Cat. # CY205

For Research Use

TaKaRa

CycleavePCR™ *Salmonella* Detection Kit Ver. 2.0

Product Manual

v201903Da

Table of Contents

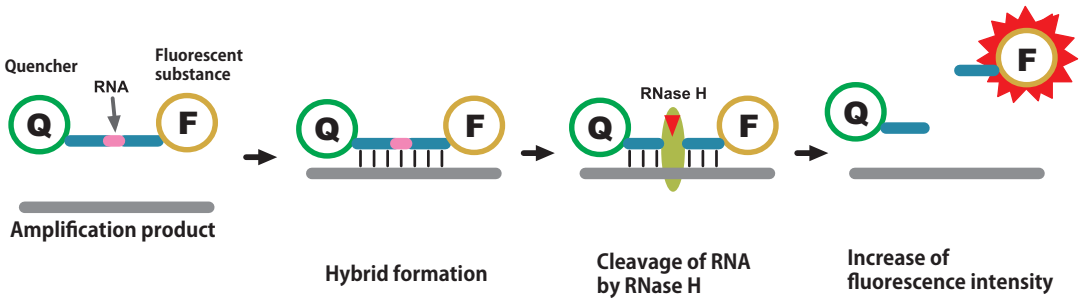
I. Description

Salmonella is a type of bacteria that belongs to the same group as *E. coli* and *Shigella*. The bacterium is widely found in nature and more than 2,500 different serotypes have been identified. *Salmonella enteritidis* is increasingly found to be the causative agent of food poisoning.

CycleavePCR *Salmonella* Detection Kit Ver. 2.0 is used to detect the invasive factor-related gene, *invA*, one of the genes present in the vast majority of *Salmonella* strains by real-time PCR. This kit enables amplification products to be detected by cycling probe technology, which provides highly sensitive detections through the combined use of a RNA/DNA chimeric probe and RNase H. This enables efficient detection of specific sequences of the gene fragment during and after amplifications. One end of the probe is labeled with a fluorescent moiety and the other end with a quencher. When intact, this probe does not emit fluorescence due to the action of the quencher. However, when it forms a hybrid with the complementary sequence of an amplification product, RNase H cleaves RNA in the chimeric probe, resulting in strong fluorescent signal emission (see Figure 1). The amount of amplified product can be monitored by measuring the intensity of emitted fluorescence.

This kit contains the FAM labeling probe for detecting *Salmonella invA* gene, internal control, and the ROX labeling probe for detecting the internal control. This kit is also able to monitor a false negative reaction through the use of internal control. The real-time detection with this kit does not require electrophoresis and the detection result can be obtained quickly .

This kit includes *TaKaRa Ex Taq* HS, a hot start PCR enzyme, which prevents non-specific amplifications caused by mispriming or primer dimer formation during reaction mixture preparation or other pre-cycling steps. The use of *TaKaRa Ex Taq* HS therefore allows high-sensitivity detections.



Principle of Cycling Probe Technology

II. Components (50 reactions, 25 μl volume)

1.	2X Cycleave Reaction Mixture	2X conc.	625 μl
2.	SIN Primer / Probe Mix (FAM, ROX labeled) *	5X conc.	250 μl
3.	dH_2O		1 ml
4.	SIN Positive Control		150 μl (30 reactions)

* Contains a fluorescent-labeled probe; store protected from light.
Primers are manufactured by SHIMADZU CORPORATION.

[Component Information]

2X Cycleave Reaction Mixture:

A PCR reaction reagent containing enzymes, buffer, dNTP mixture, and internal control DNA

SIN Primer/Probe Mix (FAM, ROX):

A mixture containing primers and probes for detecting the *invA* gene and internal control DNA.
The *invA* gene (the target gene) or the internal control DNA is amplified with the primers. The FAM-labeled probe detects the *invA* gene and the ROX-labeled probe detects the internal control DNA.

Target gene:

For this kit, the target gene is the *invA* gene.

Internal control DNA:

The internal control DNA has no sequences related to the target gene. It works to detect false negatives by its presence in every reaction in this kit. When no target is detected, a positive detection of the internal control DNA indicates the absence of PCR inhibition and indicates that the concentration of target in the sample is below the detection limit. Absence of both target and internal control DNA detections indicates that PCR did not occur properly. When there is a large amount of the target DNA, the amplification of the target is prioritized, resulting in the delay, weakening, or absence of the internal control DNA signal. In such situations, the assay can be interpreted as positive.

dH_2O:

Sterile purified water

SIN Positive Control:

A positive control for the *invA* gene

III. Storage

-20℃

IV. Materials Required but not Provided

[Instrument]

- Real-time PCR instrument
  Thermal Cycler Dice Real Time System *III* (Cat. #TP900/TP960)＊1, 2
  Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), etc.
  ＊1 Not available in the all geographic locations. Check for availability in your area.
  ＊2 0.2 ml 8-strip tubes with individual flat caps (Cat. #NJ600) for the Thermal Cycler Dice Real Time System are available for purchase. The use of such tubes is highly recommended for minimizing the risk of inter-tube contamination.
- Heat block (capable of temperature settings up to 95℃)

[Other]

- Tubes or 96 well plate for PCR
- 1,000 μl, 200 μl, 20 μl, and 10 μl micropipettes
- Micropipette tips (with hydrophobic filter)
- Benchtop centrifuge
- Refrigerated microcentrifuge (with 4℃ setting)

V. Considerations Before Use

- This kit is designed to detect bacterial DNA and can also detect non-viable bacteria. The bacterial DNA cannot be detected in some cases, when a mutation or deletion/ insertion occurs within the sequence covered by the Primer and Probe.
  (Takara Bio is not responsible for any actions taken as a result of analytical determinations made with this product.)
- When a sample is judged as positive, it should be verified also by microbioassay.

VI. Precautions

1. Operate real-time PCR amplification instruments in accordance with the manufacturer's instructions.
2. The chimeric probe and 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.
3. It is recommended to designate and physically segregate the 3 areas described below for the processes from preparation of reaction mixtures to detection. Avoid opening/closing tubes containing amplification products in any of these areas.

   Area 1: reaction mixture preparation and dispensing
   Area 2: sample preparation
   Area 3: addition of samples to reaction mixtures, reaction, and detection

   This kit allows amplification and detection to take place simultaneously in real time. Thus, no electrophoresis or other analytical methods are required after the reaction is complete. Never remove amplification products from tubes as doing so may introduce contamination.
4. Results obtained with this kit are interpreted based on analyses by a real-time PCR amplification instrument. Failure of any of the auto functions on the real-time PCR amplification instrument may lead to erroneous interpretation of results. Properly adjust the settings on the real-time PCR amplification instrument in accordance with the instrument manual when necessary.

VII. Protocols

[Overview]

1. Sample preparation
   Prepare bacterial heat-extracted samples from bacterial cultures.
2. Real-time PCR instrument setup
3. Reaction mixture preparation and reaction start
   Prepare the reaction mixture.
   ↓
   Dispense the reaction mixture into reaction tubes and add the negative control, the sample, or the positive control.
   ↓
   Set the tubes in the real-time PCR instrument, then start the reaction.
   ↓
4. Displaying results
   The amplification curves are displayed.
   ↓
   Reaction completion
   ↓
   Interpretation

VII-1. Sample Preparation (Work in Area 2)

[Preparation of heat-extracted bacterial samples]

(1) Dispense 10 μl of the bacterial culture into a 1.5 ml tube.
(2) Add 90 μl of sterile water and mix.
(3) Incubate at 95℃ for 5 min.
(4) Centrifuge at 12,000 rpm, 4℃ for 10 min, and then collect the supernatant for use as the heat-extracted sample for *invA* gene detection. Use 5 μl per reaction.

- ＊ If the PCR reaction was inhibited when using the heat-extracted samples prepared by this method, try the PCR reaction again using 10-fold and 100-fold diluted samples. Use sterile purified water to prepare the dilutions.
- ＊ Prepare bacterial cultures from food samples in accordance with the applicable standard protocol.
- ＊ Heat-extracted samples may be stored at -20℃.

VII-2. Preparation of Reaction Mixture

This kit allows the simultaneous detection of amplification products from both the *invA* gene and the internal control in a single reaction tube. To obtain accurate detection results, perform positive and negative control reactions for the *invA* gene simultaneously.

(1) Prepare the following reaction mixture on ice. (Work in Area 1)

Mix components other than the sample in volumes sufficient for the required number of tubes plus a few extra. Dispense aliquots of 20 μl in PCR tubes and cap loosely. Prepare one of the tubes as a negative control by adding 5 μl of dH_2O and then cap the tube tightly.

The required number of PCR tubes is defined as the number of samples + 2 (one for the negative control reaction and one for the positive control reaction). To maximize the reliability of result interpretation, it is recommended to run at least 2 reactions per sample.

Reagent	Volume (per reaction)	Final Conc.
2X Cycleave Reaction Mixture	12.5 μl	1X
SIN Primer/Probe Mix（5X conc.）	5 μl	1X
Sample or SIN Positive Control or dH_2O	（5 μl）＊	
○ dH_2O	2.5 μl	
Total	25 μl	

＊ Add the sample or the SIN Positive Control in step (2), not in this step.

[Precaution]
Do not touch tubes and caps with bare hands; doing so may introduce noise during detection of fluorescent signal.

(2) Add the sample (template) (Work in Area 3)

Add either the sample or the SIN Positive Control to each tube except the negative control tube and then cap tightly.

Briefly centrifuge the tubes in benchtop centrifuge and then set them in a real-time PCR instrument.

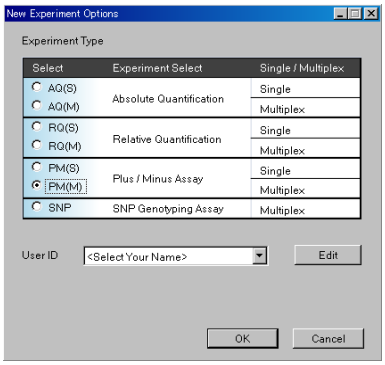
[Precaution]
Start reactions within 1 hour of preparing the reaction mixtures.

VII-3. Amplification and Detection (Work in Area 3)

For operation, see the instrument manual of the real-time PCR instrument used overview of operation and analysis are described here for the Thermal Cycler Dice Real Time System *II* and the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific).

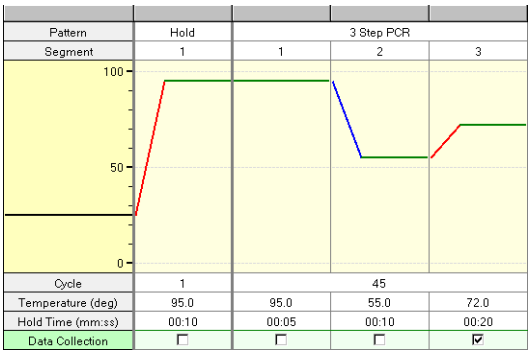
[For Thermal Cycler Dice Real Time System *II*]

(1) Open a new run file. On the "New Experiment Options" screen, select Experiment Type: "Plus/Minus Assay Multiplex"

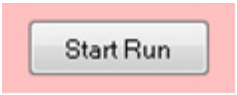


(2) On the "Thermal Profile Setup" screen, make sure both detection filters, FAM and ROX, are checked and the PCR conditions are set as follows:

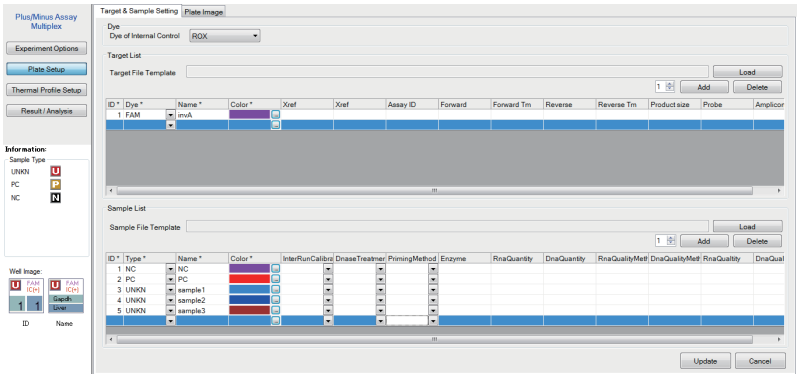
Initial denaturation (Hold)
Cycle: 1
95℃ 10 sec
3 step PCR
Cycle: 45
95℃ 5 sec
55℃ 10 sec
72℃ 20 sec (detection)



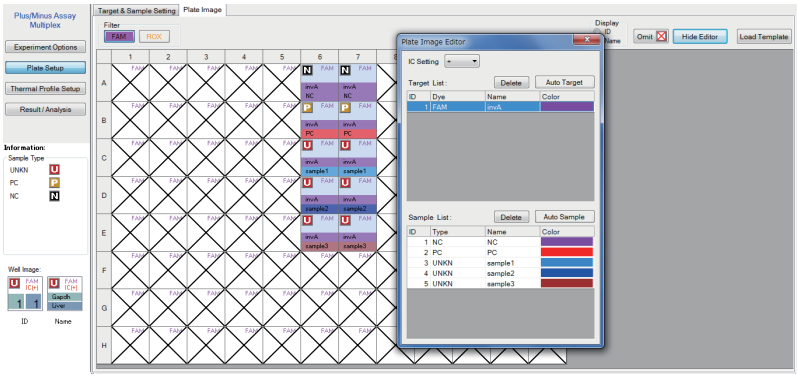
(3) Click the "Start Run" button on the bottom right-hand corner of the screen and enter a run file name to start the reactions.



(4) On the "Plate Setup" screen, enter the sample information.
For the internal control "Dye" setting, select "ROX". After targets and sample informations are entered, click the "Update" button.

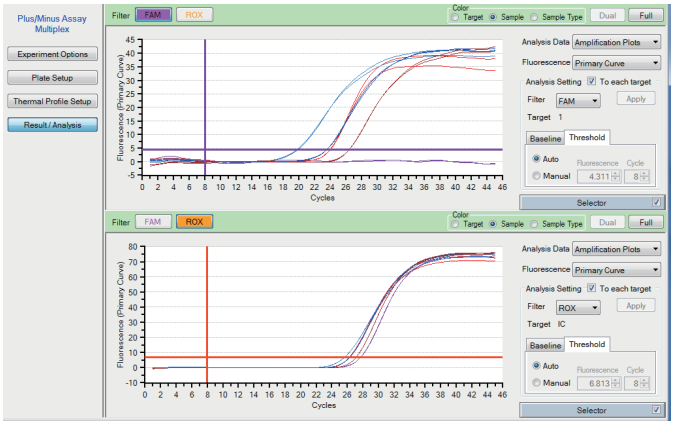


After clicking the "Plate Image" tab, select the desired wells. Select "Omit" for unused wells.



(5) Analysis of results

1. After reactions are completed, click the "Result/Analysis" button.

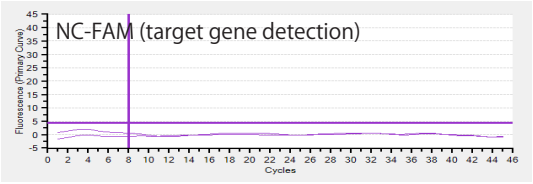


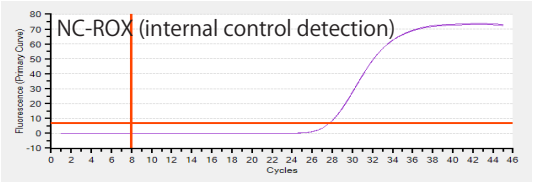
Two screens are displayed: the amplification curve of the target-detecting FAM filter at the top and the amplification curve of the internal control-detecting ROX filter at the bottom. (The threshold setting is "Auto.")

2. Check amplification curves for NC (negative control) and PC (positive control). Make sure the baseline provided by the FAM filter for NC is free of fluorescent signal changes and is below the threshold.

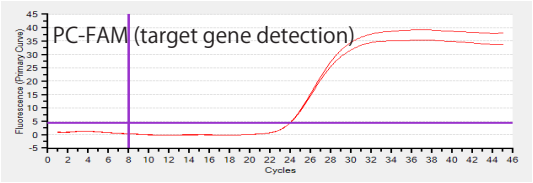
   If the threshold is exceeded, manually enter the threshold setting so that the baseline stays below the threshold.

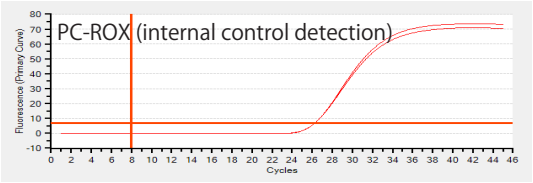
   Make sure the amplification curve for NC's ROX filter is displayed and that the curve exceeds the threshold.



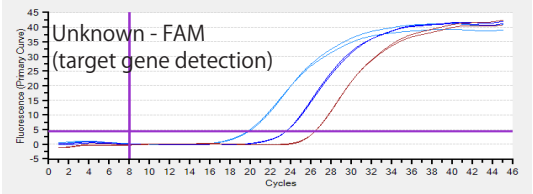


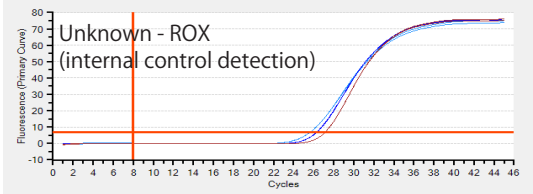
Make sure the amplification curve for PC's FAM filter is displayed and exceeds the threshold and that the amplification curve for the ROX filter is displayed and exceeds the threshold.



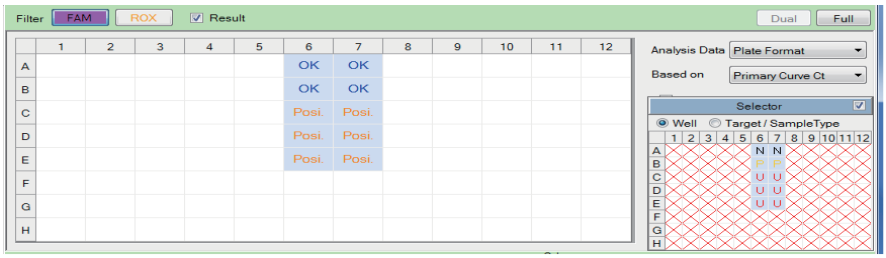


3. On the "Selector", select "U" to display the results. Then make sure the baseline and the amplification curve for the FAM and ROX filters are displayed with no abnormality.





4. On the "Analysis Data" column, display "Plate Format".



OK : The control reaction is normal (indicating the reaction performed correctly).
OUT : The control reaction is abnormal (indicating the reaction did not perform correctly).
Posi : The target gene detection is positive.
Nega : The target gene is below the limit of detection.
ND : Neither the internal control nor the target gene is detected; no interpretation available (indicating the PCR reaction failed to occur properly).
Error : Indicating different result interpretations within the same replicate.

■ Precautions for result interpretation

- When the FAM filter (target gene detection) shows an amplification curve (the result interpretation is "OUT") in the negative control reaction (NC);
  → Contamination may have occurred. Decontaminate the bench area used to prepare reaction mixtures as well as the apparatuses and instruments used. Then perform the reaction again.
- When both the FAM filter and the ROX filter (internal control detection) show no amplification curve (the result interpretation is "OUT") in the positive control reaction (PC);
  → The PCR reaction or the cycling probe detection failed to work properly. Repeat the reaction.
- When the ROX filter shows an amplification curve but the FAM filter does not show an amplification curve (the result interpretation is "OUT") in the positive control reaction (PC);
  → There may be a problem with the primer/probe mix or the positive control may have been degraded.
- When both the FAM filter and the ROX filter do not show an amplification curve (the result interpretation is "ND") in the sample reaction (UNKN);
  → The PCR or the cycling probe detection failed to work properly. Repeat the reaction.
  The sample may contain a reaction inhibitor. Perform the reaction again with diluted samples. Alternatively, prepare the sample again and then perform the reaction again.
- When the FAM filter shows an amplification curve but the ROX filter does not show an amplification curve in the sample reaction (UNKN);
  → When there is a large amount of target DNA, the amplification of the target may be prioritized, resulting in the competitive inhibition of the amplification for the internal control DNA. (The result interpretation displayed is "Posi".) The detection system performed properly.

[For Applied Biosystems 7500 Fast Real-Time PCR System]

(1) Prepare a new experiment in “Advanced Setup”.

(2) In “Experiment Properties,” select “Quantification-Standard Curve” and “TaqMan Reagents” or “Other.” (If “Other” is selected, deselect the check mark for “Include Melt Curve)”.

(3) For “Define Target” in “Plate Setup,” set “Target Name” to “SIN” “Reporter” to “FAM” and “Quencher” to “(None)”.

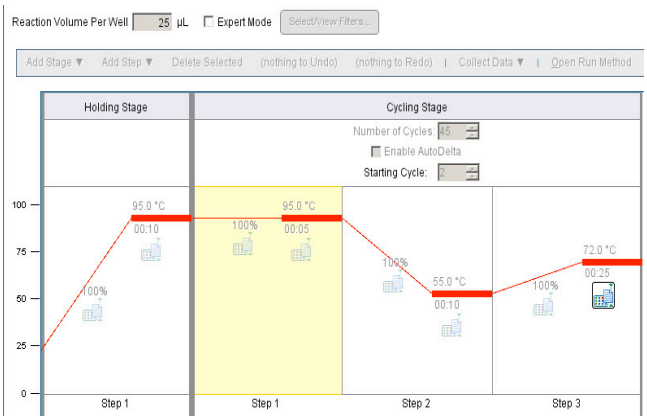
(4) For “Define Target” in “Plate Setup,” set “Target Name” to “IC” “Reporter” to “ROX” and “Quencher” to “(None)”.

(5) Enter sample names for the Negative Control (NC), Positive Control (PC) and experimental samples in “Define Samples”.

(6) Indicate the plate layout using the settings established in Steps (2), (3), (4) above. Set “Passive Reference” to “(None)”.

(7) Click the “Instrument” tab and enter the reaction conditions below.

Initial denaturation (Hold) Cycle : 1 95℃ 10 sec 3 step PCR Cycle : 45 95℃ 5 sec 55℃ 10 sec 72℃ 25 sec (detection)

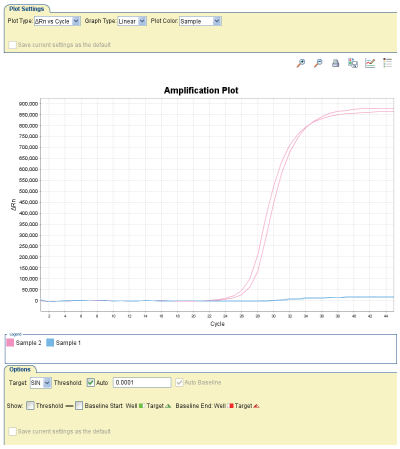


(9) Place the PCR tubes in the instrument and click the “Start” button.

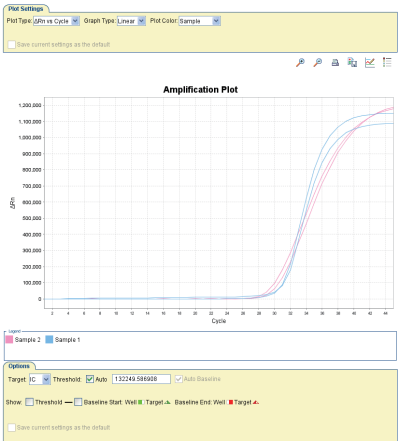
(10) After the reaction is complete, the amplification curves may be confirmed using "Amplification Plot" on the "Analysis" screen.

＊ "Threshold" and "Baseline" are to be set manually when necessary.

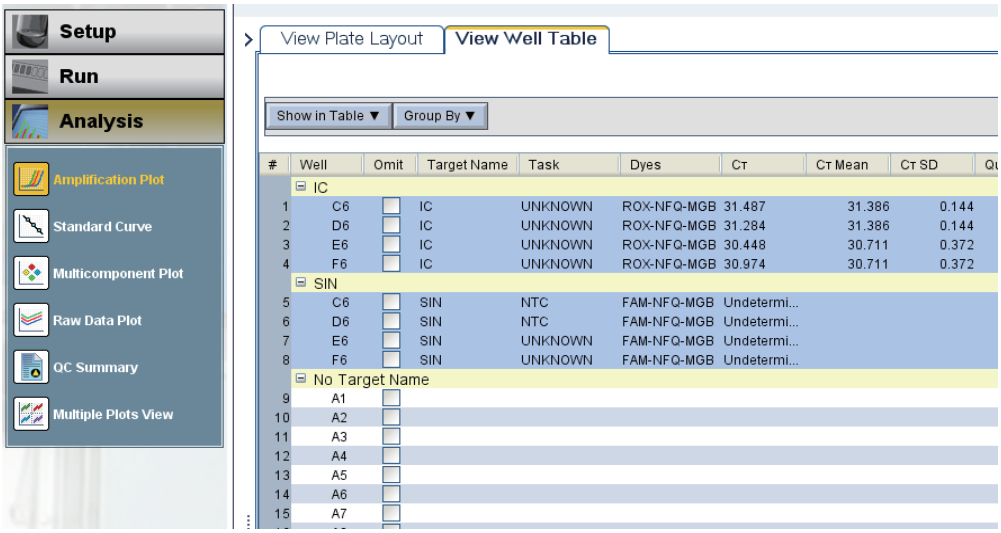
SIN detection (FAM)



Internal control DNA detection (ROX)



(11) Clicking the "View Well Table" tab will allow you to see the data obtained.



＊ The same procedure can be used for the StepOnePlus Real-time PCR System. However, since the detection sensitivity for ROX is low, the ROX (IC) amplification curve will appear small when all targets are displayed simultaneously. Analyze the FAM and ROX targets displayed separately.

VIII. Interpretation

Note : Perform a final interpretation of the assay based on all results including those of the control reactions.

Sample reaction

		ROX (internal control)	
	Amplification	(+)	(−)
FAM (*invA*)	(+)	*invA*, positive＊1	*invA*, positive＊1
	(−)	*invA*, below the limit of detection＊2	No data available＊3

Positive control reaction (using the SIN Positive Control)

		ROX (internal control)	
	Amplification	(+)	(−)
FAM (*invA*)	(+)	No problem with the reaction	No problem with the reaction
	(−)	Trouble in the reaction＊4	No data available＊3

Negative control reaction (using dH2O)

		ROX (internal control)	
	Amplification	(+)	(−)
FAM (*invA*)	(+)	Possible contamination in the reaction＊5	Possible contamination in the reaction＊5
	(−)	No problem with in the reaction	No data available＊3

＊1 Regardless of the result of the internal control DNA detection (+)/(−), *invA* is positive. Verify the absence of contamination in the reaction system based on the result of the negative control reaction.

＊2 Verify that the result is (+) for the Positive Control detection (indicating no problem with the reaction).

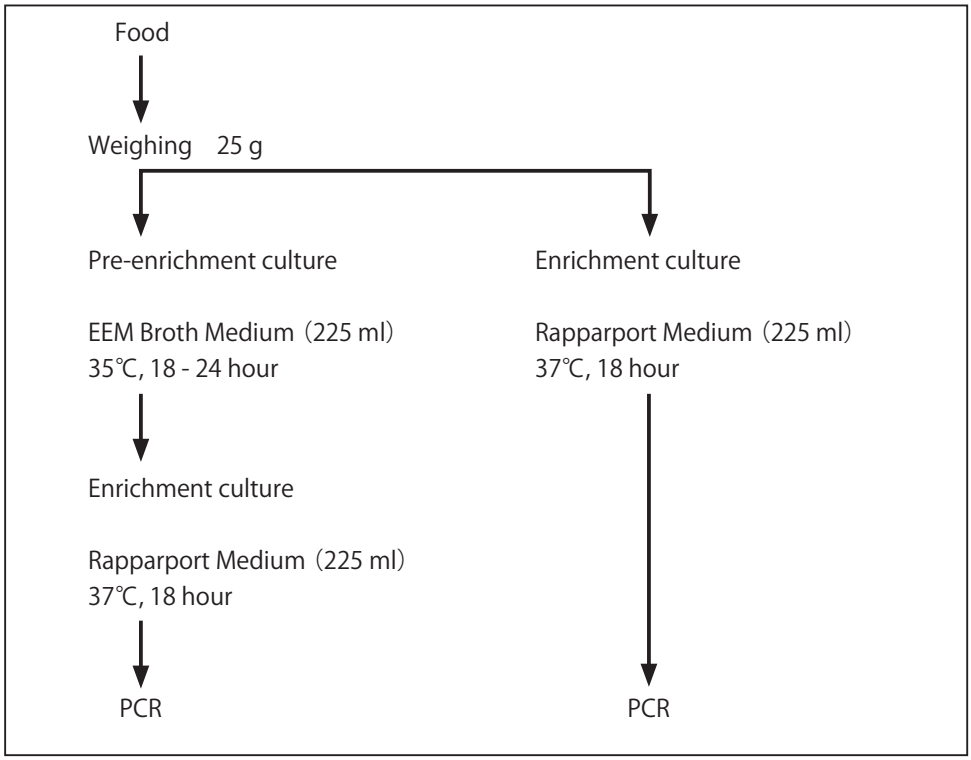
＊3 The PCR reaction or the cycling probe detection failed to work properly for some reason. Perform the reaction again. Because the sample may contain a reaction inhibitor, it may be necessary to prepare the sample again.

＊4 Either there is a problem with the SIN Primer/Probe Mix, or the SIN Positive Control is degraded.

＊5 Decontaminate the bench area and apparatuses used for preparing reaction mixtures.

IX. Experimental Example

PCR Detection Method from the Food



X. References

1) Saiki R, *et al*. *Science.* (1985) **37**: 170-172.
2) Galan J E, *et al*. *J Bacteriol*. (1992) **174**: 4338-4349.
3) Ashok K, *et al*. *Microb Pathog*. (1994) **19**: 85-95.

XI. Related Products

Thermal Cycler Dice™ Real Time System *II*（Cat. #TP900/TP960）
96 well Hi-Plate for Real Time（Cat. #NJ400）
Sealing Film for Real Time（Cat. #NJ500）
Plate Sealing Pads（Cat. #9090）
0.2 ml Hi-8-tube（Cat. #NJ300）
0.2 ml Hi-8-Flat Cap（Cat. #NJ302）
0.2 ml 8-strip tube, individual Flat Caps（Cat. #NJ600）

NOTE : This product is for research use only. It is not intended for use in therapeutic or diagnostic procedures for humans or animals. Also, do not use this product as food, cosmetic, or household item, etc.

Takara products may not be resold or transferred, modified for resale or transfer, or used to manufacture commercial products without written approval from Takara Bio Inc.

If you require licenses for other use, please contact us by phone at +81 77 565 6973 or from our website at www.takara-bio.com.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product web page. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.