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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 toxin 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 must be present in the organisms for virulence. PCR is a technique for amplifying specifically 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 denaturation, primer annealing and extension with DNA polymerase are repeated, thereby amplifying the gene fragments of interest up to 10⁶-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 using the thermal cycler for real time PCR, Smart Cycler® System (Cepheid). As this kit uses Takara's PCR enzyme efficient for Hot Start PCR, *Takara Ex Taq* R-PCR (#RR007), non-specific amplification deriving from mispriming or from primer-dimers before thermal cycling can be avoided and it achieves highly sensitive detection.

This kit employs Cycling Probe Technology for detection, which is a high-sensitive detection method utilizing a combination of chimera probe, composed of RNA and DNA, and RNase H. The specific sequence of target gene to be amplified can be detected efficiently during or after amplification by this method. The 5' end of the probe is labeled with a fluorescent substance and the 3' end is labeled with a quencher, which quenches the fluorescence emitted from the fluorescent substance at the 5' end. As long as this probe remains intact, no strong fluorescence can be emitted because of the quenching function. When this probe forms a hybrid with the complementary sequence of amplified product, RNase H specifically cuts the RNA region of this probe, resulting in emission of strong fluorescence. By measuring the intensity of emitted fluorescence, the amount of amplified product can be monitored. This kit contains two kinds of FAM labeling probes for detecting PA gene or *capA* gene, internal controls and ROX labeling probes for detecting the internal controls.

Real time PCR, in which this kit is used in combination with Smart Cycler® System, eliminates the need for electrophoresis and gives results rapidly (about 45 minutes). Moreover, this kit contains internal control to allow for monitoring false negatives.

* This kit is originally designed for quick and easy 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.

CycleavePCR *Bacillus anthracis* Detection Kit Ver.1.1 (Real Time PCR Ver.)

TAKARA

#RR029
v.0305

II. Kit contents (25 µl x 96 reactions; for each 48 reactions of PA and CAP detection)

TAKARA Ex Taq R-PCR	5 units / µl	25 µl (96 reactions)
Tli RNase H II ^{*1}	200 units / µl	50 µl (96 reactions)
5 x Reaction Mixture ^{*2}	5 x conc.	500 µl (96 reactions)
PA Primers (PA7, PA6)	3 µM each	250 µl (48 reactions)
CAP Primers (MO11, MO25)	3 µM each	250 µl (48 reactions)
PA Chimera Probe Mix (FAM, ROX)	12.5 x conc.	100 µl (48 reactions)
CAP Chimera Probe Mix (FAM, ROX)	12.5 x conc.	100 µl (48 reactions)
PA Positive Control	1 x 10 ⁴ copies / µl	20 µl (20 reactions)
CAP Positive Control	1 x 10 ⁴ copies / µl	20 µl (20 reactions)
dH ₂ O		1.3 ml

*1 Tli RNase H II is a thermostable RNase H derived from *Thermococcus litoralis*.

*2 Including dNTP Mixture and Internal Control.

*3 Be sure to store the fluorescent labeling probes in the light-shielding environment.

PA Chimera Probe Mix

	Fluorescent dye for probe	Detection channel
PA	FAM and quencher	Ch1
Internal Control	ROX and quencher	Ch4

CAP Chimera Probe Mix

	Fluorescent dye for probe	Detection channel
CAP	FAM and quencher	Ch1
Internal Control	ROX and quencher	Ch4

Primer	Sequences	Size of the amplified target products	Size of the amplified internal controls
PA Primers (PA7) (PA6)	(5'-ATCAC CAGAG GCAAG ACACC C-3') (5'-ACCAA TATCA AAGAA CGACG C-3')	211 bp	110 bp
CAP Primers (MO11) (MO25)	(5'-GACGG ATTAT GGTGC TAAG-3') (5'-CAATA GCTCC TGCTA CAAAT G-3')	179 bp	108 bp

III. Required reagents and equipment (primary items)

- [Reagents] Sterilized distilled water
- [Equipment] 1. Thermal cycler for real time PCR
Smart Cycler® System (Cepheid)
2. Special tubes for real time PCR reaction
3. Desk-top centrifuge
4. Heat block (applicable at 95°C)
- [Others] 1. Micropipets for 200 µl, 20 µl and 10µl.
2. Micropipet tips (with hydrophobic filter)

IV. Storage -20°C (for shipping and storage)

V. Precautions and warnings

1. Smart Cycler System should be operated in accordance with the instructions.
2. If a Chimera Probe Mix is decomposed due to contamination by nuclease, no correct result could be obtained. Since nuclease may invade into the probe from tester's sweat or saliva, special care must be taken during the use of this kit.
3. For the specimens determined to be positive, another microbiological test should be conducted.
4. Please divide physically the operation area into the following three parts for the procedures from preparation to detection. Do not open tubes containing amplified products within each area.

Area 1: Preparation and dispensing of reaction mixture

Area 2: Sample preparation

Area 3: Addition of sample into a reaction mixture, and perform reaction and detection.

As this kit performs amplification and detection simultaneously through real-time PCR, there is no need to use amplified product obtained from the reaction to subsequent process, such as electrophoresis, etc.

Please refrain from taking amplified products out of tubes. It can result in contamination.

VI. Protocols <Outline of protocol>

1. Sample preparation (see page 5)
 - from culture broth
 - from bacteria on plate
 - from powder sample
2. Setting of Smart Cycler® System (see pages 6-7).

Start Smart Cycler® System.

↓

Set the PCR conditions. [Define Protocols]
Set the graph view of the result. [Define Graphs]

↓

Set the parameters; number of reactions and the Protocol/Site to be used, and give a name to Run. [Create Run]

↓
3. Preparation of reaction solution and start of reaction (see page 8).

Prepare the reaction solution.

↓

Transfer the prepared solution into special reaction tubes and add a sample.

↓

Load the special tubes on Smart Cycler® System and start Run.

↓

4. Viewing of the result (see pages 9-11).
 - Select the graphs to be used. [Select Graphs]
 - ↓
 - Enter sample name. [Sample ID]
 - Set analytic parameters. [Analysis Setting]
 - ↓
 - An amplified curve is viewed on the screen in real time.
 - ↓
 - The reaction is terminated.
 - ↓
5. Judgement (see page 12).

VI-1. Sample preparation (Perform in Area 2)

[From culture broth]

- 1) Add 10 μ l of culture broth to 100 μ l of sterilized distilled water and heat the mixture at 95°C for 15 minutes.
- 2) Centrifuge, and use the supernatant for assay. Apply 1 μ l of the supernatant directly to PCR reaction.

[From bacteria on plate]

- 1) Pick a tiny amount of the bacteria with a sterilized stick, and suspend it in 100 μ l of sterilized distilled water. (The very slight amount is sufficient.)
- 2) Heat the suspension at 95°C for 15 minutes.
- 3) Centrifuge, and use the supernatant for assay. Apply 1 μ l of the supernatant directly to PCR reaction.

[From powder sample]

- 1) Suspend a tiny amount of the powder sample in 1 ml of sterilized distilled water.
- 2) After centrifugation, suspend the pellet in 1 ml of sterilized distilled water again for washing.
- 3) Centrifuge the suspension again, and finally re-suspend the pellet in 100 μ l of sterilized distilled water.
- 4) Heat the suspension at 95°C for 15 minutes.
- 5) Centrifuge, and use the supernatant for assay. Apply 1 μ l of the supernatant directly to PCR reaction.

NOTE: Samples may contain dangerous pathogenic microbes. 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.

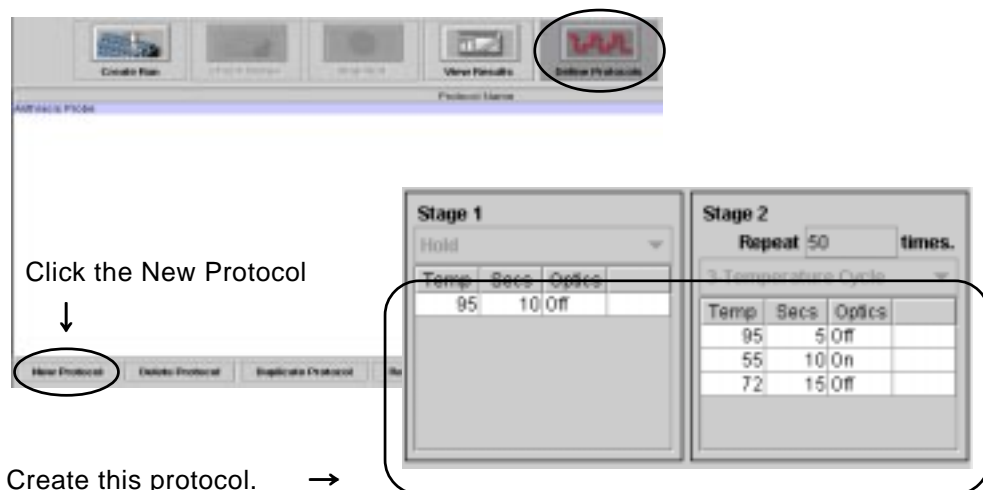
VI-2. Setting of Smart Cycler® System (Perform in Area 3)

(For more information on handling Smart Cycler® System, see the instructions supplied with it.)

(1) Start the Smart Cycler® System.

(2) Set the protocol.

Click the icon “Define Protocols” and then “New Protocol” button to create the protocol by following the steps shown below. (Since the created protocol is stored, no entry is required in subsequent reactions).



Create this protocol. →
 (PCR conditions)

(3) Set the graphs. (Since the created graphs are stored, no entry is required in subsequent reactions).

(3)-1. Set the amplified curve (FAM) for the amplified target product.

Click the icon “Define Graphs” and create the graphs by following the steps shown below.

(Since the graphs have been set under a name “FAM” at initialization, no entry is required here).



(3)-2. Set the amplified curve (ROX) for the amplified product derived from Internal Control.

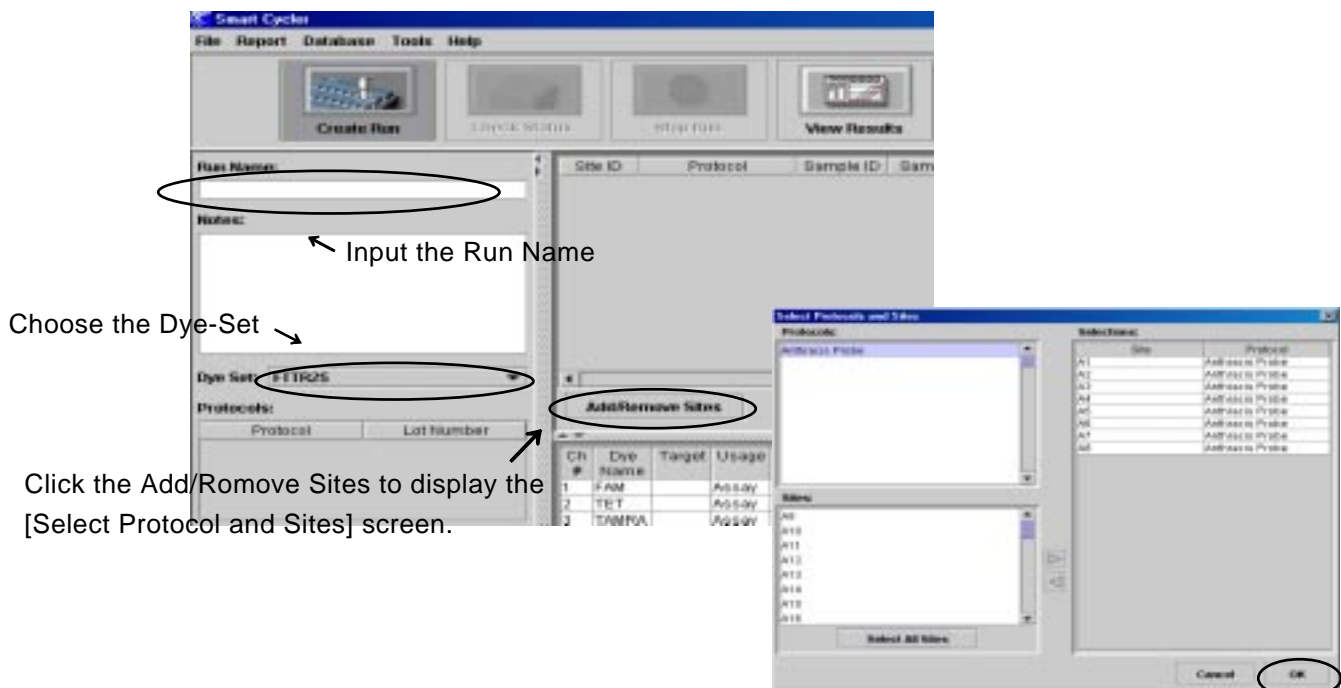
Click the icon “Define Graphs” and create the graphs by following the steps shown below.

(Since the graphs have been set under a name “ROX” at initialization, no entry is required here).



(4) Click the icon “Create Run”, enter Run Name, and select Dye-Set (FTTR25).

Click the “Add/Remove Site” button and the “Select Protocols and Sites” screen appears. From the menu, select the Site and Protocol to be used.



Choose the site and protocol to be used and click the [OK] button.

VI-3. Preparation of reaction solution and start of reaction

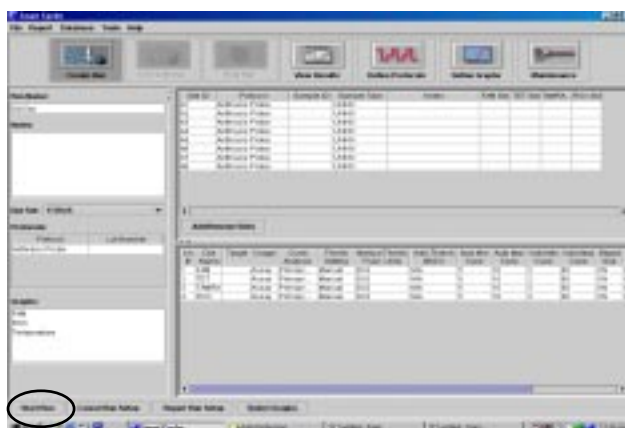
- (1) Prepare the reaction solution in accordance with the prescription described below and transfer it special tubes for real time PCR. (Perform in Area 1)
 Prepare two tubes for each specimen; one for PA detection and another for CAP detection and be sure to prepare the reaction solution separately for each tube.
 Prepare the required amount (desired number of tubes plus extra) of components other than Template DNA, transfer them into the tubes, and add Template DNA to each tube.
 Prepare the reaction solution using the sterilized distilled water instead of Template DNA as the negative control for each of PA and CAP detection.
 At the same time, it is recommended to perform the positive control reaction by using PA Positive Control and CAP Positive Control respectively.

	Volume	Final conc.
5 X Reaction Mixture	5.0 μ l	1 X conc.
PA or CAP Primers (3 μ M each)	5.0 μ l	0.6 μ M each
PA or CAP Chimera Probe Mix (12.5 X conc.)	2.0 μ l	1 X conc.
<i>TAKARA Ex Taq</i> R-PCR (5 units / μ l)	0.25 μ l	0.05 U / μ l
Tli RNase H II (200 units / μ l)	0.5 μ l	4 U / μ l
Template DNA (sample or control)	1.0 μ l	
dH ₂ O	11.25 μ l	
total		25.0 μ l

(NOTE) For PA detection, PA Primers and PA Chimera Probe Mix should be combined and for CAP detection, CAP Primers and CAP Chimera Probe Mix combined.

- (2) Addition of sample (template) (Perform in Area 3)
 Prepare one tube of negative control by adding sterilized distilled water instead of sample. For the rest tubes, add samples into the reaction mixtures prepared at the step 1). Close the tube lid tightly and centrifuge the tubes so that reaction mixture solutions are settled down in the bottom part of the tube.
- (3) Load the reaction tubes on Smart Cycler® System and click the “Start Run” button to start the reaction process.

Click the Start Run →

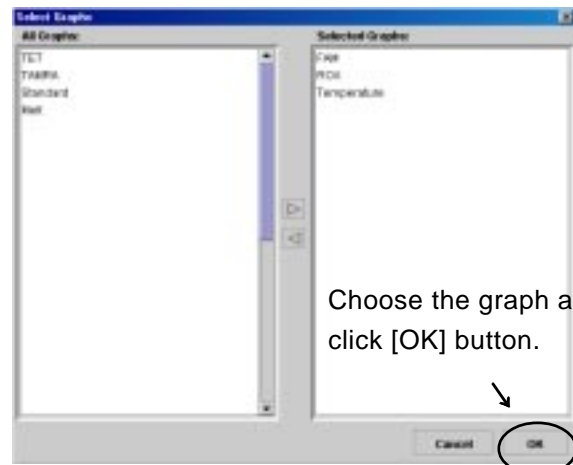


VI-4. Viewing of the result

- (1) View the View Results screen. (When the reaction process is started, the View Results screen automatically appears. If another screen is open, click the icon "View Results").
- (2) Click the "Select Graphs" button and the Select Graphs screen appears. From the menu, select FAM (the amplified curve for the amplified target product), ROX (the amplified curve for the amplified product derived from Internal Control), and Temperature (the temperature chart graph).



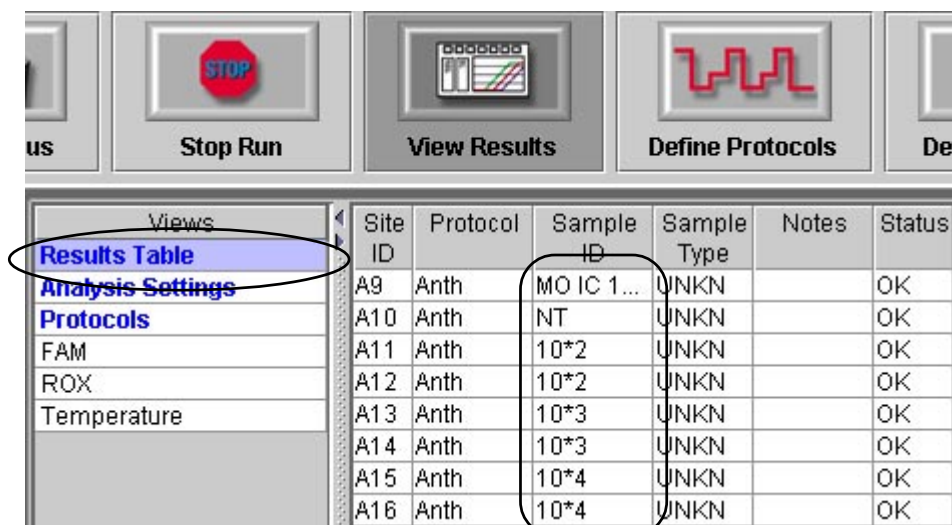
Click this button to view the Select Graphs screen. →



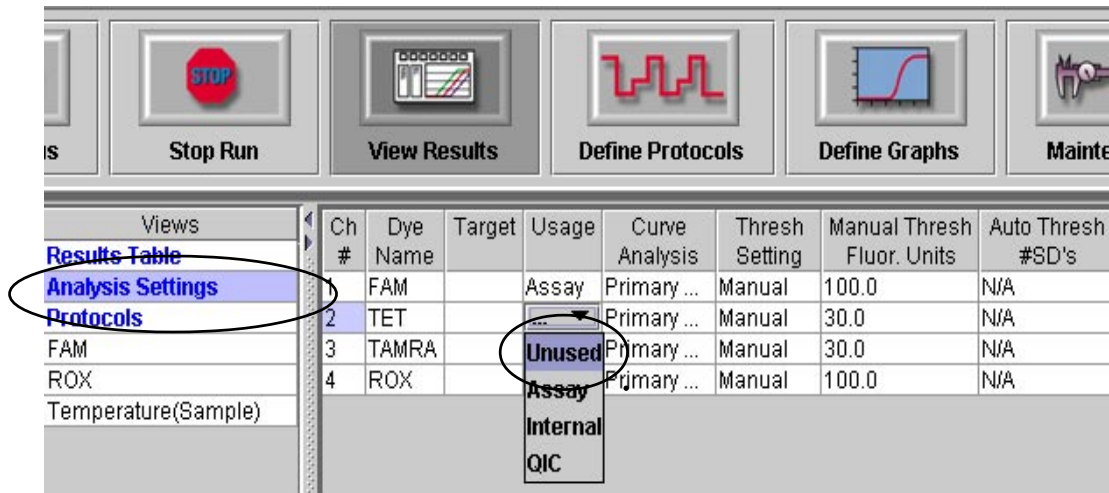
Choose the graph and click [OK] button.

* When the graphs for FAM, ROX, and Temperature have been selected at initialization, no entry is required here.

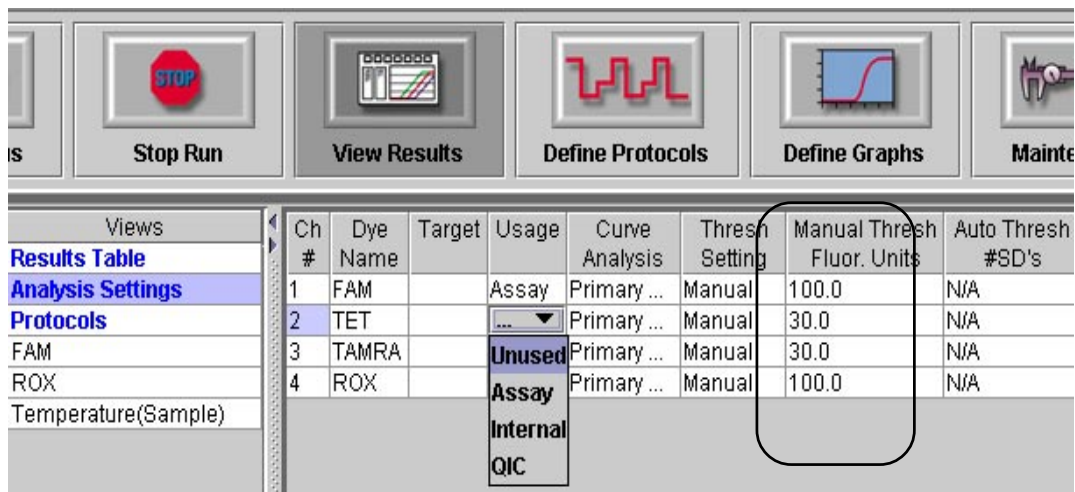
- (3) From the Views list, select "Results Table" and enter Sample ID.



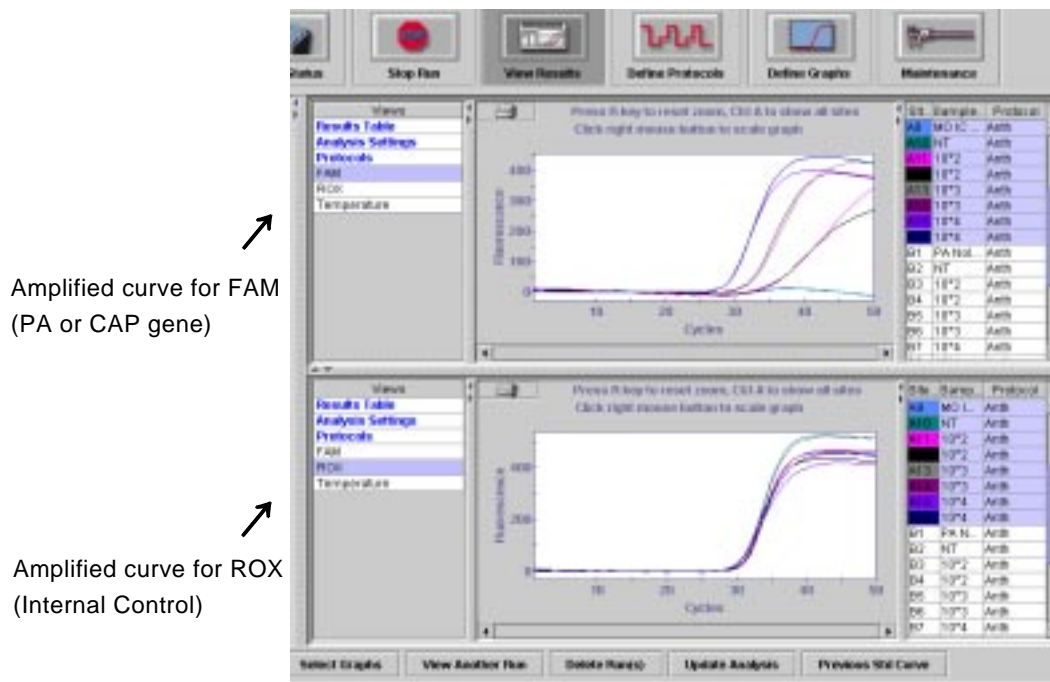
- (4) Click “Analysis Settings” for opening. Click “Usage” next to Ch#2 and Ch#3 and select “Unused” from the pull-down menu. (After this setting is activated, data on the Ch not used in Results Table is invisible.)



- (5) In the Analysis Settings screen, set Manual Thresh Fluor. Units for Ch#1 and Ch#4 to 100. This value is used as a cut-off value. (After entering data in the cells, click the “Update Analysis” button and the settings are activated.)



- (6) Select FAM in the upper Views to monitor the amplified curve for the amplified-target product in real time. (Wait for a while after the reaction process started and the graphs will be drawn.)
- (7) Select ROX in the lower Views to monitor the amplified curve for the amplified product derived from Internal Control in real time. (Wait for a while after the reaction process started and the graphs will be drawn.)



- (8) After the reaction process terminates, click “Results Table” for viewing. Look at data in the FAM Std/Res (Standard/Results) column for verifying the results. If the fluorescent signal value for amplified product derived from the PA or CAP gene is 100 or larger, “POS” is displayed in the cell of the FAM Std/Res while if it is smaller than 100, “NEG” is displayed. Similarly, the results derived from Internal Control appear in the ROX Std/Res column.



If any value appears instead of POS or NEG, see the section “Trouble shooting” on page 15.

VI-5. Judgement If in the 50-cycles real time PCR process, the fluorescent signal value for FAM detection is 100 or larger, "POS" is displayed in the cell of the FAM Std/Res (the results of detection of the fluorescent signal derived from PA or CAP gene) in the Results Table while if it is smaller than "100", NEG is displayed.

ROX Std/Res (the results of detection of the fluorescent signal derived from Internal Control) are also displayed in the same manner as those for FAM. Based on the results, judgement should be made with the reference table for judgement shown below.

(NOTE) In some cases, "POS" may appear in the cell of the FAM Std/Res or ROX Std/Res column accidentally due to the signal noises other than those from the amplified product. The shape of the amplified curve must be checked to see if a sigmoid curve has been drawn for correct determination.

→ See also the section "Trouble shooting" (2).

Quick reference table for judgement (reactions with a sample)

		ROX Std/Res (Internal Control)	
		POS	NEG
FAM Std/Res (Target)	POS	PA or CAP Positive *1	PA or CAP Positive *1
	NEG	PA or CAP Below the detection limit*2	Judgement impossible *3

Quick reference table for judgement (positive control reaction)

		ROX Std/Res (Internal Control)	
		POS	NEG
FAM Std/Res (Target)	POS	There is no problem in PA or CAP detection system	There is no problem in PA or CAP detection system
	NEG	There may be problem in PA or CAP detection system	Judgement impossible *3

Quick reference table for judgement (negative control reactions)

		ROX Std/Res (Internal Control)	
		POS	NEG
FAM Std/Res (Target)	POS	Contamination suspected *5	Contamination suspected *5
	NEG	No contamination	Judgement impossible *3

*1. The PA or CAP gene is determined to be positive whether the result of Internal Control is POS or NEG.

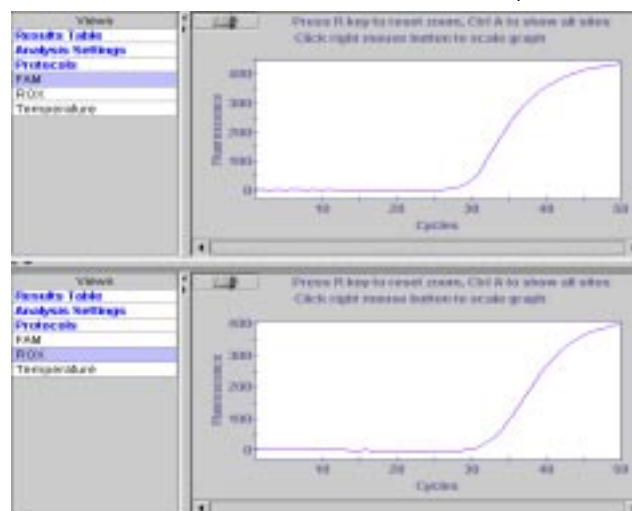
*2: Confirm that a positive control reaction is proved to be POS.

- *3. The PCR reaction or cycling probe detection process did not be correctly performed for any reason. Retry the reaction process.
- *4: It is suspected that there may be problem in Primers for target amplification or in Probe for target detection, or that Positive Control may be degraded.
- *5. Contamination is suspected. Retry after decontamination of the bench surface and the equipment used.

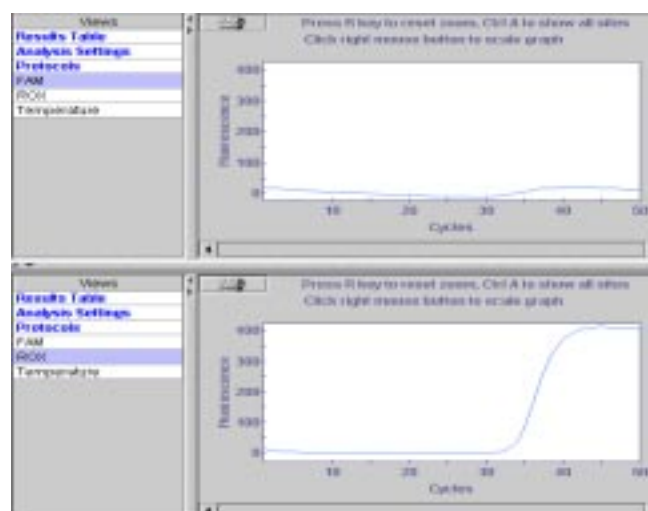
○ Reaction examples

(1) Detection of PA gene

(1)-1 Amplified curve for PA Positive Control (1×10^4 copies/ μ l) used as a template



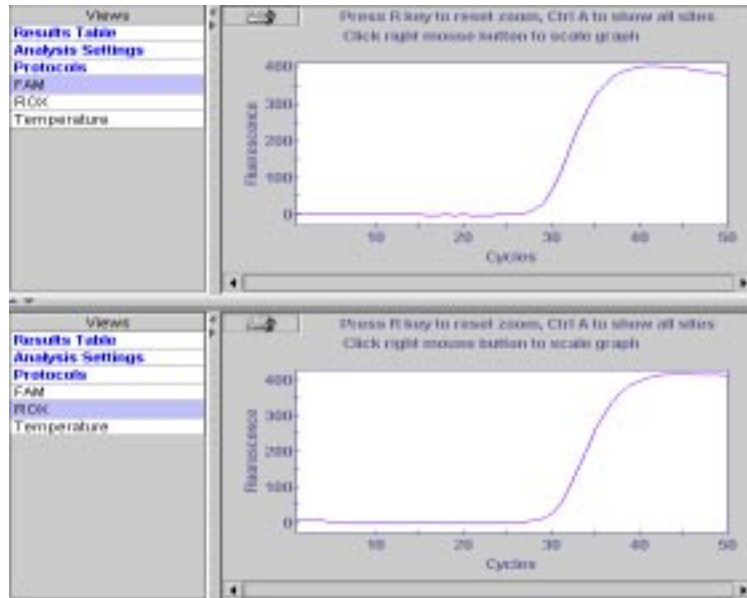
(1)-2 Amplified curve for negative control (sterilized distilled water)



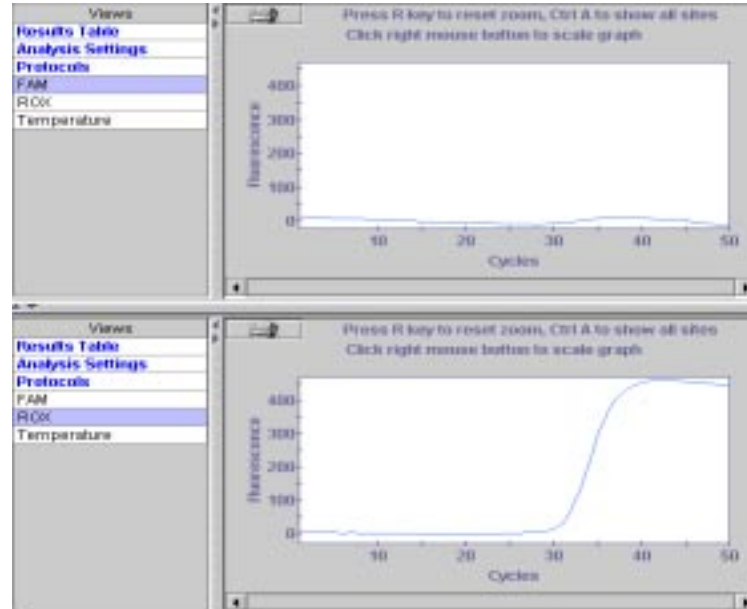
* If a weak fluorescent signal is detected, the amplified curve might be drawn. That is because the Y-axis scale on the graph is automatically adjusted to the value for the detected signal and the signal is apparently amplified, leading to a spurious amplified curve. For information on manual adjustment of the Y-axis scale, see the section "Trouble shooting" (3) on page 16.

(2) Detection of CAP gene

(2)-1 Amplified curve for CAP Positive Control (1 x 10⁴ copies/ml) used as a template



(2)-2 Amplified curve for negative control (sterilized distilled water)

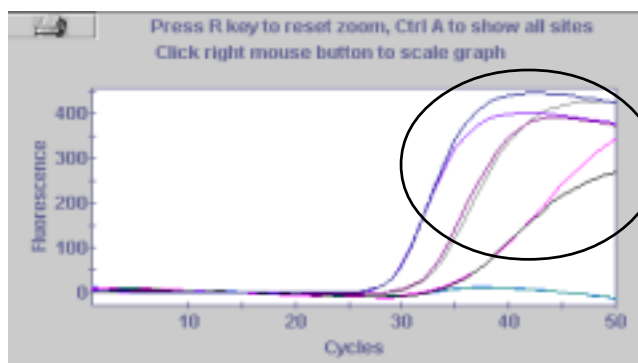


* If a weak fluorescent signal is detected, the amplified curve might be drawn. That is because the Y-axis scale on the graph is automatically adjusted to the value for the detected signal and the signal is apparently amplified, leading to a spurious amplified curve. For information on manual adjustment of the Y-axis scale, see the section "Trouble shooting" (3) on page 16.

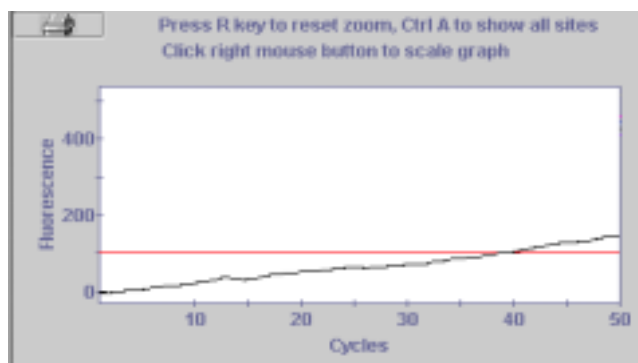
VII. Trouble shooting

- (1) A numeric value appears in the FAM or ROX Std/Res column instead of POS or NEG.
 - When Sample Type has been set to STD in Results Table, a numeric value appears.
 - All the data in the Sample Type must be set to UNKN.

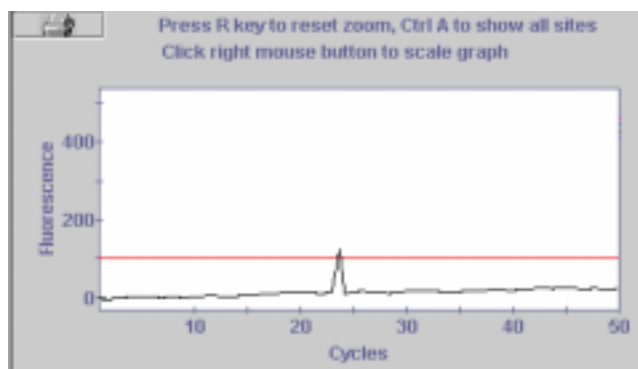
- (2) POS appears in the FAM Std/Res column accidentally due to, for example, the signal noises other than those from the amplified product.
 - Make judgement based on the shape of the amplified curve. If the judgement is difficult, retry the reaction process.



Amplified curve derived from an amplified product



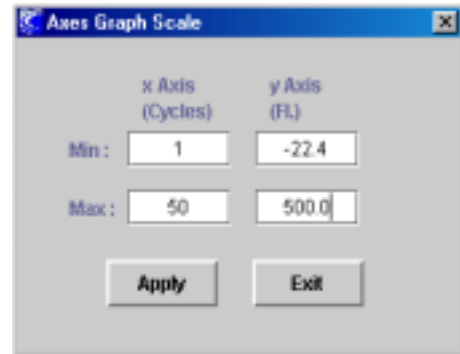
Increased signal due to any other reason than an amplified product



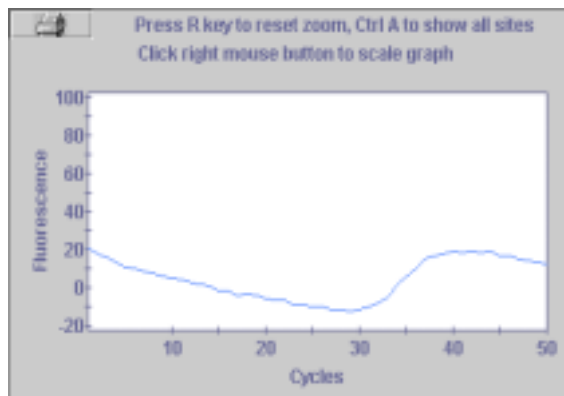
(3) The background appears to be high, because the weak fluorescent signal has been detected and the Y-axis scale on the graph has been automatically adjusted to the value for the detected signal.

→ Adjust the Y-axis scale manually.*

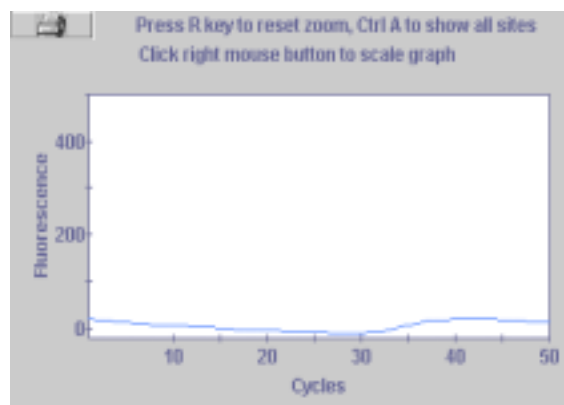
1. Click any point near the Y-axis scale with a right mouse button to open the Axes Graph Scale screen.
2. Enter an appropriate value (for example, the value along the Y-axis when the positive control reacts) in the Max field in the y Axis (F1.) and click the "Apply" button.
3. Click the "Exit" button to close the Axes Graph Scale screen.



* During reaction process, no scale can be adjusted. Scale adjustment must be done after all the reactions terminated.



Y-axis value adjusted to a weak fluorescent signal value



After the Y-axis Max value was manually set to 500

VIII. Reference

1. H.I.Cheun, S.-I.Makino, M.Watarai, T.Shirahata, I.Uchida and K.Takeshi (2001) A simple and sensitive detection system for *Bacillus anthracis* in meat and tissue. *Journal of Applied Microbiology* **91**, 421- 426.
2. S.-I.Makino, H.I.Cheun, M.Watarai, I.Uchida and K.Takeshi (2001) Detection of anthrax spores from the air by real-time PCR. *Letters in Applied Microbiology* **33**, 237- 240.
3. SOU-ICHI MAKINO, YUKIKO IINUMA-OKADA, TSUTOMU MARUYAMA, TAKAYUKI EZAKI, CHIHIRO SASAKAWA, AND MASANOSUKE YOSHIKAWA (1993) Direct Detection of *Bacillus anthracis* DNA in Animals by Polymerase Reaction. *Journal of Clinical Microbiology* **31** (3), 547- 551.
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5. S.Makino, C.Sasakawa, I.Uchida, N.Terakado and M.Yoshikawa (1988) Cloning and CO₂-dependent expression of the genetic region for encapsulation from *Bacillus anthracis*. *Molecular Microbiology* **2** (3), 371- 375.

IX. Related products

- Rapid Amplification System for real-time PCR
Smart Cycler® System (Cepheid)
- Enzyme for real-time PCR
TaKaRa Ex Taq R-PCR (#RR007)

**CycleavePCR *Bacillus anthracis* Detection Kit Ver.1.1
(Real Time PCR Ver.)**

TAKARA

#RR029
v.0305

**NOTE: For research use only.
Not for use in human and animal diagnostic or therapeutic.**

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