Cat. # R011

For Research Use

TaKaRa

TaKaRa PCR Amplification Kit

Product Manual

v201906Da

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I. Description

The TaKaRa PCR Amplification Kit is designed to perform Polymerase Chain Reaction (PCR) on all DNA templates. It includes λ DNA as a control template and control primers for amplification of specific target sequences from λ DNA (6,012 bp and 500 bp).

II. Components (100 μl PCR, 100 reactions or 50 μl PCR, 200 reactions)

1.	*TaKaRa Taq*™ DNA Polymerase*1 (5 U/μl)	50 μl (250 U)
2.	dNTP Mixture*2 (each 2.5 mM)	1.28 ml
3.	10X PCR Buffer (Mg^{2+} plus)	1 ml
	100 mM Tris-HCl (pH 8.9) 500 mM KCl 15 mM $MgCl_2$	
4.	10X PCR Buffer (Mg^{2+} free)	1 ml
	100 mM Tris-HCl (pH 8.9) 500 mM KCl	
5.	$MgCl_2$ (25 mM)	1 ml
6.	Control Template (1 μg/ml λ DNA)	100 μl
7.	Control Primer 1*3 (20 pmol/μl)	50 μl
8.	Control Primer 2*3 (20 pmol/μl)	50 μl
9.	Control Primer 3*3 (20 pmol/μl)	50 μl
10.	λ-*Eco*T14 I Marker (100 ng/μl)*4	40 μl
11.	6X Loading Buffer*5	1 ml

*1 *TaKaRa Taq* (5 U/μl)

・Form:

Supplied in	20 mM	Tris-HCl (pH 8.0)
	100 mM	KCl
	0.1 mM	EDTA
	1 mM	DTT
	0.5%	Tween 20
	0.5%	NP-40
	50%	Glycerol

・Unit definition:

One unit is the amount of enzyme that will incorporate 10 nmol of dNTP into acid-insoluble products in 30 minutes at 74℃, pH 9.3, with activated soluble salmon sperm DNA as the template-primer.

・Reaction mixture for unit definition:

25 mM	TAPS (pH 9.3 at 25℃)
50 mM	KCl
2 mM	$MgCl_2$
0.1 mM	DTT
200 μM	each dATP・dGTP・dCTP
100 μM	[^{3}H]-dTTP
0.25 mg/ml	activated salmon sperm DNA

*2 dNTP Mixture (each 2.5 mM)

dNTP Mixture is ready for use in PCR without dilution.

・Form : Dissolved in water (sodium salts), pH 7 - 9

・Purity : ≧ 98% for each dNTP

*3 Control Primer Sequences
Control Primer 1 : 5'-GATGAGTTCGTGTCCGTACAACT-3'
Control Primer 2 : 5'-CCACATCCATACCGGGTTTCAC-3'
Control Primer 3 : 5'-GGTTATCGAAATCAGCCACAGCGCC-3'

- Control Primers 1 and 2 will produce a 6,012 bp amplified DNA fragment from the Control Template (λ DNA).
- Control Primers 1 and 3 will produce a 500 bp amplified DNA fragment from the Control Template (λ DNA).

*4 λ-*Eco*T14 I Marker
Consists of a complete digest of λ cI_{857} Sam7 DNA by the restriction enzyme *Eco*T14 I.
Size marker range: 19329, 7743, 6223, 4254, 3472, 2690, 1882, 1489, 925, 421, 74 bp.
To prevent the terminal fragments of the λ DNA digest from annealing at their COS ends, heat treatment (60℃, 5 min) is needed.

*5 6X Loading Buffer

[Composition]		
	36%	Glycerol
	30 mM	EDTA
	0.05%	Bromophenol Blue
	0.035%	Xylene Cyanol

III. Storage

-20℃

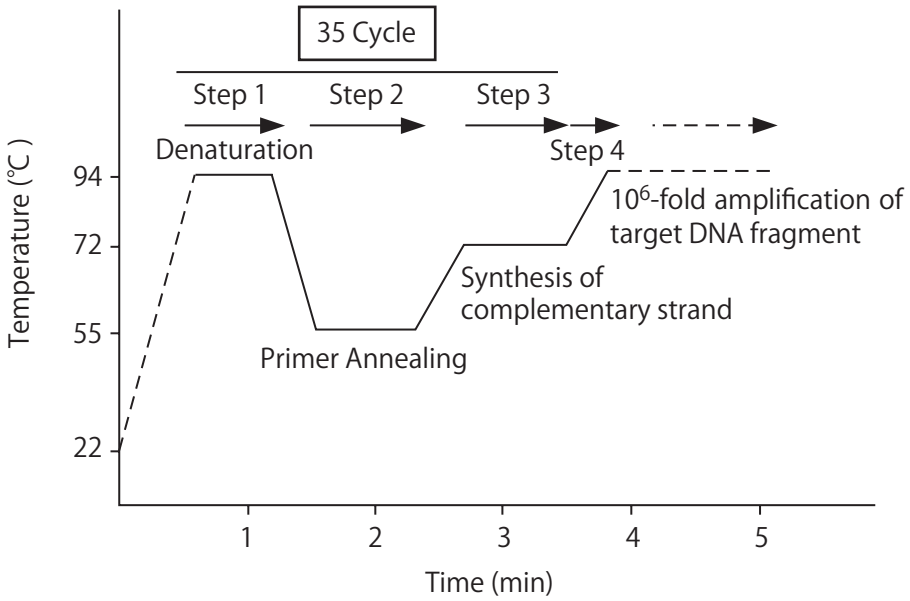
IV. Materials Required but not Provided

- Agarose gel
  PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)
  Agarose L03 [TAKARA] (Cat. #5003)
- DNA staining reagent
  SYBR® Green I Nucleic Acid Gel Stain (Cat. #5760A/5761A)*, or
  Ethidium Bromide
  Note: When using SYBR Green I for staining a gel, a filter designated for use with SYBR Green I should be used.
- Sterile purified water
- Authorized instruments for PCR
  TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600)*
  TaKaRa PCR Thermal Cycler Dice *Touch* (Cat. #TP350)*
- 0.2 ml Microtubes for PCR (made of polypropylene)
  0.2ml Single-Tube Dome Cap (Cat. #NJ204)
  0.2 ml 96 well-plate for Real Time (Frosted) (Cat. #NJ401)
  Flat cap for snap plate (Cat. #NJ720)
- Agarose gel electrophoresis apparatus
  Mupid-2 plus, Mupid-exU, Mupid-One (Mupid CO., LTD)
- Microcentrifuge
- Micropipets and pipette tips (autoclaved)

* Not available in all geographic locations. Check for availability in your area.

V. Principle

PCR is a simple and powerful method which allows *in vitro* amplification of DNA fragments through a succession of three incubation steps at different temperatures. The double-stranded DNA is heat denatured (denaturation step), the two primers complementary to the 3' boundaries of the target segment are annealed at a low temperature (annealing step), and then extended at an intermediate temperature (extension step). One set of the three consecutive steps is referred to as one cycle. The PCR process is based on the repetition of the cycle and can amplify DNA fragments. The key component of TaKaRa PCR Amplification Kit is *TaKaRa Taq* (Cat. #R001). *TaKaRa Taq* is a recombinant, thermostable, 94 kDa DNA polymerase encoded by the DNA polymerase gene of the *Thermus aquaticus* YT-1 strain which has been cloned into an *Escherichia coli* host and has essentially the same characteristics as the native *Taq* DNA polymerase.



Step 1: Denature the target double-stranded DNA fragment in the reaction mixture containing primer, dNTP, and polymerase: 94℃, 30 sec

Step 2: Anneal primer to the single-stranded DNA: 55℃, 30 sec

Step 3: Synthesize the complementary strand with DNA polymerase: 72℃, 1 min

Step 4: Return to Step 1 to denature the amplified double-stranded DNA again to yield single-stranded DNA: 94℃, 30 sec

One set of the consecutive 1 - 4 steps is referred to as one cycle; 35 cycles were performed. PCR parameters must be optimized for a specific target DNA fragment, since the most efficient conditions for PCR vary depending on the target DNA fragment.

VI. Protocol

Amplification using the TaKaRa PCR Thermal Cycler Dice

A. Control experiment

This kit includes λ DNA and primers for amplifying specific target regions of λ DNA (6,012 bp or 500 bp).

1) Prepare the PCR reaction mixture in a microtube by combining the following reagents in a total volume of 50 μl.

Reagent	Volume	Final conc.
10X PCR Buffer (Mg^{2+} plus)*	5 μl	[1X]
dNTP Mixture	4 μl	each 200 μM
Control Primer 1	0.5 μl	0.2 μM
Control Primer 2 or 3	0.5 μl	0.2 μM
TaKaRa Taq	0.25 μl	1.25 U/50 μl
Control Template	0.5 μl	0.5 ng/50 μl
Sterile purified water	39.25 μl	
Total	50 μl	

* 10X PCR Buffer (Mg^{2+} free) and $MgCl_2$ solution may be used instead of 10X PCR Buffer (Mg^{2+} plus) if necessary.

2) Place the tubes in a thermal cycler.

3) Perform the reaction under the following conditions.

・When amplifying 6,012 bp with Control Primers 1 and 2:

94℃	1 min (denaturation)	⎫ 30 cycles
68℃	4 min (annealing and extension)	⎭
72℃	5 min	1 cycle

・When amplifying 500 bp with Control Primers 1 and 3:

94℃	30 sec (denaturation)	⎫
55℃	30 sec (annealing)	⎬ 25 cycles
72℃	30 sec (extension)	⎭
72℃	2 min	1 cycle

B. Amplification of Experimental Samples

The protocol for the samples is basically the same as the control experiment described in A. The parameters of each step (temperature, time) must be optimized for specific DNA templates depending on the size of target, the target sequence, and the length of the primers.

C. Electrophoresis

1) Remove 5 - 10 μl from each PCR reaction for analysis on an agarose gel, and add 1/6 volume of 6X Loading Buffer to each sample.
2) Run the samples from Step 1 on an agarose gel. The gel composition and electrophoresis conditions will vary depending on the sizes of the PCR products.
3) After electrophoresis is complete, stain gels by soaking in SYBR Green I or Ethidium Bromide solution (1 μg/ml) for 20 - 30 min.
4) Determine the sizes of the PCR products under UV illumination.

VII. Note

1) Before adding *TaKaRa Taq*, mix the other kit components vigorously for approximately 2 sec and centrifuge. Then add *TaKaRa Taq* and mix gently by pipetting.
2) The $MgCl_2$ concentration of the supplied 10X PCR Buffer (Mg^{2+} plus) may not be the optimum concentration for amplifying DNA, depending on how the sample DNA is prepared. In this case, the optimum $MgCl_2$ concentration needs to be determined empirically by changing its concentration with supplied 10X PCR Buffer (Mg^{2+} free) and $MgCl_2$ solution.

VIII. Q & A

1. PCR Optimization

Optimal reaction conditions can vary depending on amplicon size, reaction volumes, the type of thermal cycler used, etc.

i) Cycle numbers

Set the optimum cycle number at around 25 - 30 cycles, taking into consideration the quantity or complexity of template DNA and the length of amplified DNA fragments. Using fewer cycles may not generate enough amplified product, while over cycling may produce a diffuse smear upon electrophoresis.

ii) Denaturation conditions

When using thin-wall PCR tubes, the recommended denaturation conditions are 98℃ for 10 sec or 94℃ for 20 sec. When using normal PCR tubes, the recommended denaturation conditions are 98℃ for 20 sec or 94℃ for 30 sec.
A denaturation time that is too short or a denaturation temperature that is too low may result in either diffuse smearing on an agarose gel or poor amplification efficiency. A denaturation time that is too long or a denaturation temperature that is too high may generate no identifiable product.

iii) Conditions for Annealing and Extension

Determine the optimum annealing temperature by varying temperatures in 2℃ increments over a range of 37 - 65℃. Since *TaKaRa Taq* shows sufficient activity at 60 - 68℃, Shuttle PCR (two-temperature PCR) can be performed by setting the anneal-extension temperature within this range. To carry out the combined annealing/extension at 68℃ (two-step PCR), the recommended time setting is 30 sec to 1 min per 1 kb. When temperature is set below 68℃, a longer time will be required. An annealing temperature that is too high generates no amplification products, while a temperature that is too low enhances non-specific reactions. An extension time that is too short generates no amplification products or predominantly non-specific, short products, while too long an extension time results in diffusely smeared agarose gel bands.

2. Primer Preparation

Primer specificity is very important for the generation of longer DNA amplification products. If possible, prepare primers according to the following criteria.

1) The difference between the optimum annealing temperatures of paired primers should be within 2 - 3℃.
2) Choose primers whose GC content is around 50 - 60%.
3) Avoid primer sequences that form hairpin loops or primer dimers, especially at the 3' end.

3. Primer Concentration

The optimal primer concentration will range from 0.1 μM to 1.0 μM. Below the optimal concentration, amplification yield may be poor. In contrast, at a higher concentration, non-specific reactions may outperform primer-specific amplifications.
In ordinary practice, primer concentration can be determined depending on the characteristics and amount of template DNA: low concentrations are recommended either for highly complex DNA such as human genomic DNA or for high concentrations of template DNA, whereas high concentrations are preferred for low complexity templates such as plasmid DNA or for limiting amounts of template DNA.

4. Enzyme Amount

Although 1.25 units of *TaKaRa Taq* in a 50 μl reaction is recommended, you may change the amount of enzyme used to optimize reaction conditions. The following factors should be taken into consideration: the quantity or complexity of the template DNA and the length of the amplicon. In case of excess enzyme, non-specific reactions may occur, resulting in diffusely smeared agarose gel bands. The efficiency of amplification may be diminished when the enzyme concentration is low.

5. Troubleshooting reactions that yield only a smear upon electrophoresis

Possible Causes	*Comments and suggestions*
Too much enzyme	Reduce the amount of enzyme in 0.5 unit decrements.
Insufficient denaturation time	Increase the denaturation time in 5 sec increments.
Denaturation temperature too low	Raise the denaturation temperature in 0.5℃ increments.
dNTP concentration too low	Increase the dNTP concentration in 50 μM increments.
Extension time too long	Shorten the extension time in 30 - 60 sec decrements.
Too many PCR cycles	Reduce the number of cycles in 2-cycle decrements.
Too much template	Reduce the template amount by decrements of 20%.

6. Multiple, nonspecific amplified products upon electrophoresis

Possible Causes	*Comments and suggestions*
Primer concentration too high	Decrease the primer concentration in 0.1 μM decrements.
Poor primer design	Enhance the specificity of primers by changing the complementary region of the template or by preparing longer primers (up to 25 - 30 mers).
Too much enzyme	Reduce the amount of enzyme in 0.5 unit decrements.
Too many PCR cycles	Reduce the number of cycles in 2-cycle decrements.
Annealing temperature too low	Raise the annealing temperature in 2℃ increments.
Nonspecific annealing of primers	Use Hot Start method, e.g. with Taq Antibody (Cat. #9002A/B), to avoid this phenomenon while heating PCR reactions from room temperature to the denaturation temperature (94 - 98℃)
Extension time too short	Increase the extension time in increments of 30 - 60 sec.
Poor denaturation	Raise the denaturation temperature in 0.5℃ increments and increase the denaturation time in increments of 5 sec.
Too much template	Reduce the template amount by decrements of 20%.

IX. References

1) Saiki R K, Gelfand D H, Stoffel S, Scharf S J, Higuchi R, Horn G T, Mullis K B, and Erlich H A. *Science.* (1988) **239**: 487-491.
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4) Kawasaki E S, Clark S C, Coyne M Y, Smith S D, Champlin R, Whitte O N, and McCormick F P. *Proc Natl Acad Sci USA.* (1988) **85**: 5698-5702.
5) Lawyer F C, Stoffel S, Saiki R K, Myambo K B, Drummond R, and Gelfand D H. *J Biol Chem*. (1989) **264**: 6427-6437.