 23nt	Tm : 58.8℃
	Reverse Primer : 21nt	Tm : 53.8℃

Homologous region

Forward : 81nt
Reverse : 117nt

※In this experiment, the primer sequence is included in the homologous region because the plasmid to be transferred has a homologous sequence itself.

Composition of Reaction mixture

	(μL)
Sterile distilled water	1.4
KOD One™ PCR Master Mix	2.5
3μM Primer F	0.5
3μM Primer R	0.5
50ng/μL Template	0.1
Total Volume	5.0

PCR cycle

98℃, 1min.
96℃, 10sec.
55℃, 10sec.
72℃, 10-35sec.
25cycles

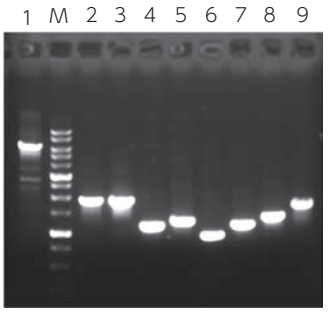
Measuring instruments

ChemiDoc MP (Bio-Rad)

After PCR, add 0.1 μL of *Dpn* I (NEB) to 5 μL of reaction solution.

↓

37℃ for 40 min (electrophoresis during this time)



1 : vector (about 6kbp)
M : 1kbp
2-9 : Insert 1uL Electrophorese (8 kinds of E.coli genes,1 kbp to 2kbp)
1μL swept

↓

Mix 1.5 μL of Vector and 1 μL of gene with 12 μL of iVEC3 strain competent cells
(The strain provided by the National Institute of Bioresources was used as a competent cell based on the cryopreservation protocol.)

↓

On ice 20 min.

↓

Add 400 μL of LB medium and incubate at 37℃ for 60 minutes.

↓

Collect the bacteria by centrifugation (12,000 ×g, 30 sec) and discard the supernatant.

↓

Suspend the bacteria in 10 μL of 10 mM Tris-HCl (pH 8.0) and immediately sow on a plate.

↓

The next day, perform colony PCR on the grown colonies using KOD One™ (total of 3 μL per sample).

Results and Discussion

One colony per gene was selected, and 7 out of 8 colonies were hit.

Comments, opinions, etc.

Switching from another company's fast PCR enzyme to KOD One™ more than doubled the efficiency of vector amplification and almost eliminated vector PCR failures. In addition, by using the iVEC3 method of transformation, we were able to complete the process from PCR to plating in 2 h.

Example 16

Mutagenesis engineering using pairs of complimentary primers

Purpose of the Experiment

Mutagenesis (three-base substitution, three-base deletion, and three-base insertion) into a plasmid of approximately 5 kb using a mutagenesis method with complementary primers.

Experimental procedure

Sample

Plasmid DNA

Sample Preparation Method

Purified from recombinant E. coli by miniprep

Primer Design ＊Forward : Fwd, Reverse : Rev

(1) Determine the mutation and position you wish to introduce.

plasmid sequence
5'-ATGCATGCATGCATGCATGCATGC**ATG**CATGCATGCATGCATGCATGCATGC-3

Mutation to be introduced
- Substitution example ATG to TGC
  5'-ATGCATGCATGCATGCATGCATGC**TGC**CATGCATGCATGCATGCATGCATGC-3
- Deletion example Remove ATG
  5'-ATGCATGCATGCATGCATGCATGC……CATGCATGCATGCATGCATGCATGC-3
- Insertion example ***AAA*** before ATG
  5'-ATGCATGCATGCATGCATGCATGC***AAA***ATGCATGCATGCATGCATGCATGCATGC-3

(2) Design Fwd primers to attach a hybridizing region of 12 to 20 bp to the plasmid on the 5′ and 3′ side, centered on the mutation site.

plasmid sequence
5'-ATGCATGCATGCATGCATGCATGC**ATG**CATGCATGCATGCATGCATGCATGC-3

5'- TGCATGCATGCATGC**TGC**CATGCATGCATGCAT -3' ⇨ Substitution Fwd Primer
(12-20bp, 12-20bp)

5'- TGCATGCATGCATGC……CATGCATGCATGCAT -3' ⇨ for deletion Fwd Primer
(12-20bp, 12-20bp)

5'- TGCATGCATGCATGC***AAA***ATGCATGCATGCATG -3' ⇨ for insertion Fwd Primer
(12-20bp, 12-20bp)

(3) Design of Rev primers
Design primers for the reverse complementary strand to the Fwd primers designed above.

5'- TGCATGCATGCATGC**TGC**CATGCATGCATGCAT -3
3'- ACGTACGTACGTACG**ACG**GTACGTACGTACGTA-5 ⇨ Substitution Rev Primer

5'- TGCATGCATGCATGC……CATGCATGCATGCAT -3
3'- ACGTACGTACGTACG……GTACGTACGTACGTA -5 ⇨ for deletion Rev Primer

5'- TGCATGCATGCATGC***AAA***ATGCATGCATGCATG -3
3'- ACGTACGTACGTACG***TTT***TACGTACGTACGTAC -5 ⇨ for insertion Fwd Primer

Composition of Reaction mixture

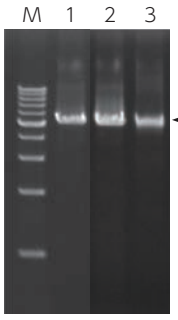
	(μL)
Sterile distilled water	21
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	1.5
10pmol / μL Primer R	1.5
50ng/ μL plasmid DNA	1
Total Volume	50

PCR
↓ ← *Dpn* I (10U/μL) 2μL
↓ [Code No. DPN-101]
37℃, 1hr.
↓
JM109 [Code No. DNA-900] is transformed

PCR cycle

98℃, 10sec. ←
60℃, 5sec. 15cycles
68℃, 25sec.
(5sec./kb)

[Electrophoretic confirmation after mutagenesis cycle]



M: 1kb DNA Ladder
1: 3-base substitution
2: 3-base deletion
3: 3-base insertion

[Mutation introduction rate and number of non-purposeful mutations for each of the 8 clones]

	mutagenic rate	number of non-purposeful mutations
3-base substitution	8/8 clones	0
3-base deletion	8/8 clones	0
and 3-base insertion	7/8 clones	0

Results and Discussion

Using KOD One™ PCR Master Mix, even a 5 kb plasmid could be mutagenized in a fast cycle with an elongation time of 25 sec. Mutagenesis was confirmed in most of the obtained colonies, and no 2nd-site mutation was observed.

Example 17

Engineering a three-nucleotide mutation into the gene sequence of the mammalian cell expression vector

Data provided by Dr. Jun Sugimoto, Assistant Professor, Department of Obstetrics and Gynecology, Graduate School of Medicine, Dentistry and Health Sciences, Hiroshima University

Purpose of the Experiment

Mutagenesis of three nucleotides inducing amino acid substitution to gene A cloned into an expression vector for mammalian cells.

Experimental Methods

Sample Type

Plasmid: Plasmid with gene A (approx. 1.8kb) cloned into a mammalian expression vector(approx. 7.1kb)

Sample Preparation Method

Plasmid DNA extracted and purified with Qiagen midi kit

Target gene name and length

Gene A : 1.8kb, Vector : 7.1kb

Primer

Primer for mutation introduction Forward (31base)
Primer for mutation introduction Reverse (31base)

Composition of Reaction mixture

	(μL)
Sterile distilled water	10.5
KOD One™ PCR Master Mix	12.5
10μM Primer F	0.5
10μM Primer R	0.5
50pg/μL plasmid DNA	1
Total Volume	25

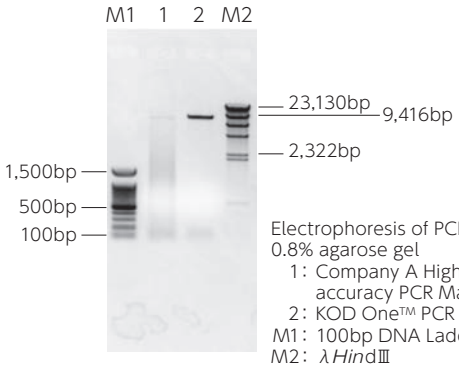
PCR cycle

98℃, 10sec.
55℃, 15sec.
72℃, 40sec.
30cycles

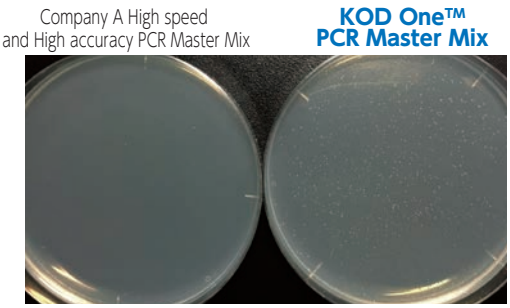
Thermal cycler

GeneAmp PCR system 9700

Results and Discussion



Electrophoresis of PCR-product (5 μL) on 0.8% agarose gel
1: Company A High speed and High accuracy PCR Master Mix
2: KOD One™ PCR Master Mix
M1: 100bp DNA Ladder
M2: λ*Hin*dⅢ



LB plate image after direct transformation of 2 μL of PCR-product into E. coli DH5α

Comments, opinions, etc.

Because of its size and characteristic sequence, it is difficult for the expression vector for mammalian cells used in our laboratory to introduce mutations directly into the cloned gene sequence. Therefore, it is necessary to introduce a mutation into a gene sequence cloned into a different vector (e.g., pGEM-T), cut out the DNA fragment containing the mutation, and reclone it into an expression vector for mammalian cells. However, the use of KOD One™ makes it possible to directly introduce the mutation, saving time and labor.

The mutation introduction efficiency was 100% among the several clones examined, and no PCR errors were observed in the neighboring sequences.

Example 18

Amplification of NGS library -Minimal amplification bias-

In the amplification of NGS libraries, it is important that there is no amplification bias owing to the sequence. KOD One™ PCR Master Mix is capable of amplifying a wide variety of sequences without bias, making it applicable to the NGS field.

A NGS library of *Thermus thermophilus* genome (GC content 70%) was amplified using KOD One™ PCR Master Mix and another company's product, and the amplified libraries were sequenced on an Illumina MiSeq®.

Composition of Reaction mixture

	(μL)
Sterile distilled water	14
KOD One™ PCR Master Mix	25
10pmol / μL Primer F	5
10pmol / μL Primer R	5
1ng/uL NGS library	1
Total	50

PCR cycle

98℃, 10sec.
60℃, 5sec.
68℃, 1sec.
} 14cycles

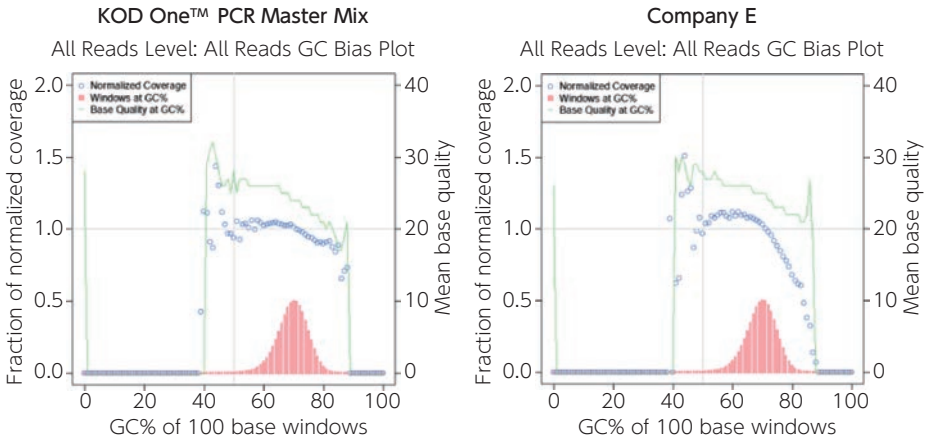
Primer

(Primer for TruSeq®)
Forward Primer : AATGATACGGCGACCACCGACATC
Reverse Primer : CAAGCAGAAGACGGCATACGAG

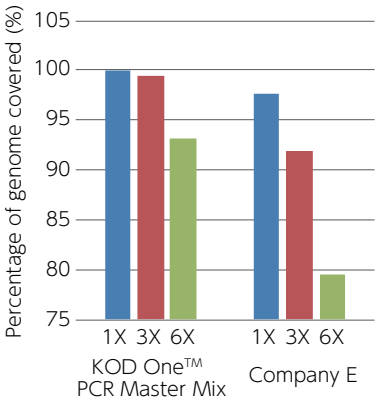
Result

The NGS libraries amplified with KOD One™ PCR Master Mix had normalized coverage close to 1 even at high GC rates This results in low bias. KOD One™ PCR Master Mix also showed better results when comparing the coverage of the same number of reads, showing high coverage even with a small number of reads. KOD One™ PCR Master Mix is also strong for the amplification of long targets, and it is expected to be applied not only to the amplification of short reads, but also to the amplification of long reads.

[Comparison of GC Bias]



[Comparison of Coverage]



GC bias plots were generated, with %GC content of 100 bp windows on the X axis. Normalized coverage is indicated by the blue circles (○), %GC of the reference sequence indicated by the red lines (-) and base quality at %GC indicated by the green line (-).

Please see below for library input amounts and cycle numbers.
(The optimal number of cycles may vary from 1 to 3 cycles depending on the sample type and mold size distribution.)

Library input amount	Number of cycle
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

Example 19

Classification of the SARS-CoV-2 sub-strain using NGS

Data provided by Miho Kuroiwa, Ph.D., Vaccine R&D Laboratory, SHIONOGI & CO., LTD.

Purpose of the Experiment

Development of technology to detect the SARS-CoV-2 sub-strain in sewage at accurate composition ratios using next generation sequencing (NGS). To confirm its accuracy, we mixed up to seven types of RNA derived from the SARS-CoV-2 sub-strain and experimentally confirmed that the exact composition ratio can be detected.

(KOD One™ was used for nested PCR for amplification of cDNA and assignment of the Illumina overhang sequences)

Sample

SARS-CoV-2 RNA

Sample template preparation

The RNA of the SARS-CoV-2 sub-strain was mixed in known proportions, and 1000 copies of the mixed RNA were used as templates for cDNA synthesis with the following S008 primers in the Reliance Select cDNA Synthesis Kit (Bio-Rad).

Target gene name and length

1st PCR : RBD region of SARS-CoV-2 spike protein (329-535aa)
2nd PCR : RBD region of SARS-CoV-2 spike protein (337-504aa)

Primer

1st PCR
S006:AACTGTGTTGCTGATTATTCTGTCC
S008:AGTTGAAATTGACACATTTG

2nd PCR
S007:TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTCTCTGCTTTACTAATGTCTATGCA
S009:GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGACTACYACTCTRTATGGTTGGT

PCR conditions

• Composition of Reaction mixture for 1st PCR

	(μL)
KOD One™ PCR Master Mix	20.6
40 μM S006 Primer	0.3
40 μM S008 Primer	0.3
cNDA solution	20
Total Volume	41.2

• 1st PCR cycle

98℃, 3sec.
98℃, 10sec. ←┐
55℃, 5sec. ┘ 10cycles
68℃, 2sec.

• Composition of Reaction mixture for 2nd PCR

	(μL)
KOD One™ PCR Master Mix	10
20 μM S013 Primer	0.5
20 μM S009 Primer	0.5
1st PCR product	3.4
Sterile distilled water	5.6
Total Volume	20.0

• 2nd PCR cycle

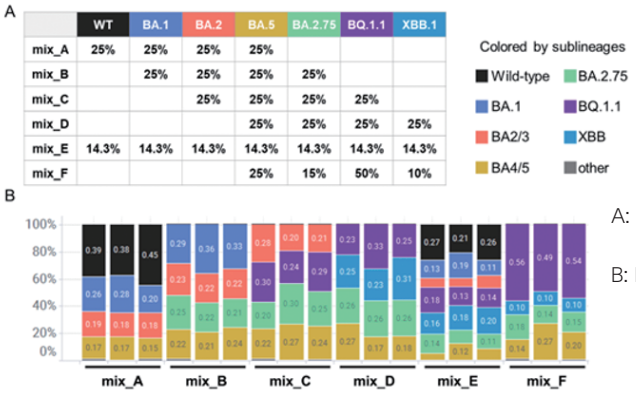
98℃, 3sec.
98℃, 10sec. ←┐
61℃, 5sec. ┘ 45cycles
68℃, 2sec.

Processing method up to NGS

The PCR amplification products were purified with Agencourt® AMPure® XP beads (Beckman Coulter) and eluted with 10 mM Tris-HCl (pH 8.5). The eluted amplified products were treated with Nextera® XT Index Kit v2 (Illumina), purified again with Agencourt® AMPure® XP beads, and submitted to MiSeq®.

Results and Discussion

The percentage of sublineages was calculated from the obtained sequence reads.



A: Theoretical mixing ratio of the SARS-CoV-2 sub-strain
B: Percentage of the SARS-CoV-2 sub-strain calculated from the NGS results

The amplicon sequencing results using KOD One™ showed that the percentage of sub-strains calculated was close to the theoretical value. KOD One™ has a low amplification bias and low mutation rate, making it ideal for amplification of sequencing targets.

<Reference>

Miho Kuroiwa, M. et al,. Targeted amplicon sequencing of wastewater samples for detecting SARS-CoV-2 variants with high sensitivity and resolution. *Science of The Total Environment*. **Volume 893**, 1 October 2023, 164766 https://doi.org/10.1016/j.scitotenv.2023.164766

MEMO

MEMO

TOYOBO
Beyond Horizons

TOYOBO CO.,LTD.
Biotechnology Overseas Sales and Marketing Department
1-13-1 Umeda, Kita-ku, Osaka, Japan 530-0001
TEL: +81-6-6348-3846
(e-mail) bio_overseas@toyobo.jp

Toyobo Research reagents website
[https://www.toyobo-global.com/products/life science/index.html]

Toyobo corporate website
[https://www.toyobo-global.com/]

Official website

THS24287/2024/12