PCR Amplification Kit Manual

Cat.# R011
v.0706

TaKaRa

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TaKaRa

Description: **PCR Amplification Kit (100µl PCR x 100 reactions)**

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

Kit Components:

1. *TaKaRa Taq* DNA Polymerase *[1](5 units/µl).....250 units
2. dNTP Mixture*[2](2.5 mM each).....1.28 ml
3. 10x PCR Buffer.....1 ml
   100 mM Tris-HCl (pH8.3 at 25℃)
   500 mM KCl
   15 mM $MgCl_2$
4. 10x PCR Buffer (-) (Mg^{2+} Free).....1 ml
   100 mM Tris-HCl (pH8.3 at 25℃)
   500 mM KCl
5. $MgCl_2$(25 mM).....1 ml
6. Control Template (1µg/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*

- Concentration: 5 units/µl
- Form: Supplied in 20 mM Tris-HCl (pH8.0), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 50% Glycerol
- Unit definition: One unit is the amount of the enzyme that will incorporate 10 nmol of dNTP into acid-insoluble products in 30 minutes at 74℃ , pH9.3, with activated soluble salmon sperm DNA as the template-primer.
- Reaction mixture for unit definition:
  25 mM TAPS (pH9.3 at 25℃)
  50 mM KCl
  2 mM $MgCl_2$
  1 mM 2-mercaptoethanol
  200 µM each dATP, dGTP, dTTP
  100 µM [α -^{32}P]dCTP
  0.25 mg/ml activated salmon sperm DNA
- Purity: Nicking activity, endonuclease and exonuclease activity were not detected after the incubation of 0.6 µg of supercoiled pBR322 DNA, 0.6 µg of λ DNA or 0.6 µg of λ -*Hin*d III digest with 10 units of this enzyme for 1 hour at 74℃ .

*2) dNTP Mixture (PCR Nucleotide Mix)
Mixture of dNTP at the equal moles, ready for use in PCR without dilution.
- Concentration : 2.5 mM of each dNTP
- pH : 7~9
- Form : Solved in water (sodium salts)
- Purity : ≧ 98% for each dNTP

*3) The sequence of Control Primer
Control Primer 1: 5'-GATGAGTTCGTGTCCGTACAACT-3'
Control Primer 2: 5'-CCACATCCATACCGGGTTTCAC-3'
Control Primer 3: 5'-GGTTATCGAAATCAGCCACAGCGCC-3'

・Control Primer 1 and 2 will result 6,012 bp amplified DNA fragment from Control Template (λ DNA).
・Control Primer 1 and 3 will result 500 bp amplified DNA fragment from Control Template (λ DNA).

*4) λ *Eco*T14 I Marker
This marker is the completely digested λ cI_{857} Sam7 DNA by restriction enzyme *Eco*T14 I.
The range of size marker: 19329, 7743, 6223, 4254, 3472, 2690, 1882, 1989, 925, 421, 74bp
Since terminal fragment of λ DNA digest is linked with COS end, heat treatment (60℃ , 5 min.) is needed.

*5) 6x Loading Buffer
Composition: 30% Glycerol, 30 mM EDTA, 0.03% Bromophenol Blue, 0.03% Xylene Cyanol.

Reagents not supplied in the kit:

-Agarose gel
ex. NuSieve® 3:1 Agarose (Lonza)
TaKaRa L03 Agarose (TaKaRa Cat.#5003)
-DNA staining reagent
[GelStar® Nucleic Acid Stain (Lonza), SYBR® Green Nucleic Acid Stain (Lonza), or Ethidium Bromide]
Note: When using GelStar® or SYBR® Green I for staining a gel, a filter designated for use with GelStar® or SYBR® Green I should be used.
-Distilled sterilized water

Equipment required:

-Authorized instruments for PCR
ex. TaKaRa PCR Thermal Cycler Dice (Cat.#TP600/TP650)
TaKaRa PCR Thermal Cycler Dice mini (Cat.#TP100)
-0.2 ml or 0.5 ml Microtubes for PCR (made of polypropylene)
ex. TaKaRa Micro PCR Tube (Cat.#9047)
TaKaRa 96 well PCR Hi-Plate (Cat.# NJ111)
TaKaRa PCR Hi-Caps (Cat.# NJ811)
-Agarose gel electrophoresis apparatus
ex. Mupid®-2 Plus (Cat.# AD110)
Mupid®-S (Cat.# AD120)
-Microcentrifuge
-Micropipets and pipette tips (autoclaved)

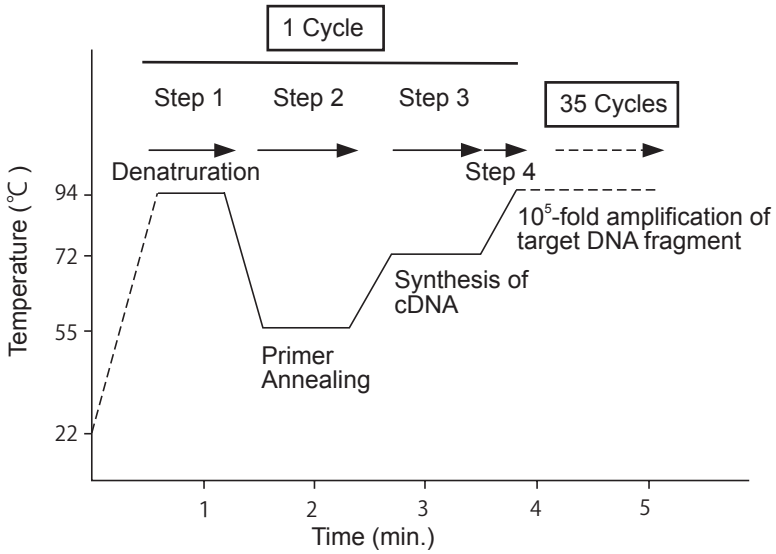
Storage: -20℃

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. (1988) *Science* **239**, 487-491.
2) Scharf, S. J., Horn, G. T. and Erlich, H. A. (1986) *Science* **233**, 1076-1078.
3) Gyllensten, U. B. and Erlich, H. A. (1988) *Proc. Natl. Acad.Sci. USA* **85**,7652-7656.
4) Kawasaki, E. S., Clark, S. C., Coyne, M. Y., Smith, S. D., Champlin, R., Whitte, O. N. and McCormick, F. P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5698-5702.
5) Lawyer, F. C., Stoffel, S., Saiki, R. K., Myambo, K. B., Drummond, R. and Gelfand, D. H. (1989) *J. Biol. Chem*. **264**, 6427-6437.

Principle:

PCR(Polymerase Chain Reaction) process 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 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. 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* (TaKaRa Cat.#R001). *TaKaRa Taq* is a recombinant, thermostable, 94kDa DNA polymerase encoded by DNA polymerase gene of the *Thermus aquaticus* YT-1 strain which has been cloned into *Escherichia coli* as the host and has been confirmed to have essentially the same characteristics as the native *Taq* DNA polymerase.



Step1: Denature the target double-stranded DNA fragment in the reaction mixture containing primer, dNTP, and polymerase : 94℃ , 30 sec.
Step2: Anneal primer to obtained single-stranded DNA : 55℃ , 30 sec.
Step3: Synthesize cDNA with DNA polymerase : 72℃ , 1 min.
Step4: 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 as one cycle and perform 25 cycles. Parameters must be optimized for a target DNA fragment as the most efficient condition for PCR varies depending on a target DNA fragment.

Protocol:
In case of using TaKaRa PCR Thermal Cycler Dice
A. Control experiment

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

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

Reagents	Volume	Final concentration
10x PCR Buffer*	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 units/50 µl
Control Template	0.5 µl	0.5 ng/50 µl
dH_2O	39.25 µl	
Total	50 µl	

*10x PCR Buffer(-) (Mg^{2+} Free) and $MgCl_2$ solution shall be used respectively instead of 10x Buffer if necessary.

2) If necessary, overlay mineral oil.
3) Place tubes in a thermal cycler.
4) Perform the reaction under the following conditions.

・When amplifying 6,012 bp with control primer 1 and 2:

94℃ , 1 min. (denaturation)
68℃ , 4 min. (annealing and extension) — 30 cycles
72℃ , 5 min. — 1 cycle

・When amplifying 500 bp with control primer 1 and 3:

94℃ , 30 sec. (denaturation)
55℃ , 30 sec. (annealing)
72℃ , 30 sec. (extension) — 25 cycles
72℃ , 2 min. — 1 cycle

B. Experiment with the samples

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 DNA templates depending on the size of target sequence and the sequence and size of primers.

C. Electrophoresis

1) Take 5~10 µl of PCR amplified samples and add its 1/6 volume of 6x Loading Buffer.
2) Pipet the samples into the agarose gel slots, and run the gel. The conditions of agarose gel vary according to sizes of amplified DNA.
3) After electrophoresis is completed, stain gels by soaking in GelStar, SYBR® Green I or Ethidium Bromide solution(1 µg/ml) for 20-30 min.
4) Verify the bands of amplified DNA under UV illumination.

Note:

1) Mix the kit components vigorously for approximately 2 sec. except *TaKaRa Taq* and centrifuge. *TaKaRa Taq* should be mixed gently by pipetting.
2) When there is a possibility that template DNA is contaminated with protease, add *TaKaRa Taq* after the first denaturation step. Protease is inactivated and will not decompose *TaKaRa Taq*.
3) $MgCl_2$ concentration of supplied 10x PCR Buffer may not be the optimum concentration for a sample DNA depending on how it 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.
4) In case of using 0.5 ml microtubes for PCR, it is recommended to prepare the reaction mixture to be the total volume of 100 μl.

Q&A

1. PCR Optimization

Optimal reaction conditions vary according to amplified sizes, reaction volumes, types of an used thermal cycler and so on.

i) Cycle numbers

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

ii) Denaturation conditions

When using thin-wall type PCR tubes, denaturation conditions recommended are either at 98℃ for 10 sec. or at 94℃ for 20 sec. When using normal PCR tubes, denaturation conditions are recommended to be either at 98℃ for 20 sec. or at 94℃ for 30 sec. A denaturation time that is too short or a denaturation temperature that is too low may cause either diffuse smearing upon electrophoresis 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 experimentally by varying temperatures in 2℃ increments over a range of 37-65℃ . As *TaKaRa Taq* shows sufficient activity at 60-68℃ , Shuttle PCR (Two Temperature PCR) can be conducted 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℃ , 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 dominantly non-specific, short products, while too long extension time causes diffusely smeared electrophoresis bands.

2. Primer preparation

Specificity of primers 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 temperature of paired primers should be within 2-3℃ .
2) Choose primers whose GC contents is around 50-60%.
3) Avoid primer sequence which form hairpin loops or primer-dimers, especially at the 3' end.

3. Primer Concentration

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

4. Enzyme Amount

Although it is recommended to use 1.25 units of *TaKaRa Taq* in a 50 μ l reaction, you may change the amount used for the optimum reaction condition. The following factors should be taken into consideration; the quantity or complexity of template DNA and the length of amplified DNA fragment. In case of excess enzyme, non-specific reactions may occur which may result in a diffuse smear upon electrophoresis. The efficiency of amplification may be diminished when the enzyme concentration is low.

5. Amplified products are entirely smeared upon electrophoresis.

Possible Causes	*Comments and suggestions*
Too much enzyme amount	Reduce the enzyme amount in 0.5 units steps.
Too short denaturation time	Increase the denaturation time by increments of 5 sec.
Too low denaturation temperature	Raise the denaturation temperature by 0.5℃ intervals.
Too low dNTP concentration	Increase the dNTP concentration in by increments of 50 μ M steps.
Too long extension time	Shorten the extension time by decrements of 30~60sec.
Too many number of PCR cycles	Reduce the number of cycles in steps of 2 cycles.
Too much template amount	Reduce the template amount by decrements of 20% of the previous one.

6. Multiple, nonspecific amplified products upon electrophoresis

Possible Causes	*Comments and suggestions*
Too high primer concentration	Decrease the primer concentration in steps of 0.1 μ M.
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 mer.
Too much enzyme amount	Reduce the enzyme amount in 0.5 units steps.
Too many number of PCR cycles	Reduce the number of cycles in steps of 2 cycles.
Too low annealing temperature	Raise the annealing temperature by 2℃ intervals.
Nonspecific annealing of primers occurs.	Use Hot Start method e.g. with *Taq* Antibody (Cat.#9002) to avoid this phenomenon during heating from room temperature to the denaturation temperature (94-98℃)
Too short extension time	Increase the extension time by increments of 15~30sec..
Poor denaturation	Raise the denaturation temperature by 0.5℃ intervals and extend the time by increments of 5 sec.
Too much template amount	Reduce the template amount by decrements of 20% of the previous one.

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