Instruction manual for KOD -Multi & Epi- 1612

F1440K

KOD -Multi & Epi-®

KME-101 200 U 200 reactions

Store at −20°C

Contents

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.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/e/bio
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

[1] Introduction

Description

KOD -Multi & Epi-® is a high-fidelity PCR enzyme based on genetically modified KOD DNA polymerase[1,2] (UKOD). This modified enzyme enables amplification from templates containing uracils (U) or using primers containing inosines (I) and uracils (U). Furthermore, addition of the Elongation Accelerator significantly reduces amplification bias during PCR. KOD -Multi & Epi-® can be applied to various purposes such as a) multiplex PCR, b) bisulfite PCR in epigenetics research, and c) metagenomics research. This enzyme is also applicable for the preparation of DNA fragments for next-generation sequencing and capillary sequencing via cloning because the enzyme exhibits about 11-fold higher PCR fidelity.

KOD -Multi & Epi-® contains two types of anti-KOD DNA polymerase antibodies that inhibit the polymerase and its 3′→5′ exonuclease activity, thus allowing for Hot Start PCR[3]. Furthermore, KOD -Multi & Epi-™ generates blunt-end PCR products because of its 3′→5′ exonuclease (proof-reading) activity.

Features

- **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.

- **Effective amplification from templates containing uracils (U)**
  Effective amplification can be achieved using bisulfite-treated template DNA that contains uracils (U).

- **Primers containing uracil (U) or Inosine (I) can be used**
  KOD -Multi & Epi-® can use primers containing inosines (I) or uracil (U), whereas conventional high-fidelity PCR enzymes cannot.

- **High fidelity**
  KOD -Multi & Epi-® exhibits approximately 11-fold higher fidelity than Taq DNA polymerase. The enzyme can be used for various purposes where this would be an advantage (*e.g.* in the preparation of long target amplicons for sequencing).

- **Applicable for amplification from crude samples**
  KOD -Multi & Epi-® can be used for amplification from various crude samples (*e.g.* blood, lysates from animal or plant sources, soil, food, etc.) because of its tolerance of the PCR inhibitors commonly present in crude samples.

- **Highly efficiency**
  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 using bisulfite-treated DNA or for the multiplexed amplification of long targets.)

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[2] Components

KOD -Multi & Epi-® (1.0 U/μL)*	200 μL × 1
2× PCR Buffer for KOD -Multi & Epi-®**	1.7 mL × 3

*The enzyme solution contains anti-KOD DNA polymerase antibodies that neutralize the polymerase and inhibit its 3′→5′ exonuclease activities.
**The 2× PCR Buffer for KOD -Multi & Epi-® contains dNTPs (dATP, dGTP, dCTP, dTTP), and Mg^{2+} at 4.0 mM (final concentration: 2.0 mM).

[3] Primer Design

(1) Singleplex PCR

- 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 μM.

(2) Multiplex PCR

- Primers should be designed according to the concepts described in **(1)**.
- The performance of each primer set should first be confirmed by singleplex PCR using the same PCR cycle plan to be used later for multiplex PCR.
- The difference in T_m between primer pairs should be as small as possible.
- Different primer sets should not exhibit cross-complementarity between targets in the same multiplex PCR reaction.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

(3) PCR for bisulfite-treated DNA

- Primers should be designed according to the same basic concepts described in **(1).** The design of primers can be facilitated by the use of specific software that allows the replacement of cytosine residues with uracil.

- The following online tool is recommended:
  MethPrimer, http://www.urogene.org/methprimer/index.html

[4] Template

The following table indicates the applicable samples and appropriate amounts for PCR (50 µL 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.)
Bisulfite-treated DNA [b]	~200 ng	
Crude sample (blood, lysates, etc.) [c]		

[a]Excessive 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]Different commercially available bisulfite kits yield different conversion efficiencies and levels of DNA degradation. Artificially methylated DNA can be used as a positive control to check the conversion efficiency of the bisulfite treatment.
[c]Various crude samples can be used as templates. For a detailed protocol for the use of crude samples, please refer to the instruction manual for KOD FX Neo.
(http://www.toyobo-global.com/seihin/xr/lifescience/support/manual/KFX-201.pdf)

[5] Cloning of PCR products

- KOD -Multi & Epi-® 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 -Multi & Epi-® 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).

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[6] Protocol

1. **Singleplex PCR**

(1) Reaction set-up

Target genes up to 40 kb in length can be amplified from human genomic DNA using singleplex PCR.

Component	Volume	Final Concentration
PCR-grade water	X µL	
2× PCR buffer for KOD -Multi Epi-™*	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 ≤ 200 ng (RNA equiv.)/50µL Crude sample ≤ 5 µL/50 µL
KOD -Multi & Epi-®	1 µL	1.0 U/50 µL
Total reaction volume	50 µL	

*2× PCR buffer for KOD -Multi & Epi-® contains dNTPs (dATP, dGTP dCTP, dTTP), and 2 mM Mg^{2+}.

Notes:

- When non-specific or smeared bands are observed, the primer concentration should be decreased in decrements of up to 0.1 µM (final).
- In cases where degenerate primers are used, the primer concentrations can be increased up to 3.0 µM (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 ≤ 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 ≤ 65°C]

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

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

2-step cycle [In cases where primers' T_m > 65°C]

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

Notes:

[a]The annealing temperature can be decreased in decrements of up to T_m−5°C to enhance amplification yield.
[b]The 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 ≥ 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.

[c]The PCR cycle number can be increased up to 40 cycles.

2. Multiplex PCR

(1) Reaction set-up

Multiple targets of up to 10 kb can be stably amplified from human genomic DNA.

Component	Volume	Final Concentration
PCR-grade water	X µL	
2× PCR buffer for KOD -Multi & Epi-®*	25 µL	1×
Primers	Y µL	0.3 µM each[#]
Template DNA	Z µL	Genomic DNA ≤ 200 ng/50 µL Plasmid DNA ≤5 0 ng/50 µL cDNA ≤ 200 ng（RNA equiv.)/50 µL Crude sample ≤ 5µL50 µL
KOD -Multi & Epi-® (1.0U/µL)*	1 µL	1.0 U/50 µL
Total reaction volume	50 µL	

*2× PCR buffer for KOD -Multi & Epi-® contains dNTPs (dATP, dGTP dCTP, dTTP), and 2 mM Mg^{2+}.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[#]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.

(2) Cycling conditions

The 3-step cycle can be applied in cases where the T_m of the primers are ≤ 65°C. The 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 ≤ 65°C]

Pre-denaturation:	94°C, 2 min.	
Denaturation:	98°C, 10 s	25 cycles[c]
Annealing:	T_m[a], 30 s	
Extension:	68°C, X s[b]	

2-step cycle [In cases where primers' Tm > 65°C]

Pre-denaturation :	94°C, 2 min	
Denaturation :	98°C, 10 s	25 cycles[c]
Extension :	68°C, X s[b]	

Notes:

[a]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°C to reduce non-specific or smeared bands. To enhance amplification, it can be decreased in decrements up to T_m−5°C.

[b]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.

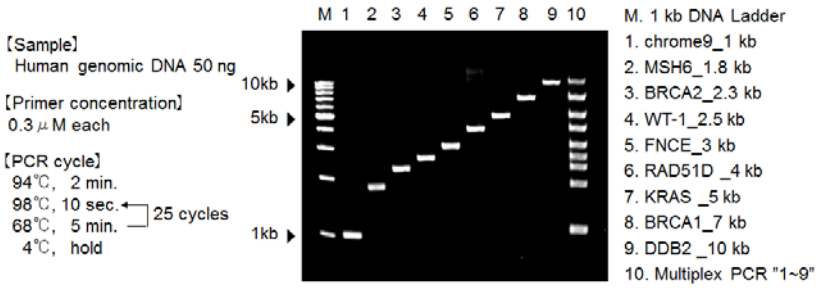
[c]The PCR cycle number can be increased up to 40 cycles.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

(3) Application data

Targets from 1 to 10 kb were amplified by singleplex or multiplex PCR using the primer pairs listed in the table below. Each target was successfully amplified by both singleplex and multiplex PCR.



Primer	Sequence	Primer length	T_m (℃)	GC(%)	Target size
Chrome9_1 kb_F primer	GAATTCCATATCTTTGCCAAACACTTGGTG	30	72.1	40.0	1 kb
Chrome9_1 kb_R primer	CCATGGGAAATGTGTTGAAGAAAACAAAGTG	31	73.5	38.7	
MSH6_1.8 kb_F primer	CAGAAGAGGAAGAAGAGATGGAGGT	25	65.9	48.0	1.8 kb
MSH6_1.8 kb_R primer	GGAGGTAAGAAGAGACAGGCAAAGT	25	65.7	48.0	
BRCA2_2.3 kb_F primer	CAGGTCTTAACCTAGCAGAGGAGGT	25	65.7	52.0	2.3 kb
BRCA2_2.3 kb_R primer	GGTTGGTCTGCCTGTAGTAATCAAG	25	65.5	48.0	
WT-1_2.5 kb_F primer	GAGGTCGAGCCACTCTTTATTACG	24	65.7	50.0	2.5 kb
WT-1_2.5 kb_R primer	TCTGACTCCCTTCGTCTAGTCTCTG	25	66.0	52.0	
FANCE_3 kb_F primer	CAGTCTTCCGTTAGATATCCTGAGC	25	64.9	48.0	3 kb
FANCE_3 kb_R primer	CTTCTGCCTAGATCTCCAGAGGATT	25	65.8	48.0	
RAD51D_4 kb_F primer	ACAGTGAGACGTGAGACCCTATCTC	25	65.7	52.0	4 kb
RAD51D_4 kb_R primer	CACAAATCTATTGCCCTGATAGCAT	25	65.7	40.0	
KRAS_5 kb_F primer	CTTCCTGTGGGCTAGAGATACACTG	25	65.9	52.0	5 kb
KRAS_5 kb_R primer	CAAGCAACTAAGGTGAGTGGAAGAG	25	66.0	48.0	
BRCA1_7 kb_F primer	GCCCTTTAAGCAAAGACAGTAGTCC	25	65.8	48.0	7 kb
BRCA1_7 kb_R primer	CATCTCTGTCTGGTCAATCCCTTAC	25	65.7	48.0	
DDB2_10 kb_F primer	GCAGCAATAGTGGAAGACTGGTTAC	25	65.7	48.0	10 kb
DDB2_10 kb_R primer	GCTAGAGCCACCATTAGACTCAGAC	25	65.2	52.0	

3. PCR using bisulfite-treated DNA

(1) Reaction set-up

Targets up to 1.5 kb can be amplified using bisulfite-treated DNA.

Component	Volume	Final Concentration
PCR grade water	X μL	
2× PCR buffer for KOD -Multi & Epi-®*	25 μL	1×
Primers	Y μL	0.3 μM each#
Bisulfite-treated DNA	Z μL	~200 ng/50 μL
KOD -Multi & Epi-® (1.0U/μL)*	1 μL	1.0 U/50 μL
Total reaction volume	50 μL	

*PCR buffer for KOD -Multi & Epi-® contains dNTPs (dATP, dGTP dCTP, dTTP), and 2 mM Mg^{2+}.

#The primer concentrations should be reduced step-wise in decrements of up to 0.1 mM (final).

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

(2) Cycling conditions

The following 3-step cycle is recommended:

Pre-denaturation:	94°C, 2 min	
Denaturation:	98°C, 10 s	↰ 40 cycles[c]
Annealing:	T_m[a], 30 s	
Extension:	68°C, X s[b]	

Notes:

[a]In cases of poor amplification, the annealing temperature can be decreased to T_m−5°C

[b]The extension time should be determined according to the following table:

	< 500 bp	500–1,000 bp	≥ 1,000 bp
Extension time	15 s	30 s	30 s/kb

In cases of poor amplification, the extension time can be extended up to two-fold.

[c]If non-specific or smeared amplification bands are observed, the number of PCR cycles should be reduced step-wise up to 30 cycles.

(3) Application data

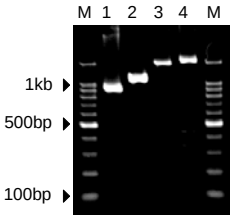
The target DNAs (917–1,583 bp) were amplified using bisulfite-treated CpG-methylated Jurkat genomic DNA. All targets were successfully amplified using KOD -Multi & Epi-®.

【Bisulfite treatment】
EpiTect® Fast DNA Bisulfite Kit (QIAGEN)

【Primer concentration】
0.3 ↑M each

【Sample】
55 ng (bisulfite-treated DNA)
/ 50 ↑l reaction

【PCR cycle】
94℃, 2 min.
98℃, 10 sec.
60℃, 30 sec.
68℃, 30 sec./ kb ↰ 40 cycles
4℃, hold



M. 100 bp DNA ladder
1. TGFß 917 bp (AT content*1 : 69.9%)
2. BRCA2 1134 bp (AT content*1 : 63.9%)
3. APOE 1487bp (AT content*1 : 69.1%)
4. TGFß 1583 bp (AT content*1 : 61.8%)

*1 The AT content after bisulfite treatment are shown.

Primer	Sequence	Primer length	T_m (℃)	GC(%)	Target size
TGF β _917 bp_F	TGGATTTTAAAGTTTTAGTTTTTTTTTAGG	29	61.6	20.7	917 bp
TGF β _917 bp_R	CAACTACTTCCAACCTCCCATAATA	25	62.8	40.0	
BRCA2_1134 bp_F	GGGGAATAGGTTTTGAGAGAATATT	25	62.2	36.0	1134 bp
BRCA2_1134 bp_R	ATACCACTAACCACATTAAACACTC	25	58.8	36.0	
APOE_1487 bp_F	GGTTTTTTTAAGTAGGGTGGTTTG	24	62.3	37.5	1487 bp
APOE_1487 bp_R	TCAAAAAACCAATTTCCTCTTTATC	25	62.0	28.0	
TGF β _1583 bp_F	TTGGAGAAAGTTGATTTAGAGTTTG	25	61.3	32.0	1583 bp
TGF β _1583 bp_R	CAACTACTTCCAACCTCCCATAATA	25	62.8	40.0	

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[7] Troubleshooting

(1) Singleplex PCR

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°C.
		In cases where degenerate primers are being used, the annealing temperature should be decreased step-wise up to T_m−5°C.
	Template DNA is of low quality and/or quantity.	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.
	Enzyme concentration is too low.	Increase enzyme concentration to 1.5–2.0 U/50 µL.
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.)
	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 guidelines. Use TArget Clone™ -Plus- (Code No. TAK-201) [see related products].

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

(2) Multiplex PCR

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 cycle step-wise up to T_m−5°C.
	Template DNA is of low quality and/or quantity.	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.	Use fresh primers.
		Redesign primers.
	Enzyme concentration is too low.	Increase enzyme concentration to 1.5–2.0 U/50 µL.
Amplification bias between targets	Cycling conditions are not suitable.	Lower annealing temperature in 3-step cycling step-wise up to T_m−5°C.
	The concentration of primers is inappropriate.	Increase the concentration of primers for the targets exhibiting faint bands, or decrease the concentration of primers for the targets exhibiting excessive amplification.
	Poor primer performance.	Decrease the difference in T_m between 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.
	Too much enzyme.	Reduce enzyme to 0.5–0.8U/50 µL reaction.

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

(3) PCR using bisulfite-treated DNA

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 cycle step-wise up to T_m−5°C.
	Template DNA is of low quality and/or quantity.	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 DNA sample.
	Poor primer performance.	Use fresh primers.
		Redesign primers.
	Enzyme concentration is too low.	Increase enzyme concentration to 1.5–2.0 U/50 µL.
Smearing/Extra bands	Cycling conditions are not suitable.	Increase the annealing temperature up to T_m+5°C.
		Decrease the number of cycles from 40 to ~30–35.
	Too much template DNA.	Reduce the amount of template DNA.
	Poor primer performance.	Use fresh primers.
		Decrease the primer concentrations step-wise from 0.3 µM to 0.1 µM.
	Low quality primers.	Redesign primers.
	Too much enzyme	Reduce enzyme to 0.5–0.8U/ 50µl reaction.
Conversion efficiency is low	Low bisulfite treatment efficiency.	Confirm the conversion efficiency using artificially methylated DNA as a positive control.
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].

JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140

[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 roducts

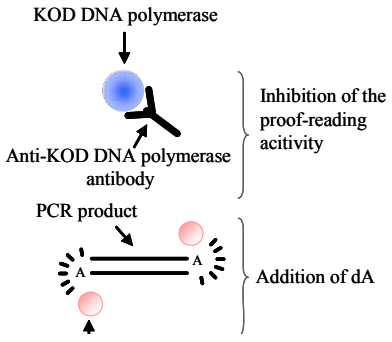
Product name	No. of Reactions	Code No.
TArget Clone -Plus-	10 reactions	TAK-201
10× A-attachment mix	25 reactions	TAK-301
Ligation high Ver.2	750 µL (100 reactions)	LGK-201

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] or KOD -Multi & Epi-® [Code No. KME-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] 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:



JAPAN
TOYOBO CO., LTD.
Tel(81)-6-6348-3888
www.toyobo.co.jp/bio/
tech_osaka@toyobo.jp

CHINA
TOYOBO Bio-Technology, CO., LTD.
Tel(86)-21-58794900.4140