PCR Protocols and Guides

Simplify your gene discovery

Merck Millipore is a division of MERCK

PCR Protocols and Guides

Introduction

Merck Millipore strives to provide up-to-date PCR protocols for your greatest experimental challenges. In this guide, we share our collective expertise on technical applications of PCR to help you achieve high fidelity gene amplification using our optimized protocols for minimal sample processing.

This guide includes information on:

- Sample preparation
- Primer design
- PCR reagent set up
- Thermocycling parameters

We've provided detailed guidelines for product usage in technical bulletins available on product-specific pages at **www.merck4chemicals.com**. Plus, our downloadable inNovations newsletters contain additional articles on specific applications. For more specific answers to your questions, please call our dedicated, experienced technical support staff at **+1 800 645 5476.**

KOD Hot Start DNA Polymerase, KOD XL DNA Polymerase, KOD Xtreme™ Hot Start DNA Polymerase, NovaTaq™ DNA Polymerase, NovaTaq™ Hot Start DNA Polymerase, One Step RT-PCR Kit

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01. User Protocol Technical Bulletins and inNovation listings

Product-specific user protocol bulletins and inNovations newsletters found on *merck4chemicals.com*

Product	Detailed Kit Protocols (Bulletins)	inNovations Issue Number
KOD DNA Polymerase	TB320	25
KOD Hot Start DNA Polymerase	TB340	17, 21 and 25
KOD Hot Start Master Mix	TB506	
KOD XL DNA Polymerase	TB342	17
KOD Xtreme™ Hot Start DNA Polymerase	TB507	28
One Step RT-PCR Master Mix	TB508	
NovaTaq™ Hot Start DNA Polymerase and Master Mix Kit	TB460	
NovaTaq™ DNA Polymerase	TB322	

02. KOD Xtreme™ Hot Start DNA Polymerase

KOD Xtreme™ Hot Start DNA Polymerase Allows Amplification from Challenging Crude Samples with Minimal Processing

Currently, typical PCR systems require extraction and purification of nucleic acid targets from samples of interest. In addition to the burden of time and cost, this sample preparation can be restricted due to low availability of initial sample specimens. The KOD Xtreme™ Hot Start DNA Polymerase system's unique formulation enables you to amplify directly from minimally processed samples; allowing highly efficient crude sample PCR target amplification. KOD Xtreme™ system is based on the ultra high fidelity, fast extension rates and processivity of DNA polymerase from *Thermococcus kodakaraensis* (KOD). Collectively, the KOD Xtreme™ system provides best in class amplification for a wide array of crude lysates (shown below) and targets up to 90% GC rich sequences resistant to PCR amplification due to hairpin secondary structures. Hairpin or stem loop type sequences often lead to truncated products or high non-specificity with other polymerases (Viswanthan 1999). In addition, with its ability to amplify long PCR targets (tens of kilobases), the KOD Xtreme™ system extends the limits of typical PCR reactions with other enzymes (shown below).

Minimal processing of typical PCR samples using KOD Xtreme™ Hot Start DNA Polymerase

Sample Preparation	Sample Type
Not required	Whole blood
Not required	Dried blood on filter paper
Not required	Cultured cells
Not required	Yeast (colony)
Not required	Bacteria (colony)
Not required	Fungus (colony)
Not required	Sperm
Not required	Plankton
Short Lysis (10 min)	Mouse tail
Short Lysis (10 min)	Plant (leaf, rice grain, etc)
Short Lysis (10 min)	Hair root

Standard Reaction Conditions

Component	Volume
PCR grade water	X µL
2x KOD Xtreme™ PCR Buffer	25 µL
2 mM dNTPs*	10 µL
10 pmol/µL Primer F	1.5 µL
10 pmol/µL Primer R	1.5 µL
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL
Genomic DNA (approx 20 ng/µL)	~200 ng
Plasmid DNA	~50 ng
cDNA	~200 ng (or RNA)
Crude Samples	2.0 µL
Total Volume	**50 µL**

*Use dNTPs supplied

Thermocycling Recommendations

A. Standard KOD Xtreme™ DNA Polymerase two-step thermocycling

Two-step Cycling (Recommended for most Applications)	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min

Repeat steps 2 and 3 for 20-35 Cycles

B. Three-step cycling recommended for low melting point (T_m) primers

1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing	T_m minus 5 °C, for 30 s
4. Extension	68 °C for 1 min/kb

Repeat steps 2-4 for 20-35 cycles

C. Step-down cycling recommended for long and difficult targets

94 °C for 2 minutes	1 cycle
98 °C for 10 s 74 °C for 1 min/kb	5 cycles
98 °C for 10 s 72 °C for 1 min/kb	5 cycles
98 °C for 10 s 70 °C for 1 min/kb	5 cycles
98 °C for 10 s 68 °C for 1 min/kb	15-25 cycles
68 °C for 7 min	1 cycle

Principles of Primer Design

- 22-35 bases with a melting temperature (T_m) over 60 °C
- For long amplification of a long target; 22-35 bases with high T_m values (up to 65 °C)
- For two-step or step-down cycling primer T_m value should be >73 °C
- If the T_m value of the primer is under 73 °C, the three-step cycle is recommended
- T_m should be calculated using Nearest Neighbor Method
- For amplification of longer targets (≥10 kb), primer oligonucleotides should be cartridge- or HPLC- purified. Primers purified by gel filtration tend to result in smeared PCR products.

Specific Application Protocols with KOD Xtreme™ Hot Start DNA Polymerase

Fast Amplification from Whole Blood Samples

STEP 1. Typical reaction setup.

Take 1-4 µL of untreated whole blood and add to 50 µL of reaction setup as below:

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer (10 µM)	1.5 µL	0.3 µM
Antisense (3') Primer (10 µM)	1.5 µL	0.3 µM
Whole Blood	1-4 µL	
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Total Volume	**50 µL**	

STEP 2. Example Thermocycling Conditions

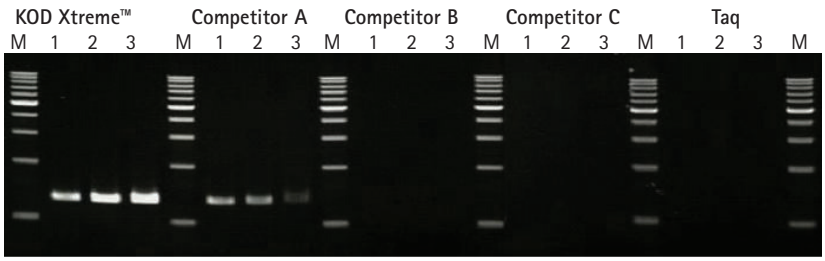
Two-step Cycling*	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min

Perform steps 2-3 for 30 cycles

* Standard tree-step cycling may also be used

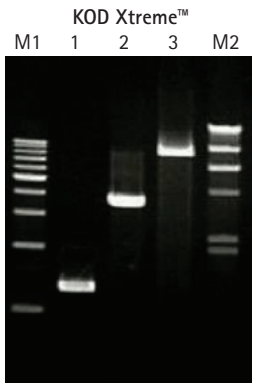
1. Polymerase activation and denaturation as above, followed by annealing [Lowest Primer T_m °C for 30 s] followed by extension [68 °C for 1 min/kb]; 20-40 cycles

Figure 1A



A. Reactions (50 µL each) were set up to amplify a 1.3 kb region of the β-globin gene from 1, 2 or 4 µL of untreated whole blood (lanes numbered 1, 2, and 3, respectively). **B.** PCR reactions were set up to amplify a 1.3 kb region, a 3.6 kb region, or an 8.5 kb region of the β-globin gene (lanes 1, 2, and 3, respectively) from 2 µL of untreated whole blood. For both A and B, PCR cycling parameters for KOD Xtreme™ were as follows: initial denaturation at 94 °C for 2 min; 30 cycles at 98 °C for 10 s, 68 °C for 1 min/kb. For polymerases from other manufacturers, optimal cycling parameters as recommended by each manufacturer were used. M and M1: 1 kb Ladder; M2: λ/Hind III Marker.

Figure 1B



Example Data:

KOD-Xtreme™ Hot Start DNA Polymerase amplification of ß-globin gene fragments from untreated Whole Blood Samples as compared with other DNA polymerases

Primer sequences used in this experiment:

<β-globin 1.3 kb>
Primer F: 5'-TTAGGCCTTAGCGGGCTTAGAC-3'
Primer R: 5'-CCAGGATTTTTGATGGGACACG-3'

<β-globin 3.6 kb>
Primer F: 5'-GGTGTTCCCTTGATGTAGCACA-3'
Primer R: 5'-ACATGTATTTGCATGGAAAACAACTC-3'

<β-globin 8.5 kb>
Primer F: 5'-TGATAGGCACTGACTCTCTGTCCCTTGGGCTGTTT-3'
Primer R: 5'-ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA-3

PCR from Dried Mouse Blood Filter Paper

STEP 1. **Blot drop of blood onto filter paper following mouse-tail venous puncture**

STEP 2. **Set up PCR reaction conditions as below for each blood sample**

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer (10 µM)	1.5 µL	0.3 µM
Antisense (3') Primer (10 µM)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Total Volume	**50 µL**	

STEP 3. **Place filter paper directly into PCR reaction (50 µL)**

PCR Tube

STEP 4. **Example Thermocycling Conditions**

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min/kb

Repeat step 2-3 for 20-35 cycles

Amplification from Mouse Tail Using Rapid Crude Lysate Preparation with Alkaline Lysis Method; Optimized Protocol for Microtube or 96-well Plate Format

STEP 1. Cut ~3 mm piece of mouse tail

STEP 2. Transfer to microfuge tube (or 96-well plate) containing 180 µL of 50 mM NaOH and vortex well

STEP 3. Incubate at 95 °C for 10 min on a thermocycler

STEP 4. Add 20 µL of 1 M Tris-HCl (pH 8.0) and vortex well (take care to avoid contamination)

STEP 5. Centrifuge lysate at 12,000 RPM for 5 mins

STEP 6. Add 0.5-2 µL of lysate to PCR reaction (50 µL) as below

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer -10 µM (10 pmol/ µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Total Volume	**50 µL**	

STEP 7. Example Thermocycling Conditions

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min/kb

Repeat step 2-3 for 30 cycles

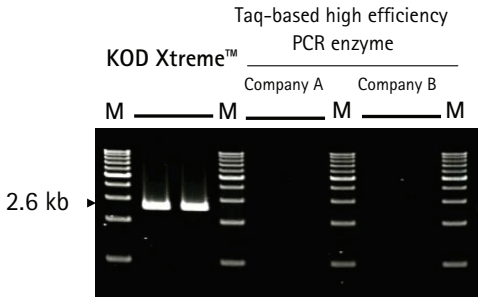
Primer sequences used in this experiment:

Primer F :5'-CCACAGAATCCAAGTCGGAACTCTTG-3'
Primer R :5'-GTAGCAGTGGTGGTATTATACATGGTG-3'

Thermocycling was performed at

1. 94 °C for 2 minutes	
2. 98 °C for 10 s	30 cycles
3. 368 °C for 2.5 mins	

0.5 µL of mouse tail lysate was added to 50 µL PCR reaction



M: 1 kb Ladder Marker

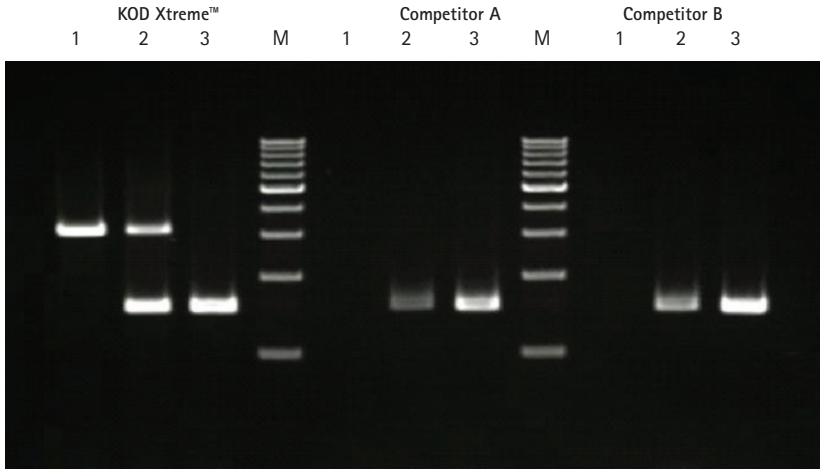
Example Data 1:

Amplification of mouse membrane glycoprotein (Thy-1) gene from mouse tail lysates prepared using alkaline lysis

Example Data 2:

Genotyping of transgenic mice using KOD Xtreme™ Hot Start DNA Polymerase using mouse tail tissue lysates

Lane	Samples
M	1 kbp Ladder
1	Tg/Tg
2	Tg/W
3	W/W



Reactions (50 µL each) were set up to amplify a 1.5 kb region or a 3.1 kb region of the wild-type or transgenic gene, respectively, from the indicated mouse tail tissue lysates*. Tissue fragments (~ 3 mm- each tail piece) were extracted in 50 mM NaOH for 10 minutes at 95 °C, neutralized with one-tenth volume of 1 M Tris-HCl, pH 8.0, spun at 12,000 rpm for 5 min, and 1 µL of each of the clarified extracts was used in the corresponding amplification reaction. PCR cycling parameters for KOD Xtreme™ were as follows: initial denaturation at 94 °C for 2 min; 30 cycles at 98 °C for 10 s, 68 °C for 3 min. For polymerases from other manufacturers, optimal cycling parameters as recommended by each manufacturer were used. Tg: transgenic; W: wild-type.

* The mouse tail samples were provided by Dr. Yugi Arai, National Cardiovascular Center, Japan.

Primer sequences used in this experiment:

Primer F: 5'-TGGACGTGAGCTTCAGCAC-3'
Primer R: 5'-AGGCCTGACAGTAGCTCAG-3'

Direct KOD Xtreme™ Hot Start DNA Polymerase PCR Using Mouse Toe

Procedure recommended for younger animals (7-10 days old)

STEP 1. **Clip small piece of mouse toe using dissection scissors (use appropriate institutional anesthesia procedures).**

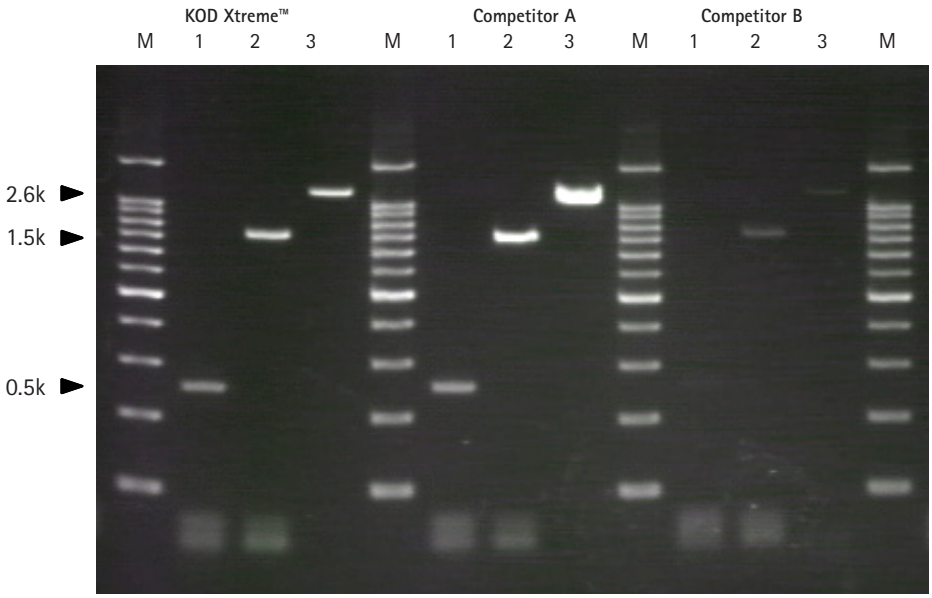
STEP 2. **Add piece of mouse toe directly into 50 µL of PCR reaction as below:**

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	11 µL	
Sense (5') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Total Volume	**50 µL**	

STEP 3. **Example Thermocycling Conditions:**

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min/kb

Repeat step 2-3 for 35 cycles



Example Data:

KOD Xtreme™ Hot Start DNA Polymerase Amplification of 3 genes using direct PCR method from mouse toe tissue using above protocol.

Lane	Samples
M	200 bp DNA ladder
1	Mouse TATA box binding protein (TBP) 0.5 kb
2	Mouse transferrin receptor (Tfr) 1.5 kb
3	Mouse membrane glycoprotein (Thy-1) 2.6 kb

Primer sequences used in this experiment:

Mouse TBP F: CAGTTGCTACTGCCTGCTGTTGTT
Mouse TBP R: GCTAGGATTAAAGACGTGCCACCA
Mouse Tfrc F: TGTGGAGGGTCAACGTGGTAGTT
Mouse Tfrc R: GTGACATTCTCAGGTGGCAGCTT
Mouse Thy-1 F02: CCACAGAATCCAAGTCGGAACTCTTG
Mouse Thy-1 R05: GTAGCAGTGGTGGTATTATACATGGTG

KOD Xtreme™ DNA Amplification of Plant Tissue Lysates

Extract using One Step (below) or homogenization method

STEP 1. Cut 3 x 3 mm of leaf tissue or ~1 grain of rice and transfer to microfuge tube.

STEP 2. Add 100 µL of Buffer A (100 mM Tris-HCL [pH 9.5], 1 M KCL and 10 mM EDTA)

STEP 3. Incubate at 95 °C for 10 min and vortex well

STEP 4. Add 1 µL of each extract to 50 µL PCR reaction

*Step 3 can be replaced by homogenization of the plant tissue in buffer A using a pestle followed by step 4 as above.

Reaction Set Up

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	10 µL	
Sense (5') Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Plant/Grain tissue supernatant	1.0 µL	
Total Volume	**50 µL**	

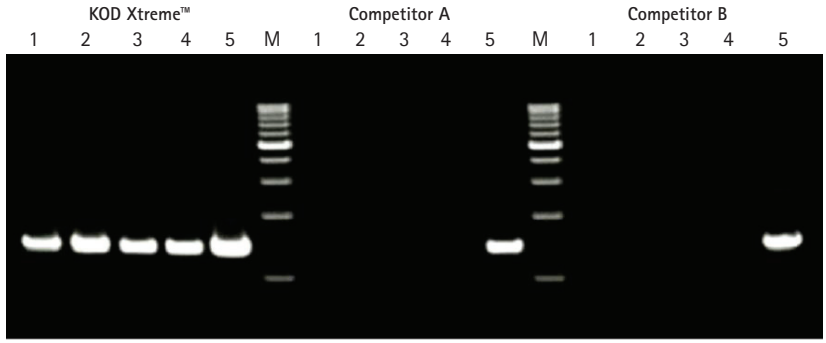
STEP 5. Example Thermocycling as follows:

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing and Extension	68 °C for 1 min/kb

Repeat steps 2 and 3 for 30 cycles

Example Data:

Performance of KOD Xtreme™ Hot Start DNA Polymerase System in Crude Lysates of different Plant Tissues



Lane	Samples
M	1 kb Ladder
1	Tomato leaf
2	Tobacco leaf
3	Rice leaf
4	Rice grain
5	Purified rice leaf DNA

Reactions (50 µL each) were set up to amplify a 1.3 kb region of the rbcL gene from each of the indicated crude plant tissue lysates. All tissues (~ 3 x 3 mm piece of leaf or one rice grain) were extracted for 10 minutes at 95 °C in 100 mM Tris-HCl (pH 9.5), 1 M KCl, 10 mM EDTA, and 1 ul of each of the extracts was used in the corresponding amplification reaction. PCR cycling parameters for KOD Xtreme™ were as follows: initial denaturation at 94 °C for 2 min; 30 cycles at 98 °C for 10 s, 68 °C for 1.5 min. For other polymerases, optimal cycling parameters as recommended by respective manufacturers were used.

Primer sequences used in this experiment:

F1: 5'-ATGTCACCACAAACAGAGACTAAAGC-3' (Tomato & Tobacco)
R1: 5'-AAGCAGCAGCTAGTTCCGGGCTCCA-3' (Tomato & Tobacco)
F2: 5'-ATGTCACCACAAACAGAAACTAAAGC-3' (Rice)
R2: 5'-AAGCTGCGGCTAGTTCAGGACTCCA-3' (Rice)

PCR Amplification From Formalin-fixed Paraffin-embedded (FFPE) Tissue Specimens Using KOD Xtreme™ Hot Start DNA Polymerase

STEP 1. Transfer formalin-fixed paraffin-embedded (FFPE) tissue (1 cm x 2 cm x 1 µm) to 1.5 mL microfuge tube

STEP 2. (De-paraffinization) Fill tube to top with xylene and leave for 5 min; remove xylene and repeat step 3x

STEP 3. Add 100% ethanol and leave for 5 min – remove ethanol and repeat 4x

STEP 4. Dehydrate tissue at 70 °C for 5 min

STEP 5. Add 300 µL of 10 mM Tris-HCl [pH 8.0] and 10 µL of Proteinase K (10 mg/mL) and incubate at 37 °C overnight

STEP 6. Heat at 70 °C for 10 min

STEP 7. Add 1 µL of lysate to 50 µL PCR reaction as below:

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	10 µL	
Sense (5') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Sample lysate	1.0 µL	
Total Volume	**50 µL**	

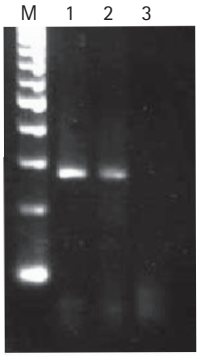
STEP 8. Example Thermocycling

Three-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Initial Denaturation	98 °C for 15 min*
3. Denaturation	98 °C for 10 s
4. Annealing	57 °C for 30 s
5. Extension	68 °C for 30 s**

Repeat step 3-5 for 38 cycles

* Initial Denaturation 7-15 min

**Extension at 1 min/kb



Example Data:

Amplification of β-globin gene (260 bp) from FFPE human tissue

Lane	Samples
M	100 bp DNA ladder
1	Sample 1
2	Sample 2
3	Distilled Water

Amplification From Complex Microbial Lysates Such as Yeast or Fungi

STEP 1. **Pick a piece of small/medium sized colony**

STEP 2. **Inoculate directly into 50 µL PCR reaction as below:**

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	11 µL	
Sense (5') Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Total Volume	**50 µL**	

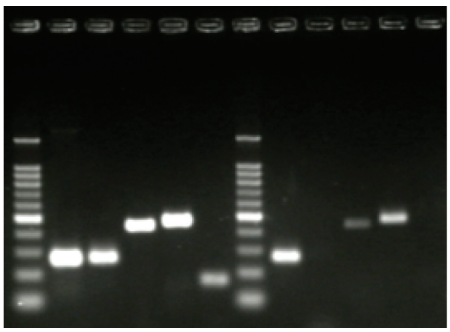
STEP 3. **Example Thermocycling as follows:**

Three-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Annealing	50 °C for 30 s
4. Extension	68 °C for 1 min

Repeat step 2-4 for 30 cycles

Example Data:

Performance of KOD Xtreme™ Hot Start DNA Polymerase System in Complex Microbial Crude Lysates



Lane	Samples
M	100 bp DNA ladder
1	*Aspergillus oryzae*
2	*Aspergillus niger*
3	*Saccharomyces cerevisiae*
4	*Schizosaccharomyces pombe*
5	*Pichia pastoris*

Primer sequences used in this experiment:

Primer F: GTAACAAGGT(T/C)TCCGT
Primer R: CGTTCTTCATCGATC

ITS-1 genes (150-470 bp) were amplified directly from small portions of colonies of fungus and yeast strains. Although fungi and yeasts have thick cell wall structures, KOD Xtreme™ Hot Start DNA polymerase successfully amplified all targets directly from the colonies.

KOD Xtreme™ Hot Start DNA Polymerase
Amplification of Long or Difficult DNA Targets

A. DNA amplification of long products from λ DNA.

STEP 1. **Add 10 ng of purified λ DNA into 50 µL PCR reaction as below:**

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	11 µL	
Sense (5') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
λ DNA	1.0 µL	10 ng
Total Volume	**50 µL**	

STEP 2. **Perform step-down cycling:**

94 °C for 2 minutes	1 cycle
98 °C for 10 s 74 °C for 1 min/kb	5 cycles
98 °C for 10 s 72 °C for 1 min/kb	5 cycles
98 °C for 10 s 70 °C for 1 min/kb	5 cycles
98 °C for 10 s 68 °C for 1 min/kb	20 cycles
68 °C for 7 min	1 cycle

PCR cycling conditions

94 °C 2 min
98 °C 10 s, 74 °C 1 min/kb (5 cycles)
98 °C 10 s, 72 °C 1 min/kb (5 cycles)
98 °C 10 s, 70 °C 1 min/kb (5 cycles)
98 °C 10 s, 68 °C 1 min/kb (20 cycles)

Primer sequences used in this experiment:

5 kb target:
F primer: 5´-CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC-3´
R primer: 5´-CGTTTGTACTCCAGCGTCTCATCTTTATGC-3´

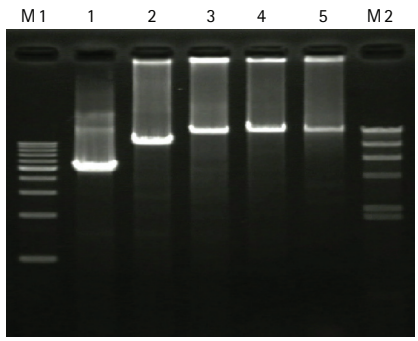
10 kb target:
F primer: 5´-CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC-3´
R primer: 5´-CATAGTCATCATCGCAGATTTCAATAACAT-3´

20 kb target:
F primer: 5´-CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC-3´
R primer: 5´-GTGCACCATGCAACATGAATAACAGTGGGTTATC-3´

30 kb target:
F primer: 5´-CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC-3´
R primer: 5´-GAAAGTTATCGCTAGTCAGTGGCCTGAAGAGACG-3´

40 kb target:

F primer: 5´-CTGATGAGTTCGTGTCCGTACAACTGGCGTAATC-3´
R primer: 5´-TAATGCAAACTACGCGCCCTCGTATCACATGG-3´



Example Data:

Long target capability: amplification of products up to 40 kb in length from λ DNA

B. Long target amplification of genomic DNA

STEP 1. Add 200 ng of purified human genomic DNA to 50 µL PCR reaction

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
Genomic DNA	X µL	200 ng
Total Volume	**50 µL**	

STEP 2. Perform thermocycling based on target size as below

1.3 kb to 8.5 kb targets

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Extension	68 °C for 1 min

Perform steps 2-3 for 30 cycles

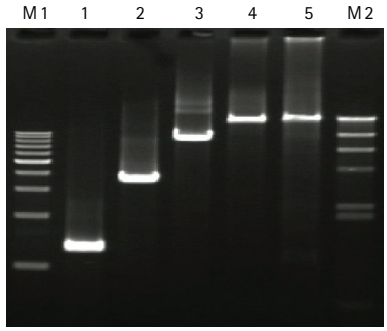
17.4 kb to 24 kb targets

94 °C for 2 minutes	1 cycle
98 °C for 10 s 74 °C for 1 min/kb	5 cycles
98 °C for 10 s 72 °C for 1 min/kb	5 cycles
98 °C for 10 s 70 °C for 1 min/kb	5 cycles
98 °C for 10 s 68 °C for 1 min/kb	20 cycles
68 °C for 7 min	1 cycle

PCR cycling conditions

1.3 to 8.5 kb targets
94 °C 2 min
98 °C 10 s, 68 °C 1 min/kb (30 cycles)

17.5 to 24 kb targets
94 °C 2 min
98 °C 10 s, 74 °C 1 min/kb (5 cycles)
98 °C 10 s, 72 °C 1 min/kb (5 cycles)
98 °C 10 s, 70 °C 1 min/kb (5 cycles)
98 °C 10s, 68 °C 1 min/kb (20 cycles)



KOD Xtreme™ Hot Start DNA Polymerase Amplification of up to 24 kb Human Genomic DNA gene targets

Example Data 1:

Amplification of β-globin and TPA genes

Primer sequences used in this experiment:

1.3 kb β-globin target
Forward primer: 5′-TTAGGCCTTAGCGGGCTTAGAC-3′
Reverse primer: 5′-CCAGGATTTTTGATGGGACACG-3′

3.6 kb β-globin target:
Forward primer: 5′-GGTGTTCCCTTGATGTAGCACA-3′
Reverse primer: 5′-ACATGTATTTGCATGGAAAACAACTC-3′

8.5 kb β-globin target:
Forward primer: 5′-TGATAGGCACTGACTCTCTGTCCCTTGGGCTGTTT-3′
Reverse primer: 5′-ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA-3′

17.5 kb β-globin target:
Forward primer: 5′-TGCACCTGCTCTGTGATTATGACTATCCCACAGTC-3′
Reverse primer: 5′-ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA-3′

24 kb tPA target:
Forward primer: 5′-CCTTCACTGTCTGCCTAACTCCTTCGTGTGTTCC-3′
Reverse primer: 5′-TGTCTCCAGCACACAGCATGTTGTCGGTGAC-3′

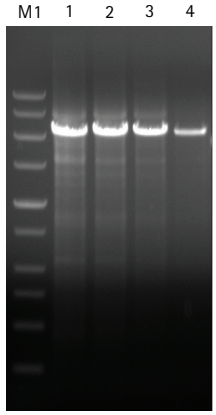
PCR cycling conditions

94 °C 2 min
98 °C 1 s, 68 °C 8.5 min (30 cycles)
4 °C Hold

Each reaction included Human Genomic DNA (Cat. No. 69237-3). Lane 1, 100 ng template; Lane 2, 50 ng template; Lane 3, 25 ng template; Lane 4, 5 ng template; Lane M1, Perfect DNA™ Markers, 0.5-12 kb (Cat. No. 69002-3). Target identity was confirmed by sequencing.

Primer sequences used in this experiment:

Forward primer: 5′-CATTGGAAGAGAGAATGTGAAGCAG-3′
Reverse primer: 5′-TCCACACTCATTGCAGACTCAGGTG-3′



Example Data 2:

Amplification of 8.4 kb p53 target from human genomic DNA

C. Long target amplification from human cDNA templates

STEP 1. Add ~200 ng of cDNA (from 50 ng of total human RNA) to 50 µL of PCR reaction:

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
cDNA	X µL	200 ng
Total Volume	**50 µL**	

STEP 2. Perform thermocycling based on target size:

6.8 kb Target

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Extension	68 °C for 7 min

Repeat steps 2 to 3 for 30 cycles

13.5 kb Target

Two-step Cycling	
1. Polymerase Activation	94 °C for 2 min
2. Denaturation	98 °C for 10 s
3. Extension	68 °C for 7 min

Repeat steps 2 to 3 for 35 cycles

Alternatively, apply step-down cycling:

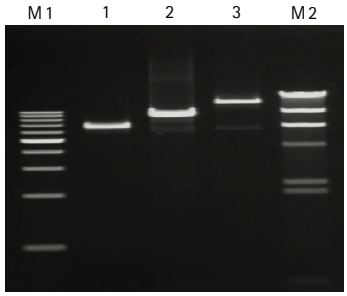
94 °C for 2 minutes	1 cycle
98 °C for 10 s 74 °C for 1 min/kb	5 cycles
98 °C for 10 s 72 °C for 1 min/kb	5 cycles
98 °C for 10 s 70 °C for 1 min/kb	5 cycles
98 °C for 10 s 68 °C for 1 min/kb	20 cycles

Primer sequences used in this experiment:

6.8 kb DNA polymerase epsilon catalytic subunit target
Forward primer: 5′-CACTTCCTCAGTTTCGGCACTCAAGCG-3′
Reverse primer: 5′-CTTCTGCAGCAGCCACTCCAGGGTCTC-3′

8.9 kb IGF2 receptor target
Forward primer: 5′-TCCCGCTCCGTCTCCACCTCCGC-3′
Reverse primer: 5′-CAGGGCGGTTTGCTTCTCAGCAATAGA-3′

13.5 kb dystrophin target
Forward primer:
5′-GCCTACTGGAGCAATAAAGTTTGAAGAACTTTTACCAGG-3′
Reverse primer: 5′-CACAACACGAAATAATGTCCAAATTAATTATGC-3′



Each reaction included cDNA derived from 50 ng total human RNA as template. A 5 µL sample of each 50 µL reaction was analyzed. Lane M1, 1 kb DNA ladder; Lane 1, 6.8 kb DNA polymerase epsilon catalytic subunit (NM_006231) target, Lane 2, 8.9 kb insulin-like growth factor 2 receptor (NM_000876) target; Lane 3, 13.5 kb dystrophin target; Lane M2, HindIII-digested l DNA markers. Identity of the dystrophin target was confirmed by sequencing.

Example Data:

KOD Xtreme™ Hot Start DNA Polymerase amplification of long targets (13.5 kb in length) from a human cDNA template.

PCR cycling conditions

6.8 kb DNA polymerase epsilon catalytic subunit target
94 °C 2 min
98 °C 10 s, 68 °C 7 min (30 cycles)

13.5 kb dystrophin target
94 °C 2 min
98 °C 10 s, 68 °C 14 min (35 cycles)

8.9 kb IGFR2 receptor target
94 °C 2 min
98 °C 10 s, 74 °C 9 min (5 cycles)
98 °C 10 s, 72 °C 9 min (5 cycles)
98 °C 10 s, 70 °C 9 min (5 cycles)
98 °C 10 s, 68 °C 9 min (20 cycles)

D. Amplification of GC-rich DNA targets

STEP 1. **Add 5 to 100 ng of DNA template to 50 µL PCR reaction as below**

STEP 2. **PCR Reaction Setup**

Component	Volume	Final Concentration
2x Xtreme™ Buffer	25 µL	1x
dNTPs (2 mM each)	10 µL	0.4 mM
PCR Grade Water	X µL	
Sense (5') Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Xtreme™ Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	
GC-rich DNA template	X µL	5 - 100 ng
Total Volume	**50 µL**	

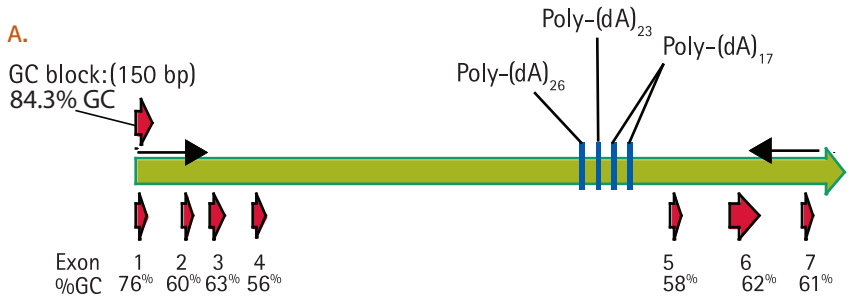
STEP 3. **Thermocycling Conditions**

94 °C for 2 minutes	1 cycle
98 °C for 1 s 74 °C for 1 min/kb	5 cycles
98 °C for 1 s 72 °C for 1 min/kb	5 cycles
98 °C for 10 s 70 °C for 1 min/kb	5 cycles
98 °C for 10 s 68 °C for 1 min/kb	15 cycles

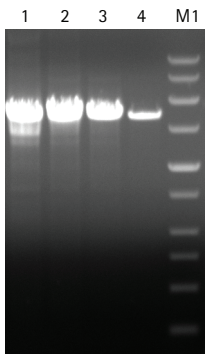
Example Data 1:

Amplification of 6.98 kb GC-rich BCL2-associated Protein X (BAX)

A.

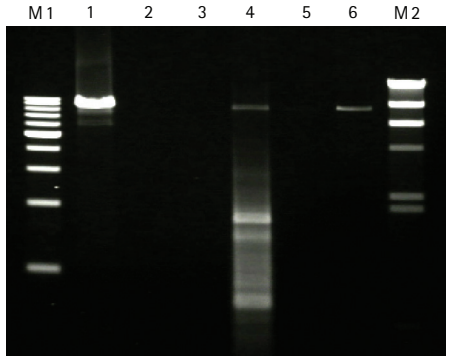


B.



The 6977 bp product is 53% GC overall. The first 529 bp are 71.8% GC and contain a segment that is 84.3% GC. The complete GC-rich region schematics and poly-(dA) stretches are shown (Panel A). Each reaction included Human Genomic DNA (Cat No. 69237). A 5 µL sample of each 50 µL reaction was analyzed (Panel A). Lane M1, Perfect DNA™ Markers, 0.5-12 kb (Cat. No. 69002-3), lanes 1-4 represent 100 ng, 50 ng, 25 ng, and 5 ng template amplification respectively (Panel B). Target identity was verified by sequencing.

Amplification of human IGF2R gene [NM_000876], which contains a 5′ region with ~90% GC content. The reactions contained cDNA derived from 50 ng HeLa cell total RNA. Lane M1, 1 kb DNA ladder; Lane 1, using KOD Xtreme™ Hot Start DNA Polymerase; Lane 2, using Polymerase ET from supplier T; Lane 3, using polymerase LT from supplier T; Lane 4, using polymerase PS from supplier T with GC Buffer; Lane 5, using polymerase LT from supplier T with GC Buffer 1; Lane 6, using polymerase LT from supplier T with GC Buffer 2; Lane M2, HindIII-digested λ DNA markers. Data contributed by Akio Sugiyama, Tsuruga Institute of Biotechnology.



Example Data 2:

Amplification of 8.9 kb GC-rich insulin growth factor 2 receptor target: KOD Xtreme™ Hot Start DNA Polymerase versus competitors' polymerases

Primer sequences used in this experiment:

Forward primer: 5′-TCCCGCTCCGTCTCCACCTCCGC-3′
Reverse primer: 5′-CAGGGCGGTTTGCTTCTCAGCAATAGA-3′

PCR cycling conditions

94 °C 2 min
98 °C 10 s, 68 °C 9 min (30 cycles)

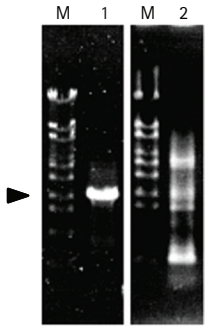
Primer sequences used in this experiment:

T_m = 63.6 °C
Size = 20 mer
Forward primer: 5′-CATTGGAAGAGAGAATGTGAAGCAG-3′
Reverse primer: 5′-TCCACACTCATTGCAGACTCAGGTG-3

PCR cycling conditions

For KOD Xtreme™ Hot Start DNA Polymerase,
98 °C 10 s, 68 °C 2 min (35 cycles)
For polymerase LT from supplier T
94 °C 1 min, 60 °C 30 s, 72 °C 2 min (35 cycles)

Reactions included 100 ng of a mouse genomic BAC as template. Lane M, EcoT141-digested λ DNA markers; Lane 1, using KOD Xtreme™ Hot Start DNA Polymerase; Lane 2, using polymerase LT from supplier T. Data provided by Mayu Yasunaga and Kenzo Sato, Tottori University Faculty of Medicine.



Example Data 3:

Amplification of 2 kb GC-rich mouse bone morphogenetic protein-2 (BMP-2) target

03. KOD Hot Start DNA Polymerase

STEP 1. Standard Reaction Setup:

Component	Volume	Final Concentration
10x Buffer for KOD Hot Start DNA Polymerase	5 µL	1x
25 mM MgSO4[a]	3 µL	1.5 mM
dNTPs (2 mM each)	5 µL	0.2 mM
PCR Grade Water	X µL	
Sense (5') Primer -10 µM (10 pmol/µL)	1.5 µL	0.3 µM
Antisense (3') Primer-10 µM (10 pmol/µL)	1.5 µL	0.3 µM
KOD Hot Start DNA Polymerase (1.0 U/µL)	1.0 µL	0.02 U/µL
Template DNA	X µL	
Total Volume	**50 µL**	

[a] To optimize for targets greater than 2 kb, final Mg^{2+} concentration may be adjusted to between 1.5 and 2.25 mM.

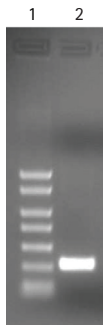
STEP 2. Thermocycling Conditions

	Target Size			
Step	<500 bp	500–1000 bp	1000–3000 bp	>3000 bp
1. Polymerase Activation	95 °C for 2 mins	95 °C for 2 mins	95 °C for 2 mins	95 °C for 2 mins
2. Denaturation	95 °C for 20 s	95 °C for 20 s	95 °C for 20 s	95 °C for 20 s
3. Annealing	[Lowest Primer T_m] °C for 10 s			
4. Extension	70 °C for 10 s/kb	70 °C for 15 s/kb	70 °C for 20 s/kb	70 °C for 25 s/kb
Repeat steps 2-4	20-40 cycles			

s/kb = Seconds per kilobase

Example Data 1:

Amplification of 335 bp fragment (CFTR exon 11) from human genomic DNA

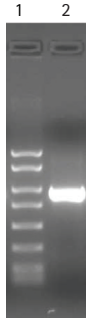


1.2 % TAE agarose gel
Lane 1: PCR Markers, 50–2000 bp (Cat. No. 69278-3)
Lane 2: 5 µL PCR reaction

1 µL of human genomic DNA (100 ng/µL) was added to a standard KOD Hot Start 50 µL PCR reaction. The reaction was incubated as follows: 95 °C for 2 min, then 30 cycles of 95 °C for 20 s, 56 °C for 10 s, and 70 °C for 4 s.

Example Data 2:

Amplification of 919 bp ORF from plasmid DNA



1.4 % TAE agarose gel
ane 1: PCR Markers, 50–2000 bp (Cat. No. 69278-3)
Lane 2: 5 µL PCR Reaction

1 µL of plasmid DNA (10 ng/µL, diluted in TE) was added to a 50 µL standard KOD Hot Start PCR reaction and subjected to thermocycling as follows: 95 °C for 2 min, then 25 cycles of 95 °C for 20 s, 55 °C for 10 s, and 70 °C for 15 s.

Ultimate Convenience: KOD Hot Start Master Mix

Pre-mixed 2x KOD Hot Start PCR components for convenience and reproducibility especially in higher throughput settings.

STEP 1. Standard Reaction Setup

Component	Volume		Final Concentration
PCR Grade Water	X µL	A µL	
Sense (5') Primer (10 µM)	1.5 µL	0.6 µL	0.3 µM
Antisense (3') Primer (10 µM)	1.5 µL	0.6 µL	0.3 µM
Template DNA	Y µL	B µL	
KOD Hot Start Master Mix (0.04 U/µL)	25 µL	10 µL	0.02 U/µL
Total Volume	**50 µL**	**20 µL**	

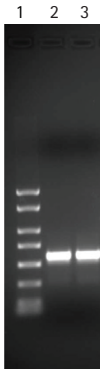
STEP 2. Thermocycling Conditions

	Target Size			
Step	<500 bp	500–1000 bp	1000–3000 bp	>3000 bp
1. Polymerase Activation	95 °C for 2 mins	95 °C for 2 mins	95 °C for 2 mins	95 °C for 2 mins
2. Denaturation	95 °C for 20 s	95 °C for 20 s	95 °C for 20 s	95 °C for 20 s
3. Annealing	[Lowest Primer T_m] °C for 10 s			
4. Extension	70 °C for 10 s/kb	70 °C for 15 s/kb	70 °C for 20 s/kb	70 °C for 25 s/kb
Repeat steps 2-4	20-40 cycles			

s/kb = Seconds per kilobase pair

1.0% TAE agarose gel
Lane 1: PCR Markers, 50–2000 bp (Cat. No. 69278-3)
Lane 2: 5 µL of 50 µL PCR Reaction
Lane 3: 5 µL of 20 µL PCR Reaction

1 µL of lambda DNA (10 ng/µL, diluted in TE) was added to a 50 µL KOD Hot Start Master Mix reaction and subjected to thermocycling as follows: 95 °C for 2 min, then 25 cycles of 95 °C for 20 s and 70 °C for 9 s.



Example Data:

Amplification of 595 bp fragment (att region) from lambda DNA

04. KOD XL DNA Polymerase

This optimized blend of KOD DNA Polymerase and a mutant form of KOD deficient in 3'-5' exonuclease activity offers reliable amplification of long and complex targets including crude samples. While other KOD formulations offer blunt ended PCR products only, KOD XL polymerase-derived amplicons will include a mixture of blunt and 3'-dA ends. Regarded as best in class for incorporation of deriviatized dNTPs.

STEP 1. Standard Reaction Conditions

Component	Volume	Final Concentration
10x buffer for KOD XL DNA Polymerase	5 µL	1x
dNTPs (2 mM each)	5 µL	0.2 mM (each)
PCR Grade Water	X µL	
Sense (5') Primer (5 pmol/µL)	2 µL	0.2-0.4 µM
Antisense (3') Primer (5 pmol/µL)	2 µL	0.2-0.4 µM
Template DNA	Y µL	
KOD XL DNA Polymerase (2.5 U/µL)	1 µL	0.05 U/µL
Total Volume	**50 µL**	

STEP 2. Thermocycling Conditions

	Target Size			
Step	<2kb	2-6kb	6-10kb	10-12kb
1. Denaturation	94 °C for 30 s	94 °C for 30 s	94 °C for 30 s	94 °C for 30 s
2. Annealing	Lowest Primer T_m - 5 °C for 5 s			
3. Extension	70-74 °C for 30-60 s/1kb	70-74 °C for 2-6 min	70-74 °C for 5-6 min	70-74 °C for 8 min
Repeat steps 1-3	25-30 cycles			
Final Extension	74 °C – 10 mins			

05. KOD DNA Polymerase

KOD DNA Polymerase is a recombinant high fidelity *Thermococcus kodakaraensis* KOD1 DNA polymerase (Nishioka 2001). Compared to competitor Taq-based or proof-reading polymerases, KOD offers superior extension speeds (2-5x faster respectively), higher processivity qualities and ultra efficient 3-5 exonuclease proofreading activity. Our standard reaction setup will offer accurate, fast and high yielding DNA amplification.

STEP 1. Standard Reaction Conditions

Component	Volume	Final Concentration
10x buffer #1 for KOD DNA Polymerase	5 µL	1x
dNTPs (2 mM each)	5 µL	0.2 mM (each)
$MgCl_2$ (10 mM)	5 µL	1 mM
PCR Grade Water	X µL	
Sense (5') Primer (5 pmol/µL)	4 µL	0.4 µM
Antisense (3') Primer (5 pmol/µL)	4 µL	0.4 µM
Template DNA	Y µL	
KOD DNA Polymerase (2.5 U/µL)	0.4 µL	0.02 U/µL
Total Volume	**50 µL**	

STEP 2. Thermocycling Conditions

For phage and plasmid DNA templates:

	Target Size			
Step	≤500 bp	1000–3000 bp	3000–5000 bp	5000–6000 bp
1. Denaturation	95 °C for 15 s	95 °C for 15 s	95 °C for 15 s	95 °C for 30 s
2. Annealing	68 °C for 1-30 s	(T_m-5) °C for 2 s	(T_m-5) °C for 5 s	(T_m-5) °C for 30 s
3. Extension	none[a]	72 °C for 20 s	72 °C for 40 s	72 °C for 60 s
Repeat steps 2-3	25	25	25-30	30

For genomic DNA templates: Up to 2 kb

Three Step Cycling	
2. Denaturation	95 °C for 15 s
3. Annealing	(T_m-5) °C for 30 s
4. Extension	72 °C for 30-60 s
Repeat steps 2-4 for 30 cycles	

For cDNA templates: Up to 2 kb

For genomic DNA templates: Up to 2 kb

Three Step Cycling	
2. Denaturation	98 °C for 20 s
3. Annealing	68 °C for 30 s
4. Extension	none[a]
Repeat steps 2-4 for 30 cycles	

[a]Primers used for two-step cycling programs must have an appropriate T_m value to anneal at 68 °C.

06. One Step RT-PCR Master Mix Kit

The One Step RT-PCR Master Mix Kit provides a convenient one-enzyme hot start system for reverse transcription (RT) of RNA to cDNA and PCR in a single tube reaction. *Thermophilus thermophilus (rTth)* DNA polymerase acts as both a thermostable RNA-dependent DNA polymerase and a DNA-dependent DNA polymerase.

Gene expression profiling (i.e. mRNA detection) by RT-PCR has become a key step in many cell culture or *in vivo* animal model experiments. The One Step procedure minimizes the risk of RNA degradation and contamination during the RT-PCR process in a single reaction for convenient setup and fast data turnaround. Use the protocols below to achieve simpler end-point PCR analysis from RNA templates with more comparative, reproducible and accurate gene expression studies. In addition, use this kit in quantitative real-time PCR settings (page 41).

STEP 1. Standard Reaction Setup

Reaction Component	Positive Control Reaction	Negative Control Reaction	Sample Reaction
2x One Step RT-PCR Master Mix	25 µL	25 µL	25 µL
50 mM $Mn(OA)_2$	2.5 µL	2.5 µL	2.5 µL
Antisense Primer (10 pmol/µL)	2 µL	2 µL	1-3 µL
Sense Primer (10 pmol/µL)	2 µL	2 µL	1-3 µL
RNA (1-1000 ng total RNA)	2 µL (*positive control RNA)	None	X µL (sample RNA)
RNAse-free water	16.5 µL	A µL	Y µL
Total Reaction	**50 µL**	**50 µL**	**50 µL**

*The Positive Control RNA is a transcribed product from the human Glyceraldehyde 3-Phosphate Dehydrogenase gene (G3PDH). Using control primers a 450 bp product will be amplified.

STEP 2. Thermocycling Conditions for RT-PCR

Three-step Cycling	
1. Polymerase Activation	90 °C for 30 s
2. Reverse Transcription	60 °C for 30 min
3. Denaturation	94 °C for 1 min
4. Denaturation	94 °C for 30 s
5. Annealing	60 °C for 30 s
6. Extension	72 °C for 1 min
Repeat steps 4-6 for 40 cycles	
7. Final Extension	60 °C for 7 min

Note: Annealing temperature for control primers (Primer F and R) is 60 °C.
For other primers, choose T_m - 5 °C.

STEP 3. Analyze results on 1–2% gel stained with ethidium bromide

(Note: this kit can also be used for real-time quantitative PCR with SYBR® or TaqMan® fluorescent technologies – see pages 41-43.)

07. NovaTaq™ Hot Start DNA Polymerase

This heat-activatable, chemically modified form of recombinant *Taq* DNA polymerase is ideal for routine PCR, offering high PCR specificity as well as yield. The NovaTaq™ Hot Start DNA Polymerase formulation is optimized for low-copy target amplification. Ideal for quantitative and high-throughput PCR applications.

STEP 1. Standard Reaction Setup

Reaction Component	Volume
PCR Grade Water	X µL
dNTP mix (10 mM each)	1 µL
Antisense Primer (5 pmol/µL)	1 µL
Sense Primer (5 pmol/µL)	1 µL
10 x NovaTaq™ Hot Start Buffer	5 µL
$MgCl_2$ (25 mM)	3-5 µL
NovaTaq™ Hot Start DNA Polymerase	0.25 µL (1.25 U)
DNA template (typically 10 ng)	1 µL
Total Reaction	**50 µL**

STEP 2. Thermocycling Conditions for RT-PCR

Three-step Cycling	
1. Polymerase Activation	95 °C for 7- 10 min
2. Denaturation	94 °C for 30 s
3. Annealing	(T_m – 5) °C for 30 s
4. Extension	72 °C for 1 min
Repeat steps 2-4 for 30 cycles	
5. Final Extension	60 °C for 10 min

Note: NovaTaq™ Hot Start DNA Polymerase may be used with most PCR cycling programs following polymerase activation step.

08. NovaTaq™ Hot Start Master Mix

NovaTaq™ Hot Start Master Mix provides a ready-to-use 2x mixture of NovaTaq™ Hot Start DNA Polymerase, ultrapure deoxynucleotides, and reaction buffer with $MgCl_2$. The Master Mix simplifies the setup for PCR, resulting in time savings, consistency, and minimal risk of contamination.

STEP 1. Reaction Setup

Reaction Component	Volume
NovaTaq™ Hot Start Master Mix	25 µL
Antisense Primer (5 pmol/µL)	1 µL
Sense Primer (5 pmol/µL)	1 µL
DNA template (typically 10 ng)	1 µL
PCR Grade Water	Y µL
Total Reaction	**50 µL**

Note: The above reaction will contain 1.5 mM final $MgCl_2$.
For some applications increased $MgCl_2$ concentrations may be optimal.

STEP 2. Thermocycling Conditions

Three-step Cycling	
1. Polymerase Activation	95 °C for 7- 10 min
2. Denaturation	94 °C for 30 s
3. Annealing	(T_m – 5) °C for 30 s
4. Extension	72 °C for 1 min
Repeat steps 2-4 for 30 cycles	
5. Final Extension	60 °C for 10 min

Note: NovaTaq™ Hot Start DNA Master mix is a robust system applicable with most PCR cycling programs following polymerase activation step.

STEP 1A. Additional $MgCl_2$ Optimization

Final $MgCl_2$ Concentration	Volume 25 mM $MgCl_2$ to add per 50 µL reaction
1.5 mM	0 µL
2.0 mM	1 µL
2.5 mM	2 µL

09. NovaTaq™ DNA Polymerases

NovaTaq™ DNA Polymerase is a premium quality recombinant form of *Thermus aquaticus* DNA polymerase. This thermostable enzyme is suitable for a wide range of routine and sensitive PCR applications.

STEP 1. Reaction Setup

Reaction Component	Volume
PCR Grade Water	40.75 µL
dNTP mix (10 mM each)	1 µL
Antisense Primer (5 pmol/µL)	1 µL
Sense Primer (5 pmol/µL)	1 µL
10 x NovaTaq™ Buffer (with $MgCl_2$)	5 µL
NovaTaq™ DNA Polymerase	0.25 µL (1.25 U)
DNA template (typically 10 ng)	1 µL
Total Reaction	**50 µL**

Note: If using NovaTaq™ Buffer without $MgCl_2$ add 25 mM $MgCl_2$ to a final concentration of 1.5-2.5 mM and decrease volume of water to compensate

STEP 2. Thermocycling Conditions

Three-step Cycling	
1. Denaturation	94 °C for 1 min
2. Annealing	(T_m – 5) °C for 1 min
3. Extension	72 °C for 2 min
Repeat steps 1-3 for 30 cycles	
4. Final Extension	72 °C for 5 min

10. FAQs

What is the difference between NovaTaq™ and KOD polymerases?

NovaTaq™ DNA Polymerase is a premium quality recombinant form of *Thermus aquaticus* DNA polymerase. The enzyme possesses 5′ to 3′ DNA polymerase activity and lacks 3′ to 5′ exonuclease activity (proofreading). The preparation is >95% pure and lacks RNase and endonuclease activities. NovaTaq™ DNA Polymerase generates PCR products with 3′- dA overhangs.

KOD is a recombinant form of *Thermococcus kodakaraensis* KOD1 DNA Polymerase. The enzyme's 3′ to 5′ exonuclease-dependent proofreading activity results in an extremely low mutation frequency. The extension speed of KOD is 2x faster than Taq, enabling shorter reaction times. KOD DNA polymerase produces blunt-ended DNA products.

Why should I use KOD DNA polymerases?

KOD DNA Polymerase is an ultra high fidelity thermostable DNA polymerase and a number of independent studies have verified the the extreme high fidelity of KOD DNA Polymerase compared to other thermophilic polymerases (Takagi 1997, Nishioka 2001, Rual 2004, Wu 2006). In addition to a low mutation frequency, the fast extension rate and high processivity of KOD result in higher yields of full-length product in fewer reaction cycles. Combined, these make KOD DNA polymerases the PCR enzyme of choice when speed and fidelity matter.

Nishioka, M. et al. 2001. *J. Biotech.* 88, 141.
Rual, J-F. et al. 2004. *Genome Res.* 14, 2128.
Takagi, M. et al. 997 *Appl. Environ. Microbiol.* 63, 4509.
Wu, G. et al. 2006. *J. Biotechnol.* 124, 496.

Are Merck Millipore's Novagen® polymerases "proofreading"? What type of proofreading mechanism do NovaTaq™ and KOD polymerases use?

KOD, KOD Hot Start, and KOD Xtreme™ polymerases all possess 3′ to 5′ exonuclease proofreading activity, making them ideal for applications where fidelity is essential, such as PCR from reverse transcription reactions and cloning. KOD XL polymerase is a mixture of traditional KOD and its exonuclease-deficient mutant. NovaTaq™ polymerase does not have proofreading activity.

A. KOD – 3′ to 5′ exonuclease proofreading activity
B. KOD Hot Start – 3′ to 5′ exonuclease proofreading activity
C. KOD XL – mixture of KOD (with exonuclease proofreading activity) and its exonuclease-deficient mutant.
D. KOD Xtreme™ – 3′ to 5′ exonuclease proofreading activity
E. NovaTaq™ – lacks 3′ to 5′ exonuclease proofreading activity
F. NovaTaq™ Hot Start – lacks 3′ to 5′ exonuclease proofreading activity

What types of ends do the various Merck Millipore Novagen® polymerases leave on PCR amplification products?

Generally, proofreading polymerases that possess 3′ to 5′ exonuclease activity will remove the 3′-dA overhangs whereas non-proofreading polymerases will not.

A. KOD – blunt ends
B. KOD Hot Start – blunt ends
C. KOD XL – mixture of blunt ends and 3′-dA overhangs
D. KOD Xtreme™ – blunt ends
E. NovaTaq™ – 3′-dA overhangs
F. NovaTaq™ Hot Start – 3′-dA overhangs

What does "hot start" mean and what are the advantages?

While it is most convenient to set up PCR reactions at ambient temperature, this can lead to mispriming events that result in non-specific amplification products. In addition, the exonuclease activity possessed by proofreading enzymes, such as KOD, can lead to primer degradation. "Hot start" means that the polymerase is not active until cycling temperatures are increased to activate the polymerase. This eliminates the mispriming and primer degradation concerns described above, resulting in greater specificity and increased target yield. In addition, "hot start" enzymes offer convenience of the room temperature reaction setup.

How does the hot start work with Merck Millipore's Novagen® polymerases?

KOD Hot Start polymerase is a premixed complex of KOD DNA Polymerase and two monoclonal antibodies. The antibodies inhibit the 3´ to 5´ exonuclease and DNA polymerase activities at ambient temperatures, providing high template specificity by preventing primer degradation and mispriming during reaction setup.

NovaTaq™ Hot Start Polymerase is a chemically modified form of Taq DNA Polymerase that is inactive at room temperature. The enzyme must be activated by heat treatment (10 minutes at 95°C), after which thermocycling can proceed.

What is the difference between KOD XL and KOD Xtreme™ Hot Start DNA Polymerases?

KOD XL polymerase is designed for accurate and rapid amplification of complex, GC-rich, and long (up to 30 kb) target DNA. KOD XL polymerase is an optimized mixture of KOD DNA Polymerase and a mutant form of KOD that is deficient in 3´ to 5´ exonuclease activity, resulting in increased efficiency and better yield of long targets. KOD XL polymerase is the only variation of KOD polymerase known to work in reactions for incorporation of modified dNTPs.

KOD Xtreme™ Hot Start polymerase is an optimized PCR system for the amplification of long or GC-rich DNA templates. The system includes an ultra high fidelity KOD DNA Polymerase complexed with two monoclonal antibodies to permit hot start thermocycling, along with specially formulated 2X Xtreme™ buffer. KOD Xtreme™ polymerase quickly and accurately amplifies genomic and phage/plasmid DNA targets up to 24 and 40 kbp, respectively. KOD Xtreme™ polymerase successfully amplifies challenging DNA templates with up to 90% GC content. Relative fidelity is higher than that of KOD XL polymerase.

Can I use KOD DNA polymerases for site-directed mutagenesis?

KOD DNA polymerases are an ideal choice for site-directed mutagenesis protocols. These polymerases possess ultrahigh fidelity, providing a reduced chance of unintentional changes. This high fidelity combined with high processivity and fast extension rate, allow for efficient amplification of plasmids over 10 kb with only the desired mutation. Below are selected citations of KOD Hot Start polymerase used for site-directed mutagenesis.

Deigendesch, N. et al. 2006 *Nucleic Acids Res.* 34, 5007
Konno, A. et al. 2007 *Mol. Biol. Evol.* 24, 2504.
Liang, J. et al. 2007 *Nucleic Acids Res.* 35, 2944.

Are there any special recommendations for using KOD with GC-rich targets?

KOD Xtreme™ Hot Start DNA Polymerases has been specifically formulated for difficult targets, including GC-rich targets. Additions, such as DMSO or other additives, are generally not needed. With other KOD polymerases, the addition of DMSO to 2-10% final concentration may decrease template secondary structure and increase yield. Final DMSO concentrations of less than 5% v/v have no effect on fidelity. The effect of DMSO above 5% v/v on enzyme fidelity has not yet been determined.

Are there any special recommendations for using KOD with long targets?

In addition to trying one of our polymerases specialized for long targets (KOD XL or KOD Xtreme™ polymerases), it is often beneficial to adjust the final Mg^{2+} concentration. Adjusting the final $MgSO_4$ concentration from 1.5 to 2.25 mM in 0.25 mM increments should be tried when suboptimal results are obtained for targets over 3000 bp. Also, the addition of DMSO to 2-10% v/v final concentration may reduce secondary structure of the template DNA and increase yield. The 2X Xtreme™ Buffer supplied with KOD Xtreme™ polymerase has been optimized for long targets. The addition of DMSO or other additives is generally not needed when using KOD Xtreme™ polymerase.

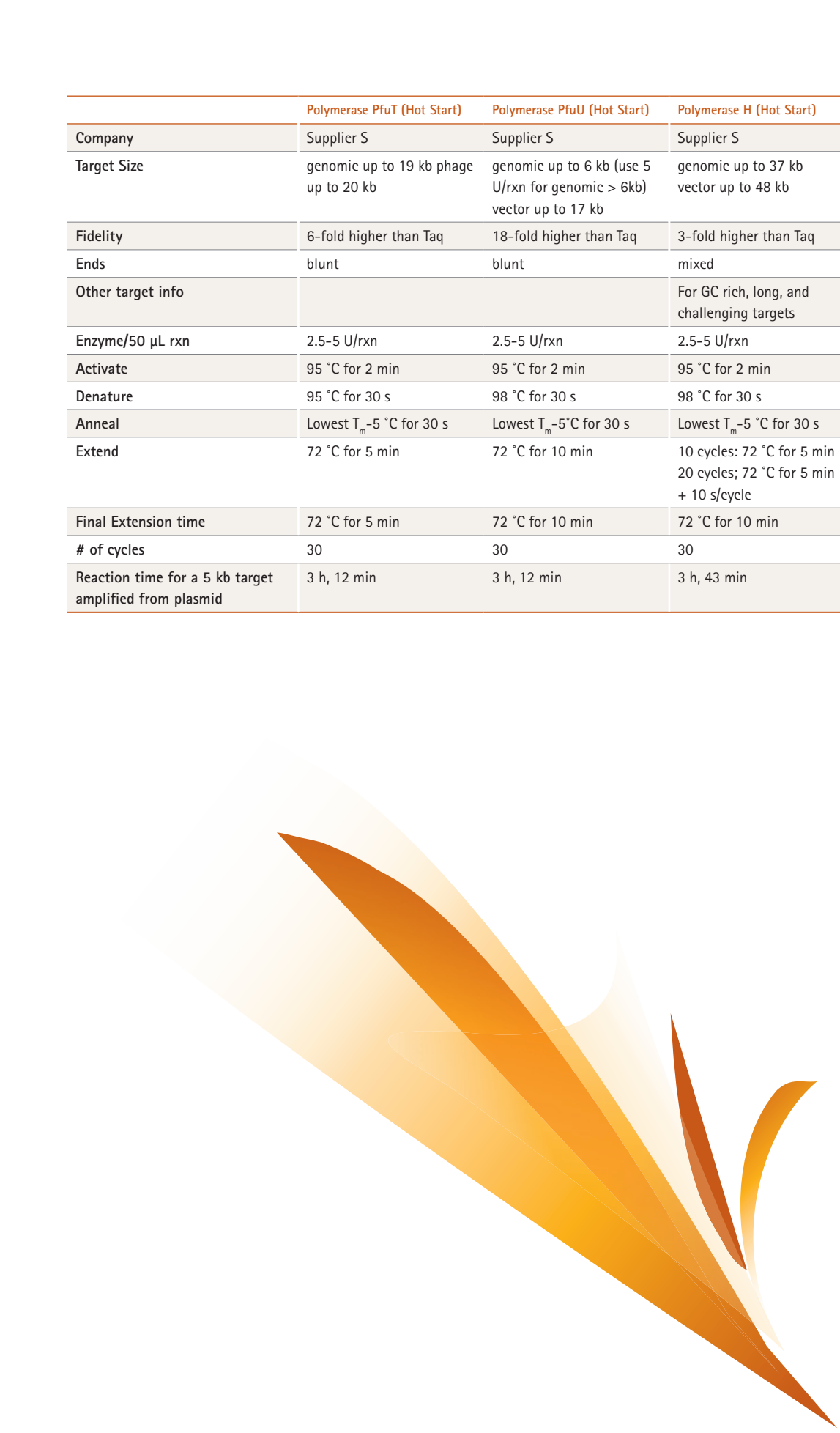
I see smearing of my PCR products after thermocycling when using KOD. What is causing this and how can I get rid of it?

Because KOD has higher processivity and faster reaction times, it is important to adjust extension times to 10-25 s/kb. Smearing can be caused by too long or short extension times. If the smear is above the target size, extension time can be reduced by 5 s/kb and/or the Mg^{2+} concentration can be reduced in 0.25 mM increments. If the smearing is below the target size, we would recommend increasing the extension time by 5 s/kb and/or increasing the Mg^{2+} concentration in 0.25 mM increments. Smearing can also sometimes be reduced by reducing the amount of template DNA added to the reaction.

If not using a hot start enzyme, smearing can be caused by exonuclease activity during reaction setup. Reactions should be set up on ice to avoid degradation.

	KOD Hot Start	KOD Xtreme™ Hot Start	Polymerase Ph (Hot Start)	Polymerase PfuF (Hot Start)
Company	Merck Millipore	Merck Millipore	Supplier N	Supplier S
Target Size	genomic up to 12 kb phage/plasmid up to 21 kb	genomic up to 24 kb phage/plasmid up to 40 kb	genomic up to 7.5 kb	genomic up to 19 kb (use 5U/rxn for genomic > 6 kb) vector up to 20 kb
Fidelity	50-fold higher than Taq	11-fold higher than Taq	52-fold higher than Taq	20-fold higher than Taq
Ends	blunt	blunt	blunt	blunt
Other target info		For targets up to 90% GC		
Enzyme/50 µL rxn	1 U/rxn	1 U/rxn	1 U/rxn	1 U/rxn
Activate	95˚C for 2 min	94 ˚C for 2 min	98˚C for 30 s	95 ˚C for 2 min
Denature	95˚C for 20 s	98 ˚C for 10 s	98˚C for 30 s	95 ˚C for 20 s
Anneal	Lowest T_m for 10 s	Lowest T_m for 30 s	T_m + 3 ˚C for 30 s	Lowest T_m-5 ˚C for 20 s
Extend	70˚C for 2 min, 5 sec	68 ˚C for 5 min	72 ˚C for 1 min, 15 s	72 ˚C for 1 min, 15 s
Final Extension time	n/a	n/a	72 ˚C for 10 min	72 ˚C for 3 min
# of cycles	25	30	30	30
Reaction time for a 5 kb target amplified from plasmid	1 h, 10 min	2 h, 51 min	1 h, 8 min	1 h, 3 min

	Polymerase PfuT (Hot Start)	Polymerase PfuU (Hot Start)	Polymerase H (Hot Start)	Polymerase PX
Company	Supplier S	Supplier S	Supplier S	Supplier I
Target Size	genomic up to 19 kb phage up to 20 kb	genomic up to 6 kb (use 5 U/rxn for genomic > 6kb) vector up to 17 kb	genomic up to 37 kb vector up to 48 kb	up to 12 kb
Fidelity	6-fold higher than Taq	18-fold higher than Taq	3-fold higher than Taq	26-fold higher than Taq
Ends	blunt	blunt	mixed	blunt
Other target info			For GC rich, long, and challenging targets	
Enzyme/50 µL rxn	2.5-5 U/rxn	2.5-5 U/rxn	2.5-5 U/rxn	1-2.5 U/rxn
Activate	95 ˚C for 2 min	95 ˚C for 2 min	95 ˚C for 2 min	94 ˚C for 2 min
Denature	95 ˚C for 30 s	98 ˚C for 30 s	98 ˚C for 30 s	94 ˚C for 15 s
Anneal	Lowest T_m-5 ˚C for 30 s	Lowest T_m-5˚C for 30 s	Lowest T_m-5 ˚C for 30 s	55 ˚C for 30 s
Extend	72 ˚C for 5 min	72 ˚C for 10 min	10 cycles: 72 ˚C for 5 min 20 cycles; 72 ˚C for 5 min + 10 s/cycle	68 ˚C for 5 min
Final Extension time	72 ˚C for 5 min	72 ˚C for 10 min	72 ˚C for 10 min	n/a
# of cycles	30	30	30	30
Reaction time for a 5 kb target amplified from plasmid	3 h, 12 min	3 h, 12 min	3 h, 43 min	2 h, 53 min



11. PCR and Product-Specific Technical Notes

1. Comparing the Speed and Product Yield of 7 High Fidelity DNA Polymerases

Keith Yaeger and Keith Fourrier - EMD Millipore Corporation

Introduction

Thermococcus kodakaraensis (strain KOD1), previously thought to be a *Pyrococcus* sp., is a hyperthermophilic archaea isolated from a solfataric hot spring on Kodakara Island, Japan (Atomi 2004). In preliminary studies to characterize the KOD1 DNA polymerase, researchers found that the enzyme had fidelity comparable to *Pfu* DNA polymerase, but with an extension rate (referred to as speed) 5 times higher and processivity 10 to 15 times higher than the *Pfu* enzyme (Takagi 1997).

When amplifying DNA for cloning, high fidelity DNA polymerases, such as KOD polymerase, are recommended. If the enzyme is also fast and can generate high yields of full-length product, fewer amplification cycles are required and the probability of obtaining error-free clones is greatly increased. Since the preliminary KOD DNA Polymerase studies, significant work has been done to optimize the PCR buffer and cycling parameters. Another improvement is KOD Hot Start DNA Polymerase, which is a premixed complex of KOD DNA Polymerase and two monoclonal antibodies. The antibodies inhibit the 3′→5′ exonuclease and DNA polymerase activities at ambient temperatures (Mizuguchi 1999), providing high template specificity by preventing primer degradation and mispriming events during reaction setup. This report evaluates the speed and product yield of KOD Hot Start DNA Polymerase in an optimized reaction buffer and compares the enzyme to 6 other commercially available high fidelity thermophilic DNA polymerases.

Materials and Methods

Thermocycler

DNA amplification was performed on MJ Research PTC-200 thermocyclers that had recently been calibrated by the manufacturer. Reactions were going to be done in tube strips, always using the same polymerase in the same location on each strip. To ensure no bias due to the location of the reaction in the strip or in the thermocycler, test reactions were performed using KOD Hot Start DNA Polymerase, and reaction yields measured. Yields were found to be comparable for all tube locations and wells tested (data not shown).

Reaction volume

Reaction volumes of 50, 25, 20, and 10 µL were tested to determine an optimum volume for consistent PCR results (data not shown). Both 50- and 25-µL reactions gave consistent results, and the 25-µL volume was used for the remaining experiments.

Template

A 919-bp fragment of human GSK3α (glycogen synthase kinase 3α) catalytic domain ORF, 54% GC-content, in an Open Biosystems cDNA plasmid (MHS1010-7507851, GeneBank BC027984) was selected as the DNA template.

Primers/reagents

Primers used were HPLC purified:

(35-mer sense) 5′-GACGACGACAAGA**TTTCCCAAGAAGTGGCTTACAC**-3′ and

(41-mer antisense) 5′-GAGGAGAAGCCCGGTCTTA**ACATCGCAGTTCATCAAAGAAG**-3′.

Bases shown in bold are homologous to the ORF. Table 1 shows the ion components used for each polymerase.

Table 1.

Reaction components and cycling profile for each DNA polymerase based on manufacturers' recommendations

	Cycling Profile 1	Cycling Profile 2	Cycling Profile 3	Cycling Profile 4
Initial Denaturation	98 °C 30 s	94 °C 2 min	95 °C 2 min	95 °C 2 min
29 cycles	98 °C 10 s 55 °C 20 s 72 °C 30 s	94 °C 15 s 52 °C 20 s 68 °C 60 s	95 °C 20 s 55 °C 20 s 72 °C 30 s	95 °C 20 s 55 °C 10 s 70 °C 15 s
Final Extension	72 °C 5 min	68 °C 5 min	72 °C 3 min	N/A

DNA Polymerase	Buffer	[Mg^{2+}] in reaction (mM) added in 1X buffer		[dNTP] (mM each)	[Primer] (mM each)	Template/ 25 µL reaction (ng)	Polymerase*/ 25 µL reaction	Cycling profile
KOD Hot Start	1x	1.5		0.2	0.3	5	0.5 U	4
KOD	1x	1.5		0.2	0.3	5	0.5 U	1
***Pyrococcus* Polymerase Hot Start (Supplier I)**	1x	1.0		0.3	0.3	5	0.5 U	2
***T. zilligii* Polymerase (Supplier I)**	1x	in buffer	1.2	0.3	0.3	5	2.5 U	2
Hot Start Polymerase Ph (Supplier N)	1x	in buffer	1.5	0.2	0.5	5	0.5 U	1
***Pfu* Hot Start Polymerase (Supplier A)**	1x	in buffer	2.0	0.25	0.2	5	.5 µL (U not given)	3
Hot Start Polymerase PS (Supplier T)	1x	in buffer	1.0	0.2	0.3	5	0.625 U	3

*Manufacturer defined units, used as recommended by manufacturer.

Cycling profiles

All 7 enzymes were tested in 4 different cycling protocols, which encompass the manufacturers' recommended cycling conditions (Table 1).

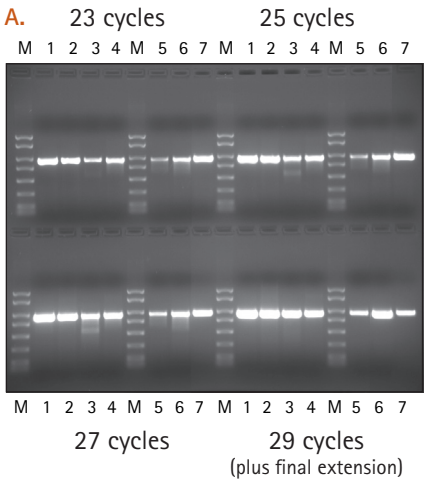
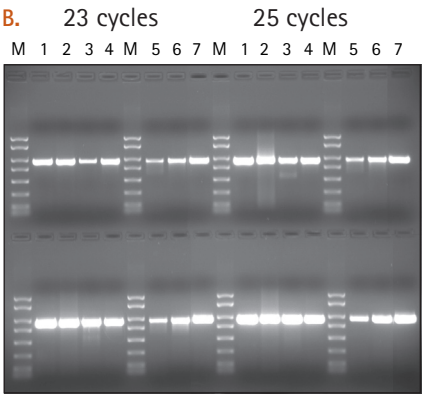
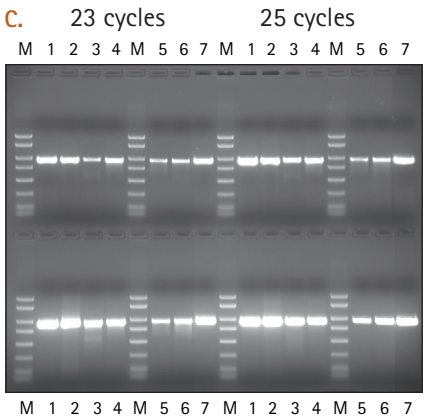


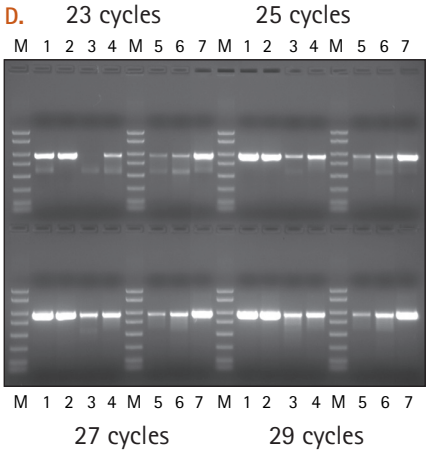
Figure 1.

PCR results from 7 high fidelity thermophilic DNA polymerases using 4 different cycling profiles.

PCR samples were removed after 23, 25, 27, and 29 cycles and 5 mL were assayed on 1.4% agarose/TAE gels. Lanes indicate the enzyme used for the reaction. Cycling profiles are defined in Table 2. (A) cycling profile 1 (Note: PicoGreen results indicate a yield increase with Hot Start Polymerase PS (Supplier T) from cycle 27 to cycle 29; the reduced band intensity here is a gel loading artifact), (B) cycling profile 2, (C) cycling profile 3, (D) cycling profile 4.







Lane	DNA Polymerase
1	KOD Hot Start
2	KOD
3	*Pyrococcus* Polymerase Hot Start (Supplier I)
4	*T. zilligii* Polymerase (Supplier I)
5	Hot Start Polymerase Ph (Supplier N)
6	*Pfu* Hot Start Polymerase (Supplier A)
7	Hot Start Polymerase PS (Supplier T)

Results and Discussion

Different Cycling Profiles

Multiple reaction strips were prepared using master mixes for each polymerase. After 19, 21, 23, 25, 27, and 29 cycles, reaction strips were removed and placed on ice. Samples (5 mL) from cycles 23, 25, 27, and 29 were assayed on 1.4% agarose/TAE gels containing ethidium bromide (Figure 1). Yield concentrations were determined on diluted samples from 19, 21, 23, 25, 27, and 29 cycles using a Quant-iT™ PicoGreen® ds DNA Assay Kit (Invitrogen) and a FLUOstar plate reader (BMG LABTECH). New primers and enzyme kits were obtained and all experiments were repeated to verify initial results and trends. Results with the new reagents were comparable to the initial experiments (data not shown).

Yields generated by each enzyme at 19, 21, 23, 25, 27, and 29 cycles were plotted for each protocol (Figure 2). Not all enzymes gave their best yield using the manufacturer's recommended cycling conditions, so the best yields for each enzyme, from any cycling protocol, were compared (Figure 2). For cloning purposes, fewer reaction cycles increase the potential for error-free clones. Cycles 19-25 have been shaded green on the graph to emphasize the reaction yields from these earlier cycles. KOD Hot Start DNA Polymerase consistently gave high yields in cycles 19-25 for all 4 profiles tested (data not shown).

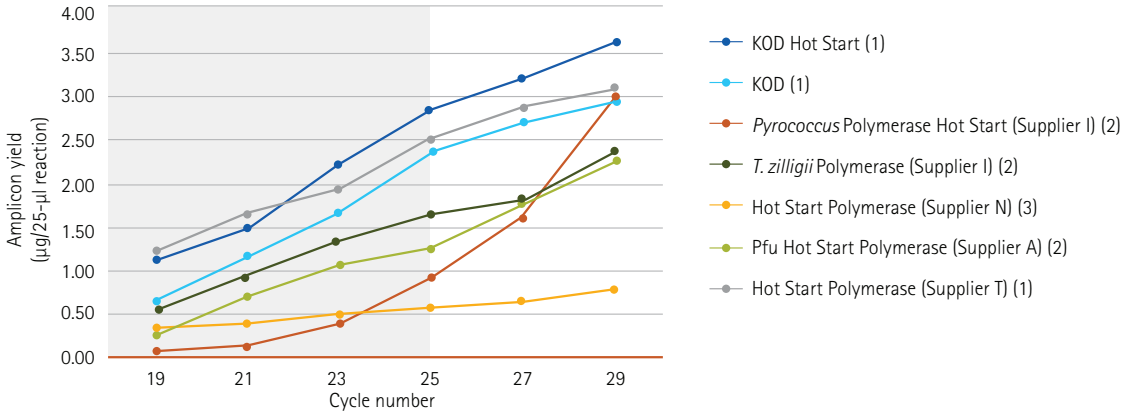


Figure 2.

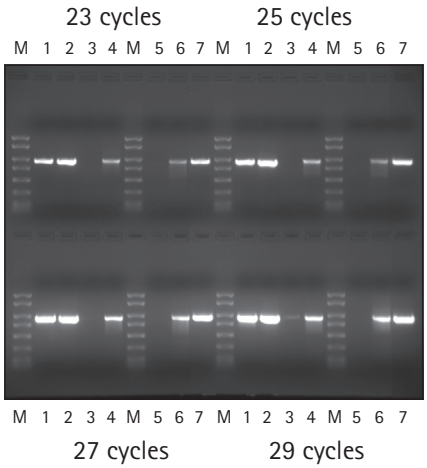
Best yield for each high fidelity thermophilic enzyme from any cycling profile

Yields were determined by PicoGreen® analysis after 19,21, 23, 25, 27, and 29 cycles for all 4 cycling profiles (Table 2). The best yield data for each enzyme, from any cycling profile, was graphed. The cycling profile that gave the best yields is identified in parentheses. The green shaded area highlights yields in cycles 19–25, which would be preferable for cloning.

Two-step PCR

Different Cycling Profiles

Longer primers (generally ≥23 bases) can increase PCR specificity and, due to higher annealing temperatures, can be used in time-saving two-step cycling profiles. KOD Hot Start DNA Polymerase and the other 6 high fidelity enzymes were tested in a two-step protocol (initial denaturation at 95 °C for 2 min, and 29 cycles of 95 °C for 20 s, 68 °C for 40 s). Figure 3 shows that not all enzymes functioned well with this two-step protocol (lanes with little or no product). Other enzymes, including the two KOD enzymes, generated high yields comparable to the three-step protocols shown in Figure 1.



Lane	DNA Polymerase
1	KOD Hot Start
2	KOD
3	*Pyrococcus* Polymerase Hot Start (Supplier I)
4	*T. zilligii* Polymerase (Supplier I)
5	Hot Start Polymerase Ph Supplier N
6	*Pfu* Hot Start Polymerase (Supplier A)
7	Hot Start Polymerase PS (Supplier T)

Figure 3.

PCR results from 7 high fidelity enzymes using a two-step cycling profile.

PCR samples were removed after 23, 25, 27 and 29 cycles of a two-step protocol and 5 mL were assayed on 1.4% agarose/TAE gels. Lanes indicate the enzyme used for the reaction.

KOD Application – Screening Plaques

Testing the ability of KOD Hot Start DNA Polymerase to amplify a variety of DNA templates, the enzyme was used in a two-step cycling profile to screen random clones from the T7Select® Human Brain cDNA Library (Merck Millipore Cat. No. 70637). Plaques were eluted in 100 µL TE (10 mM Tris-HCl, 0.1 mM EDTA) and 5 µL eluate was used for PCR. Primers were: sense primer 5´-ACT TCC AAG CGG ACC AGA TTA TCG C-3´ and antisense primer 5´-AAC CCC TCA AGA CCC GTT TAG AGG-3´. Reactions were cycled with an initial denaturation at 95 °C for 2 min, and 25 cycles of 95 °C for 20 s, 68 °C for 25 s. Of the 50 clones screened, KOD successfully amplified 49 inserts (Figure 4). Amplicons ranged in size from ~250-1800 bp.

KOD DNA polymerase (pol) fidelity in PCR has been assayed by different methods. Initial studies by Takagi et al. (1997) measured the mutation frequency in amplicons after 30 PCR cycles using a plasmid template containing the lacZ gene.

By comparing the number of white and light blue colonies (mutant) to the total number of colonies (including blue, intact lacZ colonies), they determined mutation rates of 2.8% for KOD DNA pol, 3.6% for *Pfu* DNA pol, and 48.0% for *Taq* DNA pol.

Using the same blue/white assay method, but with 25 cycles, Nishioka et al. (2001) found mutation frequencies of 0.79% for KOD DNA pol and 28.1% for *Taq* DNA pol. Rual et al. (2004) directly sequenced ~70,000 bases and determined a misincorporation rate of 1 in 35,000 nucleotides for KOD DNA pol compared to 1 in 2,000 nucleotides for Platinum® *Taq* DNA Polymerase High Fidelity in amplicons generated after 20 cycles of PCR. Discrepancies in mutation rates can be due to the different assay methods, as well as to thermal degradation of DNA at high temperatures, which is not related to enzyme function (Tindall 1988, Pienaar 2006). What stands out in these independent assays is the consistent high fidelity of KOD DNA pol.

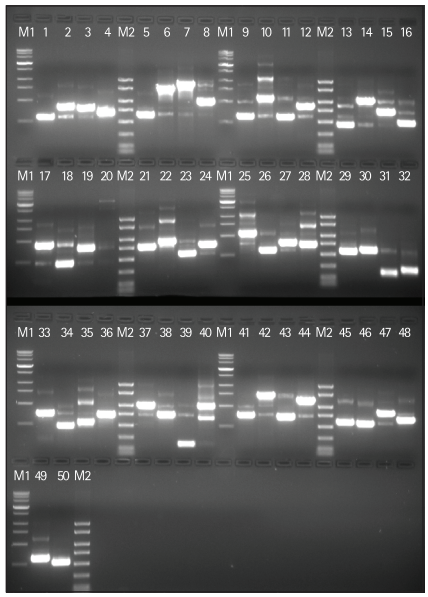


Figure 4.

Results from KOD Hot Start amplification of T7Select® Human cDNA Library clones

PCR was performed as stated in the text; 5 µL of each 25-µL reaction were assayed on a 1.4% agarose/TAE gel. KOD Hot Start successfully amplified 49 of 50 clones with amplicons ranging from ~250–1800 bp.

Lane	Sample
M1	Perfect DNA™ Markers, 0.5-12 kbp
M2	PCR Markers

Conclusion

A number of independent studies have verified the extremely high fidelity of KOD DNA Polymerase. In addition to a low mutation frequency, the fast extension rate and high processivity of the KOD enzyme result in high yields of full-length product in fewer reaction cycles. Combined, these attributes have made KOD Hot Start DNA Polymerase the PCR enzyme of choice for many routine and high throughput cloning and structural proteomics studies.

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2. Reliable and Robust Quantitative (Real-Time) PCR Amplification Using NovaTaq™ Hot Start Master Mix

Hope Schultz and Keith Yaeger – EMD Millipore Corporation

Introduction

Quantitative PCR (real-time PCR or qPCR) methods are used to determine the level of specific mRNA or DNA sequences. These methods may be used to analyze gene expression, genotyping studies, pathogen detection and more. We have demonstrated that NovaTaq™ Hot Start Master Mix can be used in real-time PCR amplification of various templates to obtain reproducible results and robust amplification of low copy number genes. In this article, a comparison of competitors' PCR mixes indicates NovaTaq™ Hot Start Master Mix provides superior amplification and specificity.

Real-time PCR Amplification from cDNA with NovaTaq™ Hot Start Master Mix

Real-Time PCR was performed using a linearized plasmid containing the glycogen synthase kinase 3 α (GSK3α) cDNA. The stock DNA at 5 × 10^8 copies/µl was 10-fold serially diluted in TE and reactions were run in triplicate. The following parameters were used on the Chromo4 Real-Time PCR Detection System (Bio-Rad): initial denaturation at 95 °C for 10 minutes; followed by 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 10 seconds for 45 cycles. The primer/probe mix was designed to target a 112 bp amplicon of GSK3α. DNA was detected using a HEX-labeled probe.

Figure 1A shows the qPCR amplification curves showing the reproducibility of the amplification using NovaTaq™ Hot Start Master Mix Kit over a range of template concentrations. In figure 1B, the cycle threshold (Ct) is plotted against DNA concentration. This yielded a standard curve with a correlation of 1, showing that NovaTaq™ Hot Start Master Mix gave linear results for 10 to 10^9 copies of the plasmid.

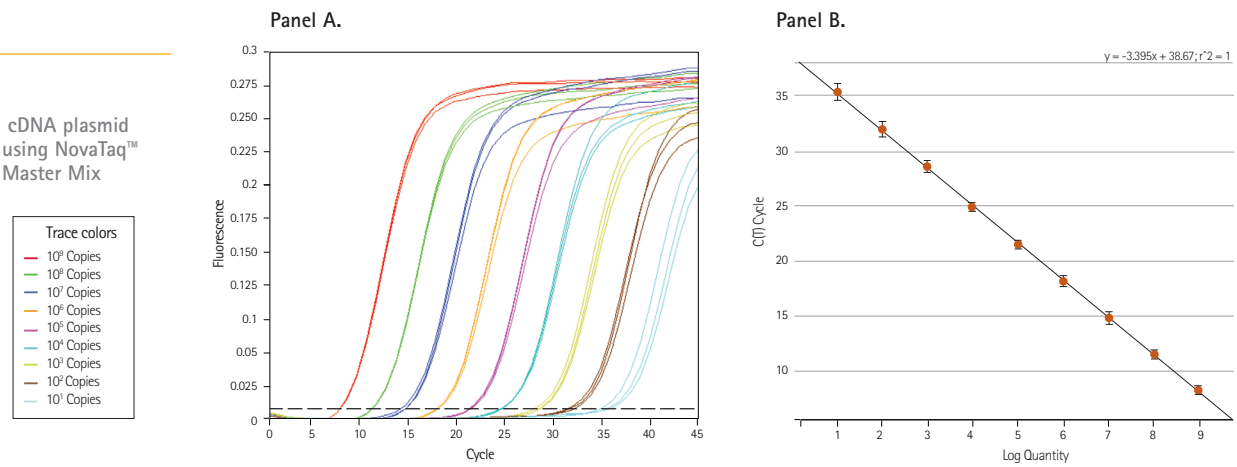


Figure 1.
Linearized cDNA plasmid amplified using NovaTaq™ Hot Start Master Mix

Quantitative Real-Time PCR Amplification from Human Genomic DNA with NovaTaq™ Hot Start Master Mix

Human genomic DNA (Merck Millipore Cat No. 69237) was serially diluted 10-fold in TE (10 mM Tris, pH 8 from 50 ng/µL. The primer/probe mix was designed to target a 139 bp amplicon of GSK3α. The cycling profile using the Bio-Rad Chromo4 Real-Time PCR Detection System was: initial denaturation at 95 °C for 10 minutes; followed by 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 10 seconds for 45 cycles. DNA was detected using a HEX-labeled probe. All reactions were performed in triplicate. Amplification was detected from as little as 3 copies per reaction as indicated by the amplification curves (Figure 2A).

Plotting Ct vs. DNA concentration (Figure 2B) yielded linear results for 0.01 to 100 ng genomic DNA. Note that 0.001 ng would correlate to about 0.3 copies of the target sequence and one of the three experiments performed in triplicate gave a signal while the other two experiments did not yield any signal.

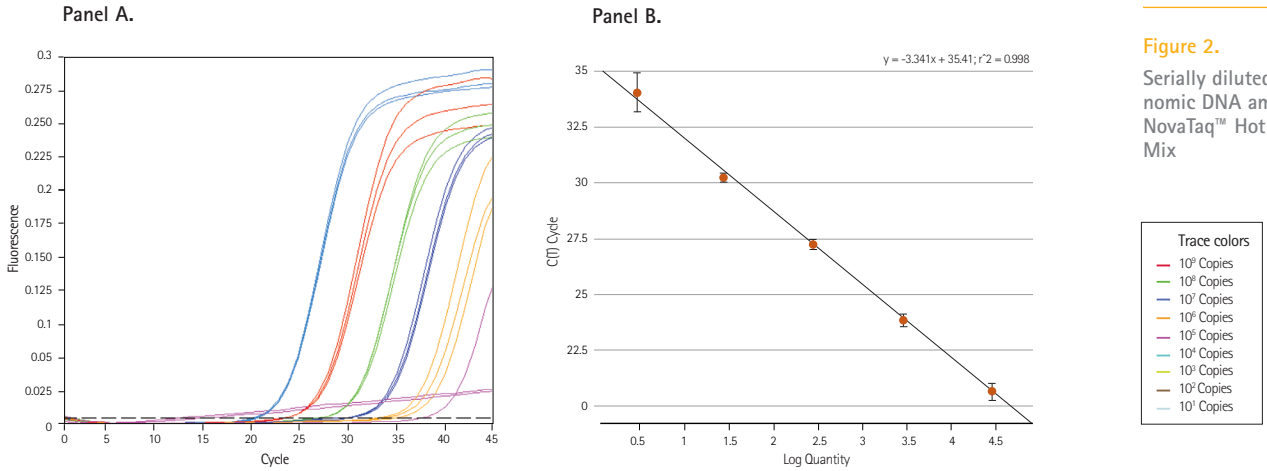


Figure 2.
Serially diluted human genomic DNA amplified using NovaTaq™ Hot Start Master Mix

Performance of NovaTaq™ Hot Start Master Mix in quantitative PCR versus PCR mixes from other suppliers.
Total RNA was isolated from mouse 3T3 cells and cDNA was synthesized using MMLV reverse transcriptase. A 122 bp region of the mouse β-actin coding sequence was amplified from 15 ng/µL of cDNA using NovaTaq™ Hot Start DNA Polymerase or qPCR mixes from the indicated manufacturer. DNA was detected using SYBR® Green dye. Each reaction was performed in triplicate. The following parameters were used with a Corbett Rotor-gene® 6000: initial denaturation at 95 °C for 10 min; followed by 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 20 s for 40 cycles. The amplification curves are shown on the right. The melting analysis (curves shown on the left) was done with a temperature range of 60 °C to 99 °C, acquiring data at each degree. A single peak in the melting curve demonstrates specificity of the reaction. Multiple peaks indicate that secondary products were also amplified.

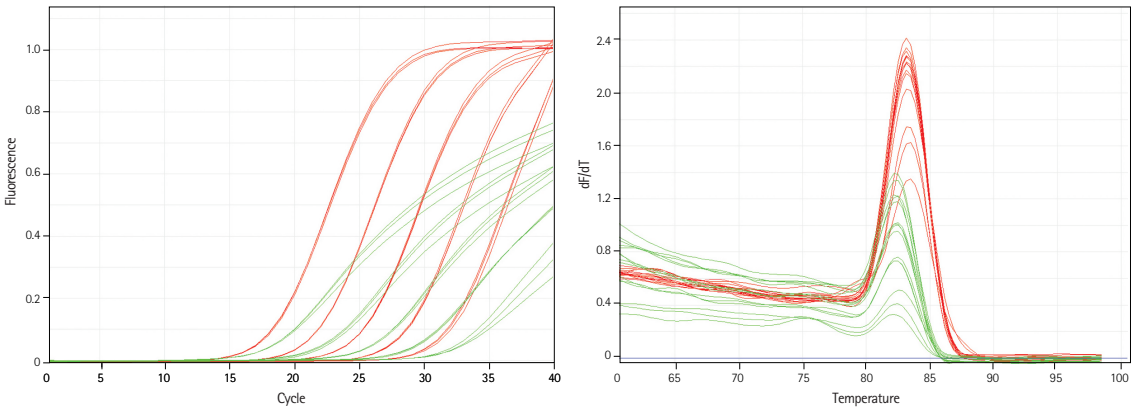


Figure 3.
NovaTaq™ Hot Start DNA Polymerase – Red
Supplier I Polymerase – Green

Supplier I Polymerase shows lower amplification efficiency as indicated by the lower fluorescence intensity.

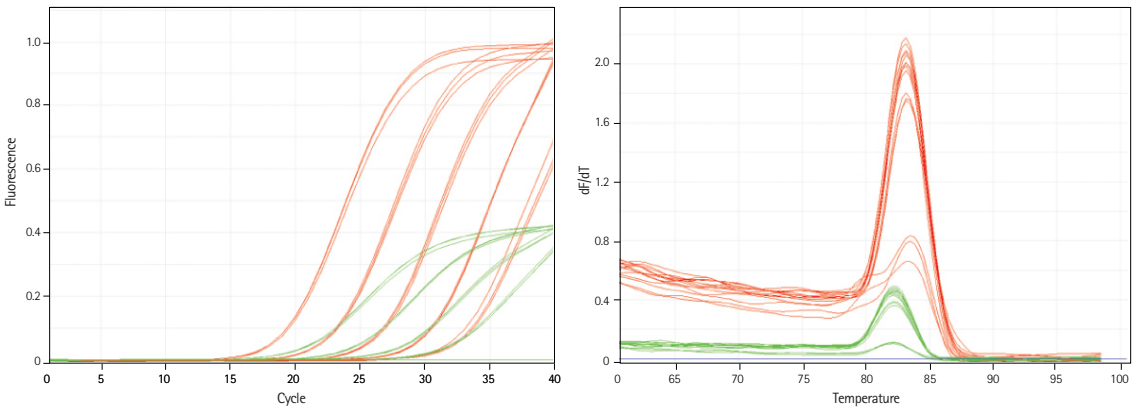


Figure 4.
NovaTaq™ Hot Start DNA Polymerase – Red
Supplier Q Polymerase – Green

NovaTaq™ Hot Start DNA Polymerase shows superior amplification efficiency as indicated by the much higher fluorescence intensity.

Figure 5.

NovaTaq™ Hot Start DNA Polymerase – Red
Supplier K Polymerase – Green

Supplier K Polymerase demonstrates poor reaction specificity as demonstrated by the multiple peaks in the melting curve.

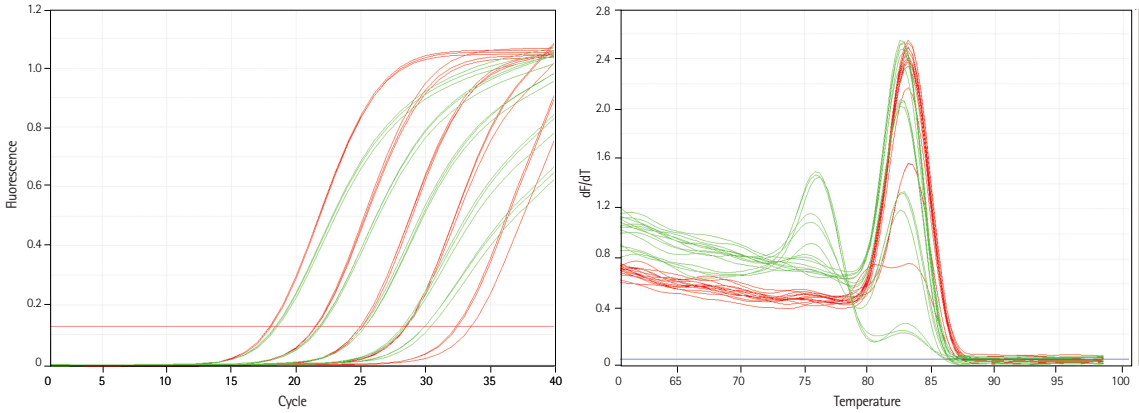
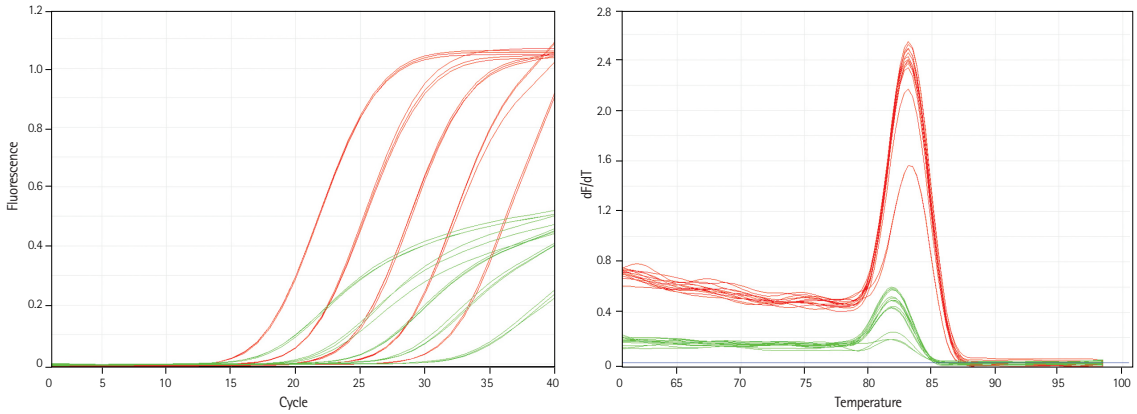


Figure 6.

NovaTaq™ Hot Start DNA Polymerase – Red
Supplier S Polymerase – Green

NovaTaq™ Hot Start DNA Polymerase shows superior amplification efficiency as indicated by the much higher fluorescence intensity.



Summary

We have demonstrated that NovaTaq™ Hot Start Master Mix Kit may be used for real-time quantitative PCR of plasmid, genomic, and first strand cDNA. The data obtained using this enzyme master mix are reproducible, indicating that the master mix can be used for the analysis of low copy number genes. NovaTaq™ Hot Start Master Mix has a higher amplification efficiency and specificity than PCR mixes from other suppliers.

3. One Step Quantitative (Real-Time) PCR Amplification of mRNA Using the One Step RT-PCR Master Mix Kit

Hope Schultz and Keith Yaeger – EMD Millipore Corporation

Introduction

Reverse transcription followed by polymerase chain reaction (RT-PCR) allows amplification and detection of very low amounts of RNA. The technique is important for gene expression profiling and RNA virus detection. In addition to using purified RNA as a template, RT-PCR kits may be used directly with crude lysates of mammalian cells, a technique that can be used to compare mRNA levels in different cells. Merck Millipore's Novagen® One Step RT-PCR Master Mix Kit allows for mRNA reverse transcription and amplification in a single tube because it contains recombinant *Thermus thermophilus* (*rTth*) DNA Polymerase, which functions as both a thermostable RNA–dependent DNA polymerase and a DNA–dependent DNA polymerase. The anti-*rTth* antibody included in the mix enhances the specificity of the reverse transcription by binding the polymerase and reducing mispriming during reaction assembly. The high optimal temperature for polymerization by *rTth* polymerase in reverse transcription offers the advantage of increased success by reducing secondary mRNA structure and the single reaction setup reduces chances of contaminating the samples with exogenous DNA, RNA or nucleases, allowing for sensitive detection of RNA. One Step RT-PCR Master Mix Kit is compatible with SYBR® Green, providing fluorescent signal to detect the amplified region and melt analysis to confirm homogeneity of product. In this article, the One Step RT-PCR Master Mix Kit was used to amplify reverse-transcribed mRNA directly from mammalian extracts. Duplex RT-PCR using the same crude extract demonstrates that the One Step RT-PCR Master Mix can be used to analyze two targets in a single reaction when probes are used as the detection method.

Quantitative (Real–Time) PCR from Crude Lysate

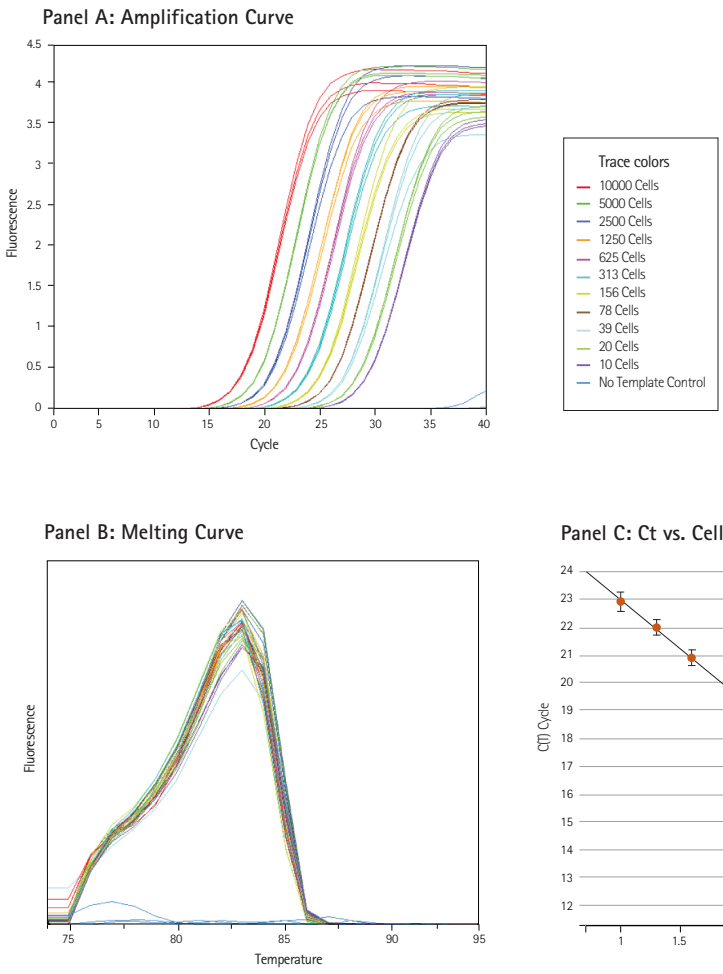
To test the ability of the One Step RT-PCR Master Mix Kit to amplify mRNA from HeLa cell lysates in a quantitative (real-time) PCR reaction using SYBR® Green I for detection, HeLa cells ($5X10^5$ cells) were cultured in DMEM, pelleted, and stored at -70 °C. The cell pellet was treated with 50 µL of CytoBuster™ Protein Extraction Reagent (Merck Millipore, Cat. No. 71009) to lyse cells and 40 units RNase Inhibitor (Merck Millipore, Cat. No. 556881). 50 µL of TE (10 mM Tris, pH 8, 1 mM EDTA) + 0.02% Triton® X-100 was added to yield a lysate containing 5000 cell equivalents/µL. The lysate was two-fold serially diluted in TE + 0.02 % Triton® X-100, and 2 µL of each dilution was used as template in 50 µL RT-PCR reactions that were done in triplicate. The primers used targeted a 99 bp amplicon from exon 3 to exon 4 of cyclophilin B (PPIB, genomic accession NT_010194.16). PCR was performed using a Chromo4 Real-Time PCR Detection System (Bio-Rad) and SYBR® Green I was used from Invitrogen. Table 1 contains the cycling parameters.

Cycling Step	Time/temperature
1. Polymerase Activation	90 °C for 30 s
2. Reverse Transcription	60 °C for 15 min
3. Denaturation	94 °C for 30 s
4. Denaturation	95 °C for 1 s
5. Annealing	50 °C for 15 s
6. Extension	72 °C for 5 s
Repeat Steps 4-6 for 40 cycles	

Table 1.
Thermocycling parameters

We observed excellent yield and reproducibility as shown in the amplification curves (Figure 1A). The signals titrated from 10,000 to 10 cells as shown by the standard curve with a correlation of 0.999 (Figure 1C). The melting curve (Figure 1B) shows that a single product was amplified.

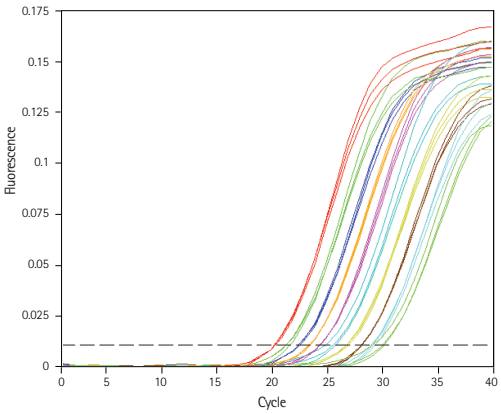
Figure 1.
PPIB mRNA amplified from crude HeLa lysates



Duplex Quantitative (Real-Time) PCR with One-Step RT-PCR Master Mix

The template used in the duplex RT- PCR experiment was the same HeLa cell preparation used in the SYBR® Green I experiments described above. Primer Set I targeted a 99 bp amplicon spanning from exon 3 to exon 4 of PPIB. Primer Set II targeted an 112bp amplicon spanning from exon 4 to exon 5 of glycogen synthase kinase 3 alpha (GSK3α, genomic accession NC_000019). FAM Dye was used to detect PPIB and HEX dye was used to detect GSK3α. Reactions were performed in triplicate using the Chromo4 Real-Time PCR Detection System (Bio-Rad) with the cycling conditions described in Table 1. The plate was read after step 5 for these experiments. Figures 2 and 3 shows that PPIB and GSK3α are efficiently amplified in the same reaction and the signal titrates well from 5000 to 10 cells with a standard curve with a correlation coefficient of 0.997.

Panel A: Amplification Curve



Panel B: Ct vs. Cell Number

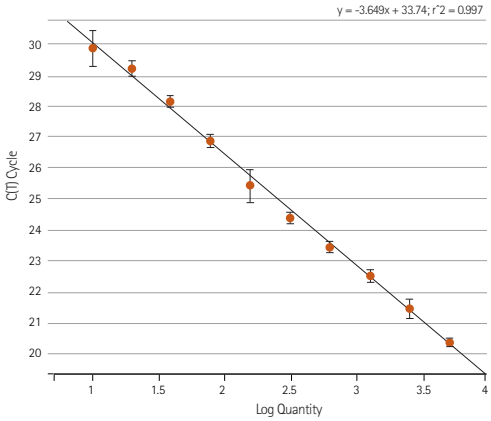
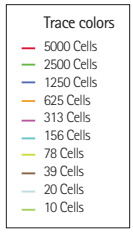
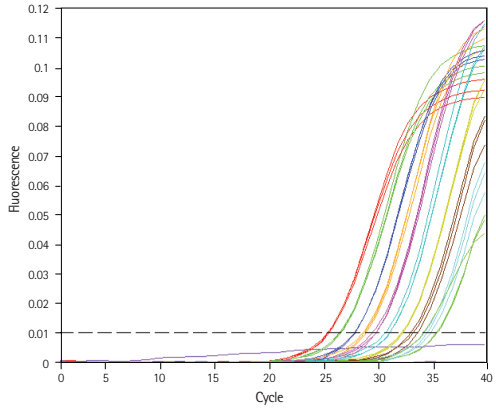


Figure 2.

PPIB mRNA amplified from crude HeLa cell lysates using the One Step RT-PCR Master Mix Kit and FAM Dye.



Panel A: Amplification Curve



Panel B: Ct vs. Cell Number

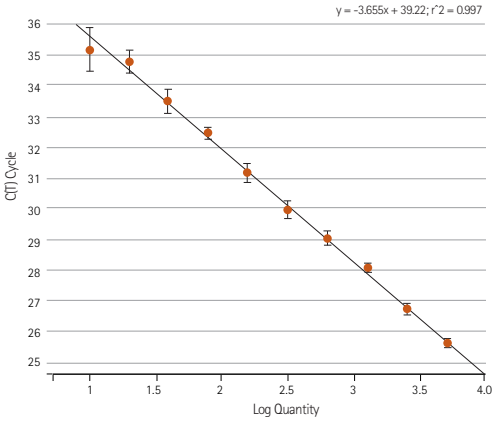
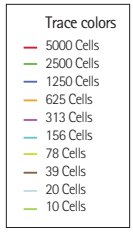


Figure 3.

GSK3α mRNA amplified from crude HeLa cell lysates using the One Step RT-PCR Master Mix Kit and HEX Dye.



Summary

Quantitative (real-time) reverse transcription PCR is a powerful technique in functional proteomics.

Merck Millipore's Novagen® One Step RT-PCR Master Mix Kit provides results that are reproducible and robust.

4. Detection of Shiga Toxin-producing *E. coli* Using Multiplex Direct Colony PCR with KOD XL DNA Polymerase

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Introduction

Certain strains of *E. coli* are known to produce a family of related toxins, referred to as Shiga toxin 1 (*Stx1*, encoded by *stx1*) and Shiga toxin 2 (*Stx2*, encoded by *stx2*). Shiga toxin-producing *E. coli* (STEC), represented by serotype O157:H7, has a strong infectious capacity and pathogenicity. In recent years, this bacterium has been affecting an increasing number of victims, resulting in life-threatening illness such as hemorrhagic colitis, hemolytic- uremic syndrome, and thrombotic thrombocytopenic purpura[1]. The morbidity and mortality associated with STEC disease highlight the threat these organisms pose to public health.

For this reason, there is an increasing demand for fast and efficient methods for the detection of virulent strains of STEC in fecal samples and in meat and dairy products. PCR is generally considered the most sensitive means for determining if a food or fecal sample contains STEC. A multiplex PCR method developed by Paton and Paton[2] enables simultaneous determination of *stx1*, *stx2*, and correlated genes that encode accessory STEC virulence factors, such as *eaeA* and *hlyA*, in crude DNA extracts from primary fecal cultures.

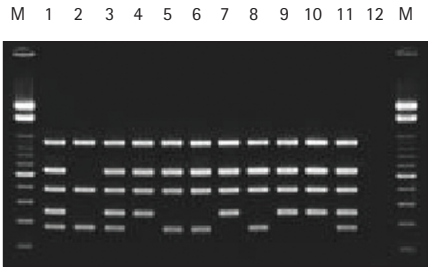
In this study, we developed a rapid typing system for STEC that improves upon the original multiplex PCR assay. With our method, a bacterial colony from a food or fecal culture was used directly as the template. In addition, four target genes were examined for the presence of the IS1203v insertion sequence discovered in *stx2* genes[3,4] with IS1203v-specific primers. To reduce the time needed for the PCR, Taq DNA polymerase was replaced with the faster KOD XL DNA Polymerase*. Multiplex CD-PCR analysis for eleven STEC strains and one control K-12 strain isolated at Kanagawa Prefecture, Japan, between 1996 and 1999 was performed (Figure 1).

Component	Concentration
PCR buffer for KOD XL	1X
dNTP mix	0.2 mM
stx1 primers (stx1-F + stx1-R)	0.2 µM each
stx2 primers (stx2-F + stx2-R)	0.2 µM each
eaeA primers (eaeA-F + eaeA-R)	0.2 µM each
hlyA primers (hlyA-F + hlyA-R)	0.2 µM each
IS1203v primers (1203v-F + 1203v-R)	0.1 µM each
Bacterial cells	approximately 10^4 cfu
KOD XL DNA Polymerase	2.5 U

[1] When using KOD XL DNA Polymerase, set up the PCR on ice.

Table 1 identifies the final concentrations of the PCR components PCR were performed using the following conditions: initial denaturation at 94 °C for 5 minutes, 30 cycles of 98 °C for 15 seconds, 60 °C for 5 seconds, and 74 °C for 30 seconds. After the PCR, one-tenth of the reaction solution was analyzed by agarose gel electrophoresis (2% TAE gel, Figure 1).

The results of the multiplex PCR showed a clear amplification for each target: 180 bp for *stx1*, 255 bp for *stx2*, 384 bp for *eaeA*, 534 bp for *hlyA*, and 910 bp for *IS1203v*, in 11 STEC strains (Figure 1). Amplification of the target genes from the control K-12 strain was negative. The results clearly demonstrated that this system is effective for STEC typing.



Lane	Sample
M	Markers (100-bp ladder)
1–11	STEC strains
12	K-12 strain

Figure 1.

STEC identification by CD-PCR

Eleven STEC strains and one K-12 strain were used for CD-PCR. Reaction products were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Summary

By using KOD XL DNA Polymerase, PCR was completed in less than 1.5 hours, a significant time savings compared to the nearly 3.5 hours required by the original method, representing a great advantage when multiple specimens require quick processing.

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12. Troubleshooting Guides

(I) KOD Xtreme™ Hot Start DNA Polymerase

Symptom	Possible cause	Solution
No PCR product	Annealing temperature is too high	Lower annealing temperature in 3 °C increments
	PCR primers are not long enough	Use primers longer than 21 bases
Low yield	Suboptimal PCR conditions	Increase number of cycles
Smearing	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 10 s/kbp
Smearing above target size	Extension times too long	Reduce extension time 10 s/kbp
Primer dimers	Primers are complementary to each	Design primers that do not form hairpins (are not self complementary) or dimers (are not complementary to each other)
	Primer concentration is too high	Reduce primer concentration
	Annealing temperature too low	Raise annealing temperature
Unexpected mutation	Denaturation step is too long	Lower denaturation time to 5 s or 1 s.

(II) KOD Hot Start DNA Polymerase

Symptom	Possible cause	Solution
No PCR product	Extension time is too long	Lower extension time to 15 s/kpb
	Too much secondary structure in template DNA	Add DMSO to a final concentration of 5–10% v/v
	PCR primers are not long enough	Use primers longer than 21 bases
	Annealing temperature is too high	Lower annealing temperature in 3 °C decrements
Low yield	High GC content	Add DMSO to a final concentration of 5–10% v/v
	Suboptimal PCR conditions	Increase final $MgSO_4$ concentration in 0.25 mM increments
	Long target DNA	Increase final $MgSO_4$ concentration in 0.25 mM increments
Smearing	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 5 s/kbp
	$MgSO_4$ concentration too low	Increase final $MgSO_4$ concentration 0.25 mM increments
Smearing above target size	Extension times too long	Reduce extension time 5 s/kbp
	$MgSO_4$ concentration too high	Decrease final $MgSO_4$ concentration in 0.25 mM increments
Primer dimers	Primers are complementary to each other	Design primers that are not self-complementary or complementary to each other
	Primer concentration is too high	Reduce primer concentration
	Annealing temperature too low	Raise annealing temperature

(III) KOD Hot Start Master Mix

Symptom	Possible cause	Solution
No PCR product	Extension time is too long	Lower extension time to 15 s/kpb
	Too much secondary structure in template DNA	Add DMSO to a final concentration of 5–10% v/v
	PCR primers are not long enough	Use primers longer than 21 bases
	Annealing temperature is too high	Lower annealing temperature in 3 °C steps
Low yield	High GC content	Add DMSO to a final concentration of 5–10% v/v.
	Incomplete extension	Increase extension time 5 to 10 s/kbp
	Inefficient polymerization	Increase number of cycles
Smearing	Too much template DNA	Reduce the amount of template DNA
Smearing below target size	Extension times are too short	Increase extension time 5 s/kbp
Smearing above target size	Extension times too long	Reduce extension time 5 s/kbp
	Extension temperature too high	Reduce extension temperature 3 to 5 °C
Primer dimers	Primers are complementary to each other	Design primers that are not self-complementary or complementary to each other
	Primer concentration is too high	Reduce primer concentration
	Annealing temperature too low	Raise annealing temperature

(IV) KOD XL DNA Polymerase

Symptom	Possible cause	Solution
No PCR product	PCR primers are not long enough	Use primers longer than 21 bases.
Smear instead of distinctive DNA band on agarose gel	Reactions were not set up on ice	The reaction should be set up on ice and the KOD XL should be added last to the PCR reaction mix to prevent degradation of the primers and template.
	Suboptimal PCR conditions	Reduce the units of KOD XL DNA Polymerase and/or decrease the extension time.
Low yield	High GC content/long target DNA	Add DMSO to a final concentration of 2–5%. DMSO concentration less than 5% does not change enzyme fidelity.
Multiple Bands	Long target DNA	For target DNA over 8 kbp, increasing the dNTP concentration to 0.35 mM may reduce the appearance of multiple bands.

(V) KOD DNA Polymerase

Symptom	Possible cause	Solution
No PCR product	Target size too large	Use a smaller target size. KOD amplifies up to 2 kbp genomic DNA and up to 6 kpb plasmid and phage DNA targets.
Smear instead of distinctive DNA band on agarose gel	Reactions were not set up on ice	The reaction should be set up on ice and the KOD should be added last to the PCR reaction mix to prevent degradation of primers and template.
	Suboptimal PCR conditions	Decrease annealing and extension times according to the table on page 2. KOD extension rate is faster than other thermostable polymerases and longer extension times can cause smearing.
Low yield	High GC content	Add DMSO to a final concentration of 2–5%. DMSO does not change enzyme fidelity.
	Long target/genomic DNA	Using PCR Buffer #2 may enhance the quality and quantity of the PCR product. Add DMSO to a final concentration of 2–5%. DMSO does not change enzyme fidelity.
	Low amount of template	For plasmid or phage DNA 0.006–6 ng of template is adequate. Genomic and cDNA templates may require up to 12 ng.

(VI) One Step RT-PCR Master Mix Kit

Symptom	Possible cause	Suggestion
Target cDNA band is not observed	Enzymes are inactivated due to incorrect storage.	Use the positive control RNA and primers to test the enzyme performance.
	The annealing temperature is not optimal	Change the annealing temperature to 5 °C below the T_m value.
	Primer sequence is incorrect	Redesign primers to be more complementary to the target gene. The 3' end of the primers should be completely complementary to the template. The primers should have a GC content of 40–60%. Check that the primer sequences are not self complementary, especially at the 3' end.
	The reaction conditions and/or cycling times are not optimal	The amplification conditions described on p 3 are optimal for 0.1–1 kbp products. For a longer product increase reverse-transcription time and the PCR extension time. Note that the limit for robust amplification is ~1 kbp.
	Template RNA is poor quality and/or degraded	Perform gel analysis of the template RNA to determine the RNA quality. The A_{260}/A_{280} ratio should be >1.7. SDS, NaCl, heparin and guanidine thiocyanate from RNA purification methods can interfere with RT-PCR. Reduce the volume of RNA, perform additional purification steps or change purification method to avoid interference. Use RNase-free reagents and equipment.
	Insufficient template RNA	Increase the amount of template RNA in the reaction (up to 1000 ng).
Multiple products are observed from the RT-PCR reaction	The primer sequence is not specific to the desired target	Redesign the primers.
	The annealing temperature is not optimal	Change the annealing temperature to 5 °C below the T_m value.
	The T_m value of the primers is low	Use primers with higher T_m value.
	Number of cycles is too high	Try reducing the number of cycles; optimal range is 20–50 cycles. Increase the amount of template RNA.
	$Mn(OAc)_2$ concentration is insufficient	Usually 2.5 mM is optimal for RT-PCR but a range between 1–4 mM may be used.
	Genomic DNA contaminationindicated by higher molecular weight product than expected.	Note that genomic DNA may amplify with gene specific primers. Perform a control reaction using a DNA polymerase without reverse transcriptase activity (e.g. NovaTaq™ DNA polymerase) instead of r*Tth* DNA Polymerase. If amplification from the NovaTaq™ reaction occurs, then the template is contaminated with DNA.
	Multiple cDNA products may be obtained from multigene families or alternatively spliced genes	Redesign primers to ensure specificity, as appropriate. Sequence products to verify identity.

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(I) KOD Xtreme™ Hot Start DNA Polymerase

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(III) KOD Hot Start Master Mix

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Gene cloning	Kim, T. S., Maeda, A., Maeda, T., Heinlein, C., Kedishvili, N., Palczewski, K., and Nelson, P. S. (2005) *J. Biol. Chem.* 280, 8964–8704.
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Construction of knock-out targeting vector	Kim, T. S., Maeda, A., Maeda, T., Heinlein, C., Kedishvili, N., Palczewski, K., and Nelson, P. S. (2005) *J. Biol. Chem.* 280, 8964–8704.
Gene cloning	Herrin, B. R., Groger, A. L., and Justement, L. B. (2005) *J. Biol. Chem.* 280, 3507–3515.
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