Cat. # RR027

For Research Use

TaKaRa

TaKaRa *Bacillus anthracis* PCR Detection Kit

Product Manual

v202601w

Table of Contents

I. Description

Anthrax (*Bacillus anthracis*) is a spore-forming, aerobic, gram-positive *bacillus* (1-2 x 5-10 μm). The virulence is due to the presence of two plasmids (pX01 and pX02). Plasmid pX01 encodes three toxic components (PA: protective antigen, LF: lethal factor, EF: edema factor). Plasmid pX02 encodes a group of genes involved in capsule synthesis (*capA*, *capB*, and *capC*). Both of these two plasmids are responsible for virulence.
PCR is a technique for amplifying exclusively the fragments of the genes of interest in a short period of time using a trace amount of DNA as template. The cycle comprising three steps of thermal denaturation, primer annealing and extension with DNA polymerase are repeated, thereby amplifying the gene fragments of interest up to 1,000,000-fold in quite a short period of time.
This kit is designed to detect the PA gene contained in plasmid pX01 and the *capA* gene in plasmid pX02 by PCR amplification and agarose gel electropheresis.
As this kit utilizes efficient PCR enzyme for hot start PCR, *TaKaRa Ex Taq*® Hot Start Version (Cat. #RR006), the kit can prevent nonspecific amplification due to mispriming and/or formation of primer dimers during reaction set-up before thermal cycling and it achieves highly sensitive detection.
Moreover, this kit contains internal control to allow for monitoring false negatives.

* This kit is originally designed for a primary quick detection.
For the final judgement of the presence of anthrax, it is recommended to combine with a result of a general microbiological test, such as gram staining.

The construction of this kit was realized by the courtesy of Dr. Sou-ichi Makino, Obihiro University of Agriculture and Veterinary Medicine.

II. Components (For 48 tests)

1.	*TaKaRa Ex Taq* HS (5 units/μl)	12.5 μl
2.	5X Reaction Mixture*1	500 μl
3.	PA Primers (PA7, PA6) (10 μM each)	200 μl
4.	CAP Primers (MO11, MO12) (10 μM each)	200 μl
5.	100 bp DNA Ladder (650 ng/5 μl)	50 μl
6.	6X Loading Buffer*2	60 μl

*1 dNTP Mixture and Internal Control are included.
*2 36% Glycerol, 30 mM EDTA, 0.05% Bromophenol Blue, 0.035% Xylene Cyanol

Primer	Sequences	Size of the amplified target products	Size of the amplified internal controls
PA Primers			
(PA7)	(5'-ATCAC CAGAG GCAAG ACACC C-3')	211 bp	409 bp
(PA6)	(5'-ACCAA TATCA AAGAA CGACG C-3')		
CAP Primers			
(MO11)	(5'-GACGG ATTAT GGTGC TAAG-3')	591 bp	98 bp
(MO12)	(5'-GCACT GGCAA CTGGT TTTG-3')		

III. Storage -20℃

IV. Materials Required but not Provided

[Reagents]

1. Sterile purified water
2. PrimeGel™ Agarose PCR-Sieve (Cat. #5810A)*
3. Electrophoresis buffer
   Tris-Borate-EDTA Buffer (TBE) Powder, pH8.3 (Cat. #T9121)* etc.
4. DNA Stainer
   SYBR™ Green I Nucleic Acid Gel Stain (Cat. #5760A/5761A)*

[Equipment]

1. Authorized thermal cycler
   Clontech PCR Thermal Cycler GP (Cat. #WN400)*
   TaKaRa PCR Thermal Cycler Dice™ Gradient (Cat. #TP600: discontinued)*
   TaKaRa PCR Thermal Cycler Dice *Touch* (Cat. #TP350: discontinued)*
2. Electrophoresis apparatus
3. Power supply
4. Ultraviolet transilluminator (Wavelength 300 nm)
5. Polaroid camera or digital imaging system to photograph stained gel
6. Heating block (applicable at 95°C)
7. Refrigerate centrifuge, compatible with 1.5 ml tubes

[Others]

1. 0.2 ml PCR tube
2. Micropippet
3. Micropippet tips (with hydrophobic filter)
4. Tray for staining agarose gel

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

V. Precautions for Use

1. Primers are susceptible to degradation by nuclease and, if degraded, cannot provide accurate detections. Take care to avoid nuclease contamination from sources such as perspiration or saliva introduced during sample handling.
2. Samples that test positive should be subjected to an additional microbiological test to verify the result.
3. We recommend designating and physically segregating the 4 areas described below for the indicated processes.
   - ○ Area 1: Preparation of reaction solution to tubes
   - ○ Area 2: Preparation of samples
   - ○ Area 3: Addition of samples to reaction solution and will do the reaction
   - ○ Area 4: Detection by electrophoresis

   Do not open the cap of tubes containing amplified PCR product in areas other than Area 4.

VI. 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), and the two primers complementary to the 3' region of the target segment are annealed at low temperature (annealing step). The primer is 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 exponentially, up to 10^6-fold amplification in only a few hours.

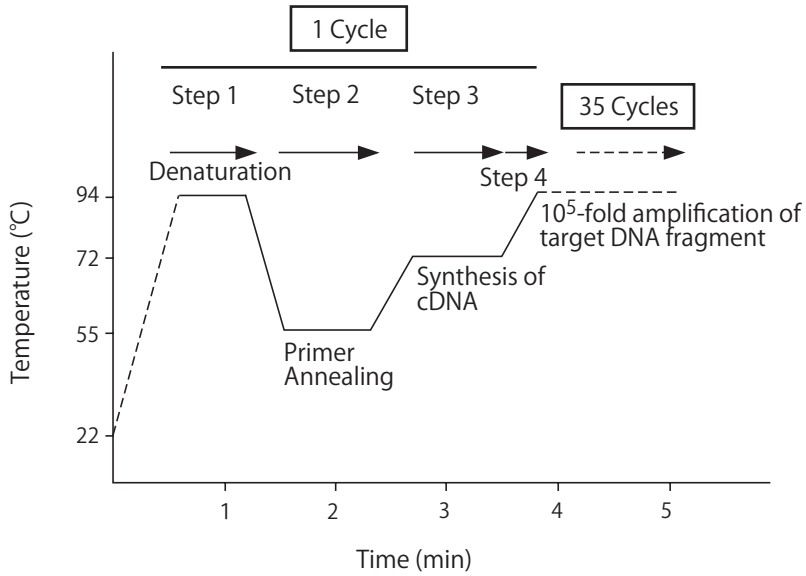


Figure 1. Process of DNA amplification by PCR

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

Step 2: Anneal primer to obtained single-stranded DNA.

Step 3: Synthesize DNA with DNA polymerase.

Step 4: Return to Step 1 to denature the synthesized double-stranded DNA again to yield single-stranded DNA.

One set of the consecutive 1 - 4 steps is referred as one cycle.
Perform 35 cycles.

VII. Protocols

1. Sample preparation

[from bacterial culture]

1) Add 10 μl of bacterial culture to 100 μl of sterile purified water and heat the mixture at 95℃ for 15 min.
2) Centrifuge, and use the supernatant for assay. Apply 1 μl of the supernatant directly to PCR reaction.

[from bacteria on agar plate]

1) Pick a tiny amount of the bacteria with a sterile stick, and suspend it in 100 μl of sterile purified water. (Trace amount is sufficient.)
2) Heat the suspension at 95℃ for 15 min.
3) Centrifuge, and use the supernatant for assay. Apply 1 μl of the supernatant directly to PCR reaction.

[from powder sample]

1) Suspend a tiny amount of the sample powder in 1 ml of sterile purified water.
2) After centrifugation, suspend the pellet in 1 ml of sterile purified water again for washing.
3) Centrifuge again, and finally re-suspend the pellet in 100 μl of sterile purified water.
4) Heat the suspension at 95℃ for 15 min.
5) Centrifuge, and use the supernatant for assay. Apply 1 μl of the supernatant directly to PCR reaction.

Note: Samples may contain dangerous pathogenic substances. So very careful attention must be paid during the operation. After the operation, all tools, instruments, cultures and solutions should be treated according to the safety instructions pre-defined at each laboratory.
When a sample is judged as positive, it should be verified also by microbioassay.

2. PCR

1) Prepare the following reaction solution each 0.2 ml PCR tube.

Reagent	Volume	Final conc.
5X Reaction Mixture	10 μl	1X
CAP Primers (10 μM)	4 μl	0.8 μM each
PA Primers (10 μM)	4 μl	0.8 μM each
Template*	1 μl	
TaKaRa Ex Taq HS (5 U/μl)	0.25 μl	0.025 U/μl
Sterile purified water	30.75 μl	
Total	50 μl	

* Prepare one tube including sterile purified water instead of template. This is used as negative control.

2) Reaction conditions

95℃ 2 min
↓
95℃ 15 sec ┐
60℃ 15 sec ├ 35 cycles
72℃ 30 sec ┘
↓
72℃ 5 min

Apply 5 - 10 μl to the subsequent gel electrophoresis analysis. Reactant can be stored at 4℃ or -20℃.

3. Preparation of agarose gel.

1) Dispense electrophoresis buffer into a triangle flask and slowly add PrimeGel Agarose PCR-Sieve to the concentration of 3% (w/v) with mixing.
2) Heat for 2 - 3 min in a microwave. After heating, mix well and confirm that the agarose is uniformly solved. Heat the slurry again for the minimum time required to allow all of the grains of agarose to dissolve.
3) Set up the gel board.
4) After the agarose gel solution cools to 50 - 60℃, pour the solution into the gel board and insert a comb to generate wells. Leave for 30 min - 1 hour at room temperature and harden the gel.

   When staining the gel with ethidium bromide before applying samples.
   Cool the solution to 50 - 60℃ and add ethidium bromide solution in a final concentration of 0.5 μg/ml and mix gently to be dissolved uniformly.
5) Remove the comb carefully not to tear the wells or break the gel.
6) Mount the gel in a electrophoresis tank.
7) Pour the electrophoresis buffer into the electrophoresis tank so that the gel is completely immersed.

4. Gel electrophoresis

1) Connect the electrical leads taking care not to mistake anode and cathode. As the DNA is negatively charged, it migrates from cathode to anode.
2) Add 1 - 2 μl of 6X loading Buffer to each tube containing 5 - 10 μl of PCR reactant and mix. Slowly load the mixture into the wells of the submerged gel using a micropipette. DNA marker (2.5 μl 100 bp DNA Ladder + 0.5 μl 6X Loading Buffer) should be loaded into wells on both the right and left sides of the gel.
3) Apply a constant voltage of 50 - 150 V and run the gel until the Bromophenol Blue* have migrated 3 - 4 cm in front of the comb.

* Bromophenol Blue migrates faster.

5. Verification of stained band

Note: When the gel has been prestained with ethidium bromide, perform only 3).

1) Prepare enough 1 μg/ml ethidium bromide solution or SYBR Green I* solution (pre-diluted by 10^4-fold with TE buffer or electrophoresis buffer) to submerge the gel, and place it in a staining tray.
2) Place the gel in the tray and leave it without agitation for 20 - 30 min.
3) Set the gel on an ultraviolet transilluminator and photograph the gel.* Verify the size of reactant bands by comparing with DNA marker.

* When using SYBR Green I for staining, a filter designated for use with SYBR Green I should be used.

CAUTION : Gloves should be worn in handling Ethidium bromide, SYBR Green I, and the stained gel.

VIII. Interpretation

When PA (protective antigen) gene exists in the sample, the band of 211 bp would appear.
When CAP gene (involved in capsule synthesis) exists in the sample, the band of 591 bp would appear.
When amplifying internal control, 409 bp band would appear for PA Primers, and 98 bp band would appear for CAP Primers.

(1) In case that each gene is positive or under detection limit. (Figure 2)

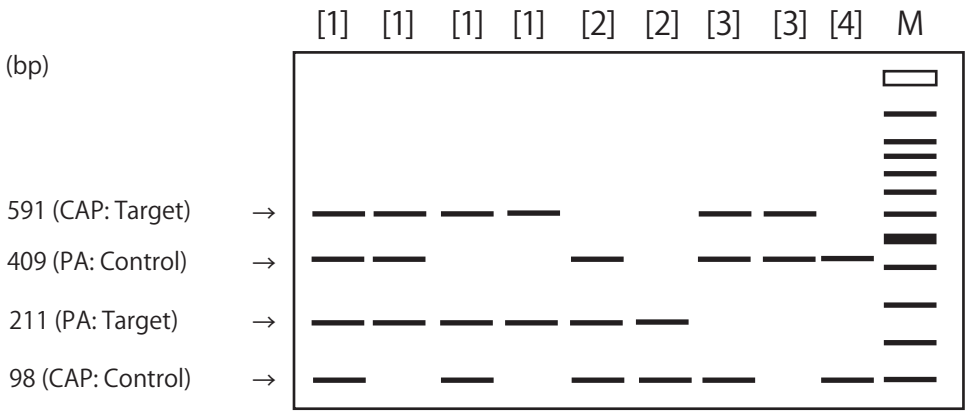


Figure 2. Electrophoresis result

Electrophoresis result	Interpretation
[1] Both bands of 211 bp and 591 bp appear.	Regardless of the existence of band of internal control (409 bp and 98 bp), positive for both PA and CAP genes. When PA gene and CAP gene exist in a sample in a large amount, the band of internal control would disappear.
[2] Band of 211 bp appears.	Regardless of the existence of band of internal control (409 bp), positive for PA gene. When many PA gene exists in a sample in a large amount, the band of internal control (409 bp) may disappear.
[3] Band of 591 bp appears.	Regardless of the existence of band of internal control (98 bp), positive for CAP gene. When many CAP gene exists in a sample in a large amount, the band of internal control (98 bp) may disappear.
[4] Neither 211 bp nor 591 bp appears. But internal control bands for both genes (409 bp and 98 bp) appears.	Below the detection limit for both PA and CAP genes.

(2) In case that one or both gene can not be judged.

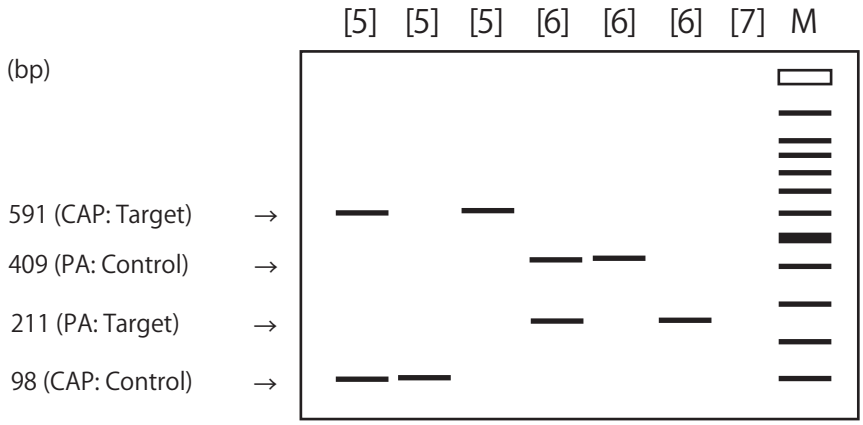


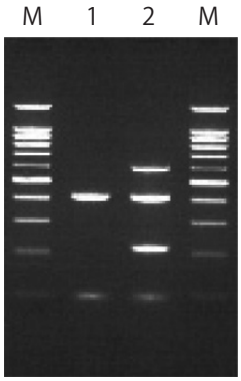
Figure 3. Electrophoresis result (2)

Electrophoresis result	Interpretation
[5] Neither band of 211 bp nor internal control (409 bp) appears.	Inconclusive whether is positive or lower than detection limit for PA gene. * Reaction might not be performed correctly. Retry the experiment. (Possible to judge for CAP gene.)
[6] Neither band of 591 bp nor internal control (98 bp) appears.	Inconclusive whether is positive or lower than detection limit for CAP gene. * Reaction might not be performed correctly. Retry the experiment. (Possible to judge for PA gene.)
[7] No band appear.	Inconclusive about both PA and CAP genes. * Reaction might not be performed correctly. Retry the experiment.

(3) Reaction of negative control

- Only 409 bp, 98 bp band appears;	PCR reaction was performed correctly.
- 211 bp, 591 bp band appears;	Contamination is suspected.
- Either 409 bp or 98 bp band does not appear; Or neither bands appears.	There may have been incorrect operation during the protocol. Or primers may have been degraded. Or enzyme may have been inactivated.

IX. Experimental Example



M:	100 bp DNA Ladder
1:	Negative Control
2:	CAP&PA Positive Control

CAP Positive Control = *cap* region (M24150)[4] cloned into pHY300PLK
PA Positive Control = *pagA* gene (M22589) cloned into pHY300PLK

X. References

1) H. I. Cheun, S. -I. Makino, M. Watarai, T. Shirahata, I. Uchida, and K. Takeshi. A simple and sensitive detection system for *Bacillus anthracis* in meat and tissue. *Journal of Applied Microbiology.* (2001) **91**: 421-426.
2) S. -I. Makino, H. I. Cheun, M. Watarai, I. Uchida, and K. Takeshi. Detection of anthrax spores from the air by real-time PCR. *Letters in Applied Microbiology.* (2001) **33**: 237-240.
3) S. -I Makino, Y. Iinuma-Okada, T. Maruyama, T. Ezaki, C. Sasakawa, and M. Yoshikawa. Direct Detection of *Bacillus anthracis* DNA in Animals by Polymerase Chain Reaction. *Journal of Clinical Microbiology.* (1993) **31**: 547-551.
4) S. -I. Makino, I. Uchida, N. Terakado, C. Sasakawa, and M. Yoshikawa. Molecular Characterization and Protein Analysis of the *cap* Region, Which Is Essential for Encapsulation in *Bacillus anthracis*. *Journal of Bacteriology.* (1989) **171**: 722-730.
5) S. Makino, C. Sasakawa, I. Uchida, N. Terakado, and M. Yoshikawa. Cloning and CO_2-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. *Molecular Microbiology.* (1988) **2**: 371-375.