Cat. # RR250A

For Research Use

TaKaRa

TaKaRa qPCR *Norovirus* (GI/GII) Typing Kit

Product Manual

v202108Da

Table of Contents

I. Description

Norovirus (NoV) is the major causative agent of acute viral gastroenteritis. Vomit or feces from infected individuals, as well as bivalve mollusks (e.g., oysters) contaminated with NoV, are suspected as the source of infection. The main symptoms of NoV include vomiting, diarrhea, abdominal pain, and fever (37 - 38°C) after a latent period of several days.

NoV has a positive-sense single-stranded RNA genome, and has cup-shaped structural proteins in its surface. Since current culture techniques do not readily permit the isolation and detection of NoV, laboratory diagnoses have relied on techniques such as reverse transcription PCR and real-time PCR. NoV is divided into five genogroups (I through V). Studies have indicated that genogroups I and II (GI and GII) infect humans.

This kit uses real-time PCR with probes and primers for detecting GI and GII. Real-time PCR methods are simple, rapid, and highly specific. They have been used in recent years in laboratory diagnosis of various pathogens, including NoV in foods. This kit uses the same probe sequences provided in Norovirus Detection Method, a notification issued by the Inspection and Safety Division, Department of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare of Japan (Notification No. 1105001 of ISD-DFS; latest version, Notification 1022 No. 1 of ISD-DFS, October 22, 2013; hereafter called the "official method").

A combination of *TaKaRa Ex Taq*® HS, a hot-start PCR enzyme formulated with an anti-*Taq* antibody, and a buffer optimized for real-time PCR allows for high amplification efficiency and high detection sensitivity while reducing non-specific amplification. This kit is suitable for high-speed PCR and allows for accurate target quantification and detection over a broad dynamic range for highly reproducible and reliable real-time PCR analyses.

Amplification products are detected using the TaqMan probe, the same label used in the official method. The TaqMan probe is a modified oligonucleotide with a 5′ fluorophore (e.g., FAM) and a 3′ quencher (e.g., TAMRA). Under annealing conditions, the TaqMan probe hybridizes specifically to the template DNA, but the quencher suppresses fluorescence. During the extension phase of the reaction, the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase degrades the hybridized TaqMan probe, releasing quencher suppression and allowing fluorescence detection (see Figure 1). By measuring the intensity of the emitted fluorescence, the amount of amplified product can be monitored.

This product was developed based on studies conducted in collaboration with the Gunma Prefectural Institute of Public Health and Environmental Sciences.

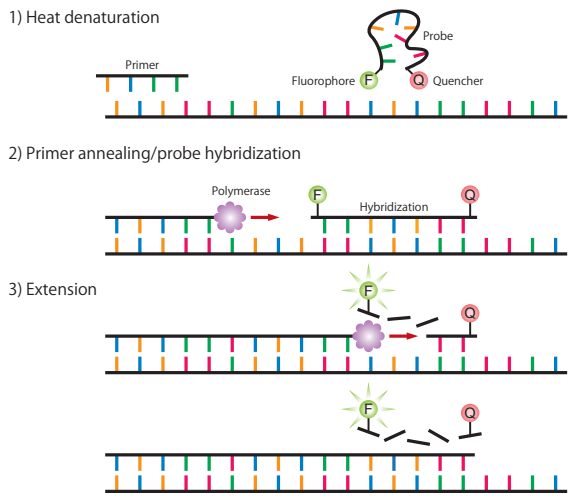


Figure 1. Principle of probe technology

II. Components (for 50 RT reactions, 50 GI reactions*1, 50 GII reactions*1)

1.	5X PrimeScript™ Buffer (NV)*2, 3		200 µl
2.	PrimeScript RT Enzyme Mix (NV)*2, 4		50 µl
3.	Random 6-mers (100 µM)*2		50 µl
4.	*Premix Ex Taq* (NV) (2X conc.)*5		625 µl x 2
5.	GI Primer Mix*6		50 µl
6.	GII Primer Mix*6		50 µl
7.	GI Probe Mix*6, 7		50 µl
8.	GII Probe Mix*6, 7		50 µl
9.	ROX Reference Dye (50X conc.)*8		50 µl
10.	ROX Reference Dye II (50X conc.)*8		50 µl
11.	RNase Free dH_2O		1 ml
12.	GI Positive Control DNA*9	4 x 10^6 copies/µl	20 µl
13.	GII Positive Control DNA*9	4 x 10^6 copies/µl	20 µl
14.	EASY Dilution (for Real Time PCR)*10		700 µl

*1 This kit allows up to 12 samples to be tested assuming that the standard curve is generated based on 8 serially diluted concentrations in triplicate (n = 3) and that the experimental samples and the negative control are tested in duplicate (n = 2).

*2 Reagent for reverse transcription.

*3 Contains dNTP Mixture and Mg^{2+}.

*4 Contains an RNase inhibitor.

*5 Contains *TaKaRa Ex Taq* HS, dNTP Mixture, and Mg^{2+}.

*6 Has the same nucleotide sequence as provided in the official method.

*7 Protect this component from light, it contains a fluorescently labeled probe.

*8 Use with an instrument that corrects fluorescent signal between wells, such as the real-time PCR instrument of Applied Biosystems.

◆ Use the ROX Reference Dye
- Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)

◆ Use the ROX Reference Dye II
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)

◆ Do not use this component
- Thermal Cycler Dice™ Real Time System *II* (Cat. #TP900/TP960: discontinued)

*9 Use this component to create a standard curve. No reverse transcription is required since it is a DNA control plasmid. Use the same method as that for the G1 and G2 control plasmids in the official method. Take care to avoid repeated freeze-thaw cycles.

*10 Use this component as a diluent to make serial dilutions of the positive control DNA.

III. Storage

-20℃

Premix Ex Taq (NV) (2X conc.), once thawed, should be stored at 4℃ and used within 6 months. For long-term storage, keep frozen at -20℃.
After opening, store components 12 and 13 away from the rest of the components to avoid cross-contaminating other reagents with positive control DNA.

IV. Materials Required but not Provided

【 Reagents 】
RNA extraction reagents

【 Apparatus 】
- 200 μl, 20 μl, and 10 μl micropipettes
- Micropipette tips with hydrophobic filters

【 Instruments 】
Real-time PCR thermal cycler system
- Thermal Cycler Dice Real Time System III with PC (Cat. #TP970)
- Thermal Cycler Dice Real Time System *Lite* (TP700/TP760: discontinued)
- Thermal Cycler Dice Real Time System *II* (Cat. #TP900/TP960: discontinued)
  0.1 ml 8-strip tube, individual Flat Caps (Cat. #NJ902)
  0.2 ml 8-strip tube, individual Flat Caps (Cat. #NJ600) for the Thermal Cycler Dice Real Time System Series are available for purchase. The use of these tubes is highly recommended for minimizing the risk of cross-contamination between tubes.
- Applied Biosystems StepOnePlus Real-Time PCR System, Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific), etc.

V. Precautions for Use

This section describes precautions for using this kit. **Make sure to read these instructions before use.**

1. Intended use: This kit is for analysis of environmental and food samples. It is intended for research use only.
2. Results of assay: This kit is designed to detect Norovirus GI/GII genes and can also detect inactivated viruses. (Takara Bio is not responsible for any actions taken as a result of analytical determinations made with this product.)
3. Disposal: Samples should be handled according to regulations governing the use of potentially infectious materials. Dispose of materials according to the safety regulations for your facility and in accordance with any applicable local, state, or federal regulations. Keep the work area sanitized at all times and sterilize all samples and equipment used during the experiment. Sterilization may be conducted by autoclaving at 121℃ for at least 20 min or by treatment with 2.5% sodium hypochlorite, followed by processing materials according to guidelines for potentially infectious waste. Process and dispose of plastic reagent containers and instruments according to regulations concerning the handling of hazardous materials.

VI. Protocol Precautions

1. Briefly centrifuge the PrimeScript RT Enzyme Mix (NV) before use to collect contents at the bottom of the tube. The enzyme is in a high-viscosity 50% glycerol solution. Pipette slowly and carefully.
2. When dispensing reagents, use fresh disposable tips to avoid cross-contamination between samples.
3. If nuclease contamination of samples, probes, or primers occurs, target PCR products will not be properly detected. Human perspiration and saliva may be a source of nuclease contamination. Wear disposable gloves and a face mask while performing the procedure.
4. *Premix Ex Taq* (NV) contains enzymes. Before use, make sure the reagent is evenly mixed by gently turning it upside down several times without creating bubbles; if inadequately mixed, it may not provide sufficient reactivity. Do not mix by vortexing.

   When stored frozen at -20℃, *Premix Ex Taq* (NV) may precipitate over time. Gently warm it by hand or let it briefly stand at room temperature, then invert the tube several times to dissolve the precipitate completely. Make sure it is evenly mixed before use.
5. We recommend that the following three areas be established in the laboratory working space and that they are physically separated from each other. Avoid opening or closing tubes containing amplification products in 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 the amplification reaction and detection to take place in real time. Therefore, the amplification products require no electrophoresis or other treatment after the reaction is complete. Never remove amplification products from tubes, as doing so may cause nucleic acid contamination in the laboratory workspace.
6. Operate real-time PCR devices in accordance with the user manual provided by the manufacturer.
7. When analyzing the results of real-time PCR, failure of various types of 'Auto' functions of real-time PCR instrument systems or software may cause incorrect analyses. If necessary, operate the real-time PCR instrument system with manual settings according to the instrument user manual.
8. This kit may not be able to detect NoVs with novel mutations in a region corresponding to the primer or the probe sequence.

VII. Quantitative Analysis with GI Positive Control DNA and GII Positive Control DNA

The GI and GII Positive Control DNA supplied with this kit are plasmids containing a NoV sequence synthesized by Takara Bio.

The DNA concentration of the positive control plasmids have been adjusted to give the same quantification results as those given by the G1 and G2 control plasmids in the official method. Thus, the same method as the official method applies.

Quantitative analyses can be achieved by generating a standard curve based on serial 10-fold dilutions of GI or GII Positive Control DNA prepared with EASY Dilution (for Real Time PCR). The quantifiable range depends on the data obtained. Repeated freeze-thaw cycles may affect the quantitative performance of the positive control plasmids.

VIII. Protocol

<Quantitative Analysis with Positive Control DNA>

Protocol overview

1. Sample preparation
2. Reverse transcription reaction
3. Real-time PCR instrument setup
4. Preparation of reaction mixtures and start of the reaction

   Prepare samples for the standard curve by making serial dilutions of GI and GII Positive Control DNA.
   ↓
   Prepare reaction mixtures.
   ↓
   Dispense the reaction mixture into reaction tubes and add either the negative control (sterile water), a sample for the standard curve, or an experimental sample.
   ↓
   Set the reaction tubes in the real-time PCR instrument and start the reaction.

5. Displaying results

   The amplification curves are displayed in real time on the instrument system's screen.
   ↓
   Reaction is complete.
   ↓
   Use the plotted standard curve to determine the number of NoV copies in an experimental sample.

VIII-1. Sample preparation (work in Area 2)

When preparing nucleic acid samples carefully avoid nuclease contamination by perspiration or saliva by wearing a mask, wearing a clean pair of disposable gloves, and designating a laboratory bench exclusively for preparing RNA samples.

Whenever possible, use disposable plastic laboratory supplies. RNase-OFF® (Cat. #9037), an RNase decontamination solution, is recommended for the removal of RNase on laboratory bench tops, apparatuses, and tubes. In addition, when using non-disposable materials (e.g., plastic and glass), designate them exclusively for work with RNA. Do not use them for other experiments.

Prepare RNA samples from specimens (e.g., fecal samples) in accordance with the procedure provided in the official method. Handle patients' fecal samples in a safety cabinet and take precautions to avoid infection.

VIII-2. Reverse transcription

(1) Prepare the following reaction mixture on ice. (Work in Area 1)
Add all components other than the sample RNA. Prepare a sufficient volume of master mix for the required number of tubes plus a few extra to account for pipette error. Dispense 10 μl aliquots into 0.2 ml microtubes.

Reagent	Volume (per reaction)
5X PrimeScript Buffer (NV)	4 μl
PrimeScript RT Enzyme Mix (NV)	1 μl
Random 6-mers (100 μM)	1 μl
Sample RNA	10 μl
RNase Free dH_2O	4 μl
Total	20 μl

(2) Add the sample RNA to the reaction tubes. Start the reverse transcription (RT) reaction using the following settings. (Work in Area 3)
Using a thermal cycler is recommended.

37℃ 15 min (Reverse transcription reaction)
85℃ 5 sec (Heat-inactivation of reverse transcriptase)
4℃

Note: After the RT reaction is complete, the reaction mixture may be stored at -20℃ until qPCR is performed.

VIII-3. Preparation of samples for standard curves (work in Area 3)

Prepare separate sets of samples for GI and GII reactions.

(1) Dispense 45 μl aliquots of EASY Dilution into 7 microfuge tubes (1.5 ml). Prepare 7 microfuge tubes each for the GI and GII Positive Control reactions.

(2) Transfer 20 μl of GI and GII Positive Control DNA, respectively, into new, separate 0.2-ml tubes.

(3) Incubate the tubes at 98℃ for 5 min Cool the tubes quickly by placing them in an ice bath.

(4) Dispense 5 μl of each heat-treated GI and GII Positive Control DNA (4×10^6 copies/μl) into the tubes from Step (1) to prepare a dilution with a concentration of 4×10^5 copies/μl.

(5) Repeat Step (4) to prepare serial dilutions until reaching a concentration of 4×10^{-1} copies/μl, as indicated in the table below.
The serial dilutions prepared will range in concentration from 4×10^{-1} copies/μl to 4×10^6 copies/μl.

1.	4×10^6 copies/μl	(GI or GII Positive Control DNA stock solution)
2.	4×10^5 copies/μl	(5 μl of GI or GII Positive Control DNA stock solution + 45 μl EASY Dilution)
3.	4×10^4 copies/μl	(5 μl of 4×10^5 copies/μl-solution + 45 μl EASY Dilution)
4.	4×10^3 copies/μl	(5 μl of 4×10^4 copies/μl-solution + 45 μl EASY Dilution)
5.	4×10^2 copies/μl	(5 μl of 4×10^3 copies/μl-solution + 45 μl EASY Dilution)
6.	4×10^1 copies/μl	(5 μl of 4×10^2 copies/μl-solution + 45 μl EASY Dilution)
7.	4×10^0 copies/μl	(5 μl of 4×10^1 copies/μl-solution + 45 μl EASY Dilution)
8.	4×10^{-1} copies/μl	(5 μl of 4 copies/μl-solution + 45 μl EASY Dilution)

NOTE: Plot standard curves based on the set of 8 serial dilutions. Use 2.5 μl each per reaction. Three replicates (n = 3) are recommended.

VIII-4. Preparation and initiation of real-time PCR reaction

Use this kit to simultaneously generate standard curves from the positive control DNA, and detect signal from experimental samples and the negative control (sterile water).

(1) Prepare the following reaction mixtures on ice. (Work in Area 1)
Prepare separate mixtures for the GI reaction and the GII reaction.

Add all components except template, preparing sufficient volume of master mixes for the required number of tubes plus a few extra to account for pipetting error.
The number of reaction tubes required = the number of samples + the number of standard samples for standard curve + the number of negative control reactions.
Perform at least 2 replicates (n ≧2) for experimental samples and the negative control and 3 replicates (n = 3) for standard curve samples (8 serial dilutions).
Dispense 22.5 μl aliquots of the appropriate master mix into reaction tubes and cap lightly. Add 2.5 μl of sterile water to the reaction tubes for the negative control and cap tightly.

Reagent	Volume (per reaction)	Final Conc.
Premix Ex Taq (NV) (2X conc.)	12.5 μl	1X
GI or GII Primer Mix	1 μl	
GI or GII Probe Mix	1 μl	
ROX Reference Dye＊1 or ROX Reference Dye II＊1 or dH_2O	0.5 μl	
cDNA sample＊2 or standard sample for standard curve＊2 or sterile water (negative control)	2.5 μl	
dH_2O	7.5 μl	
Total	25 μl	

＊1 Use ROX Reference Dye with the StepOnePlus Real-Time PCR System and ROX Reference Dye II with the Applied Biosystems 7500 Fast Real-Time PCR System.
Add dH_2O when using the Thermal Cycler Dice Real Time System //.

＊2 The cDNA sample and the samples for standard curve should be added in Step (2), NOT here in this step.

【Caution】

Because real-time PCR relies on the optical measurement of fluorescence, take care that the tubes do not become dirty. Wear gloves when handling PCR tubes.

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

Add 2.5 μl of the cDNA sample or the standard curve sample into a corresponding tube containing the mixture prepared in Step (1) and cap tightly. Do not add anything to the negative control tubes.

Spin down briefly in a microcentrifuge for 0.2 ml tubes. Set tubes in the real-time PCR instrument.

Note: Start the PCR reaction within 1 hour of preparing the reaction mixture.

VIII-5. Amplification and detection by real-time PCR and quantitative analysis (Work in Area 3)

Operating procedures differ depending on the real-time PCR instrument. For specific operating procedures, refer to manufacturer protocols provided in the instrument user manual.

An overview of operation and quantitative analysis for the Thermal Cycler Dice Real Time System *II* and for the Applied Biosystems 7500 Fast Real-Time PCR System is described in this manual.

<For Thermal Cycler Dice Real Time System *II*>

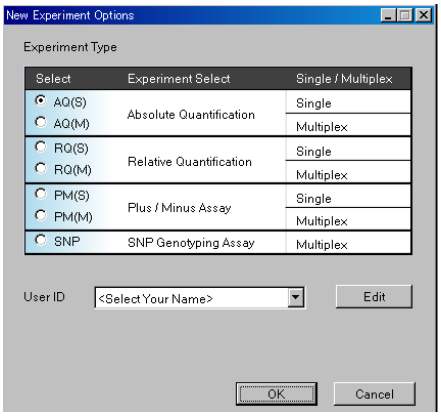
Example of Absolute Quantification

Perform the GI reaction and GII reaction, respectively, on the samples as described below in accordance with the official method.

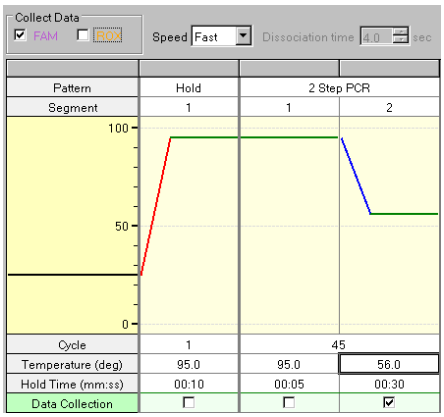
Samples for standard curve: 1 copy - 1 x 10^7 copies (n = 3)
Experimental samples and negative control (n = 2)

Generate a standard curve for both the GI and GII reactions. Test the experimental samples and the negative control with both the GI and GII reactions.

(1) Open a new run file. On the "New Experiment Options" screen, select the analysis type "AQ(s), Absolute Quantification, Single" and click the "OK" button.



(2) Set the PCR conditions on the "Thermal Profile Setup" screen.



Hold
Number of cycles: 1
95°C 10 sec

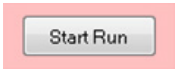
2-step PCR
Number of cycles: 45
95°C 5 sec
56°C 30 sec (detection)

Remove the check mark "☑" for ROX under "Collect Data".

How to change the default settings:
Double-click the parameter you would like to change and input the desired value directly, or change the value using the arrow keys.

Parameter	Change
Hold	95℃, 30 sec → 10 sec
2-Step PCR	60℃ → 56℃
Number of cycles	40 cycles → 45 cycles

(3) Click the "Start Run" button on the bottom right-hand side of the screen to start the reaction.



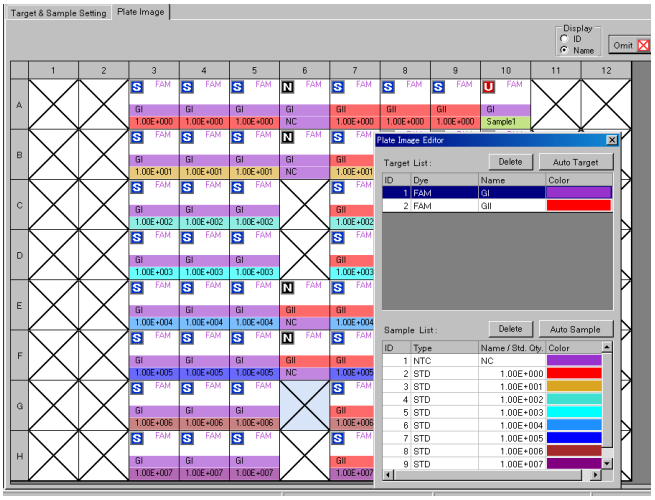
(4) On the "Plate Setup" screen, make the Target List and Sample List.

1) Target List setup:
Set the detection filter and target name.
1. Add a line in the Target List by clicking the "Add" button. (Click the "Delete button" to delete a line.)
2. Select "FAM" from the "Dye" drop-down menu.
3. Input Target Name, G1, or GII, in the "Name" menu.
(4. Color can be changed, if required.)

2) Sample List setup:
1. Select the desired well and select the sample type from the drop-down menu.
NTC [No Template Control]: Control reaction—no template added
STD [Standard]: Sample for standard curve
NAC [No Amplification Control]: Control reaction—no enzymes added
UNKN [Unknown]: Unknown sample to be tested
2. Input the Sample Name in the "Name" menu.
(3. Color can be changed, if needed.)

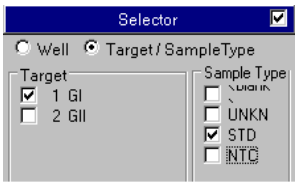
3) Click the "Update" button.

4) Plate Image setup:
   1. On the "Plate Image" screen, select the "Target" on the "Plate Image Editor" for the desired wells.
   2. Select "Sample" on the "Plate Image Editor".
   3. For unused wells, select "Omit".

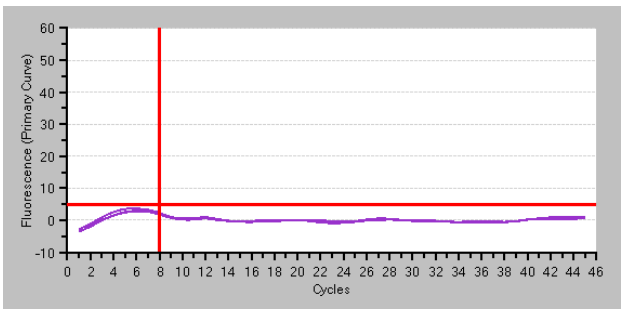


(5) Analyze the results.
After the reactions are complete, click the "Result/Analysis" button.

1) Check the amplification curves:
Select the FAM detection filter and check the amplification curves for the NTC (negative control) and the STD (standard curve using positive control DNA).
Check the curves for GI and GII, respectively.
To display the GI or GII curves, select wells from the "Selector" directly or select from the targets displayed under "Target/Sample Type".



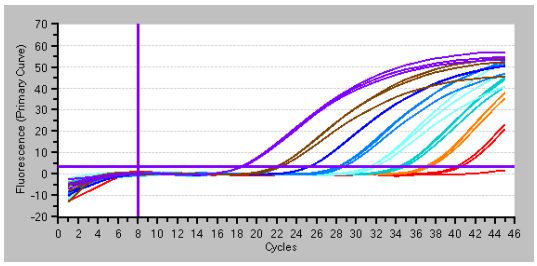
1. Display the NTC amplification curve:
Go to "Selector" and select "NTC".



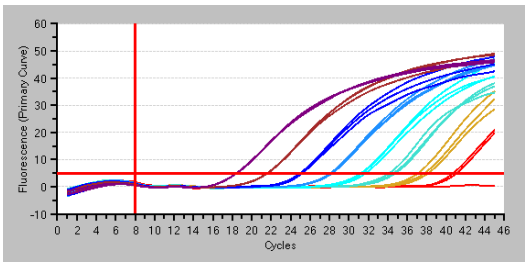
Make sure the value for the FAM filter does not exceed the threshold value.

2. Display the amplification curves for the standards:
   Go to "Selector" and select "STD".

GI reaction



GII reaction



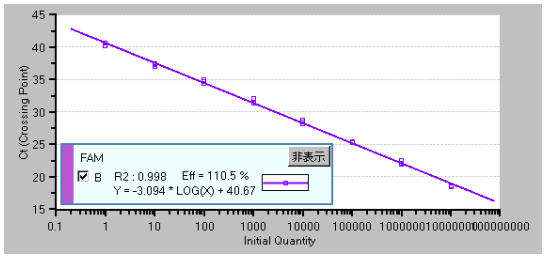
Make sure the amplification curves for the FAM filter are plotted for each reaction and that the curves exceed the threshold.

Note: The single-copy amplification curve may not exceed the threshold.

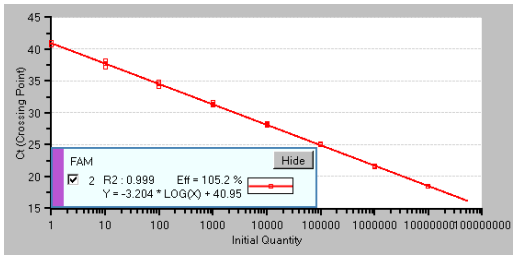
3. Check the sample amplification curves for experimental samples by selecting "UNKN" in the "Selector".

2) Check the standard curve:
From the "Analysis Data" dropdown menu, select "Standard Curve".
Check the standard curves for GI and GII, respectively.

GI standard curve



GII standard curve



Values that deviated from the standard curve may be omitted, if necessary.

1. Go to "Selector" to select the wells to be omitted.
2. Right-click and select "Omit" and "Set" to exclude the selected wells from analysis. The excluded wells may be included for analysis later by selecting "Omit" and "Reset".

(6) Display the results.
Display the reports in text:

1) From the "Analysis Data" menu select "Text Report" to display a text report in the graph displaying area.

2) Go to "Selector" to select wells to be displayed.

3) Go to "Data Set" to select data sets to be displayed.

Data Set of Each Well:	Analytical results for individual wells
Data Set of Replicate:	Analytical results for individual replicates (combination of targets and samples in the same group; e.g., mean and standard deviation)
Select all:	Display all analytical results

4) From the check boxes under "Show Items", select the items to be displayed in the detailed item list.

Analysis Setting:	Items related to analytical parameters
CP Method Data:	Analytical results based on the Crossing Point method
SDM Method Data:	Analytical results based on the 2nd Derivative Maximum method

5) Changes may be made to the display of each data element through the check boxes on the detailed item list.

6) Data can be sorted by a particular item by clicking that item on the text report.

Example: GI reaction standard curve and experimental samples

Sample Type	Target Name	Sample ID	Sample Name	Ct(CP)	Ct Avg.(CP)	Init Qty	Qty(CP)	Qty Avg.(CP)
NTC	GI	1		–	–	–	–	–
NTC	GI	1		–	–	–	–	–
STD	GI	2		40.21	40.45	1.000E+000	1.451E+000	1.237E+000
STD	GI	2		–	40.45	1.000E+000	–	1.237E+000
STD	GI	2		40.68	40.45	1.000E+000	1.023E+000	1.237E+000
STD	GI	3		37.07	37.30	1.000E+001	1.501E+001	1.275E+001
STD	GI	3		37.55	37.30	1.000E+001	1.050E+001	1.275E+001
STD	GI	3		37.29	37.30	1.000E+001	1.275E+001	1.275E+001
STD	GI	4		34.70	34.71	1.000E+002	8.759E+001	8.817E+001
STD	GI	4		35.02	34.71	1.000E+002	6.903E+001	8.817E+001
STD	GI	4		34.42	34.71	1.000E+002	1.079E+002	8.817E+001
STD	GI	5		31.47	31.68	1.000E+003	9.692E+002	8.492E+002
STD	GI	5		32.11	31.68	1.000E+003	6.020E+002	8.492E+002
STD	GI	5		31.46	31.68	1.000E+003	9.765E+002	8.492E+002
STD	GI	6		28.45	28.53	1.000E+004	9.173E+003	8.765E+003
STD	GI	6		28.87	28.53	1.000E+004	6.711E+003	8.765E+003
STD	GI	6		28.28	28.53	1.000E+004	1.041E+004	8.765E+003
STD	GI	7		25.43	25.41	1.000E+005	8.681E+004	8.792E+004
STD	GI	7		25.44	25.41	1.000E+005	8.617E+004	8.792E+004
STD	GI	7		25.37	25.41	1.000E+005	9.078E+004	8.792E+004
STD	GI	8		22.06	22.25	1.000E+006	1.066E+006	9.409E+005
STD	GI	8		22.57	22.25	1.000E+006	7.294E+005	9.409E+005
STD	GI	8		22.11	22.25	1.000E+006	1.027E+006	9.409E+005
STD	GI	9		18.59	18.60	1.000E+007	1.410E+007	1.398E+007
STD	GI	9		18.69	18.60	1.000E+007	1.309E+007	1.398E+007
STD	GI	9		18.53	18.60	1.000E+007	1.475E+007	1.398E+007
UNKN	GI	10	Sample1	25.04	25.04	–	1.160E+005	1.160E+005
UNKN	GI	10	Sample1	25.04	25.04	–	1.160E+005	1.160E+005

In the example of GI detection shown, detections were successful in 2 reaction series for the single-copy standard curve in triplicate (n = 3).

A standard curve based on the Ct values of all standard samples was used to determine the copy number in each experimental sample. The mean Ct (CP) in Sample 1 (n = 2) was 25.04, equivalent to a quantitative mean (CP) of 1.16×10^5 copies.

<For Applied Biosystems 7500 Fast Real-Time PCR System>

When using the StepOnePlus Real-Time PCR System, reactions may be performed using the same settings as those for the Applied Biosystems 7500 Fast Real-Time PCR System.

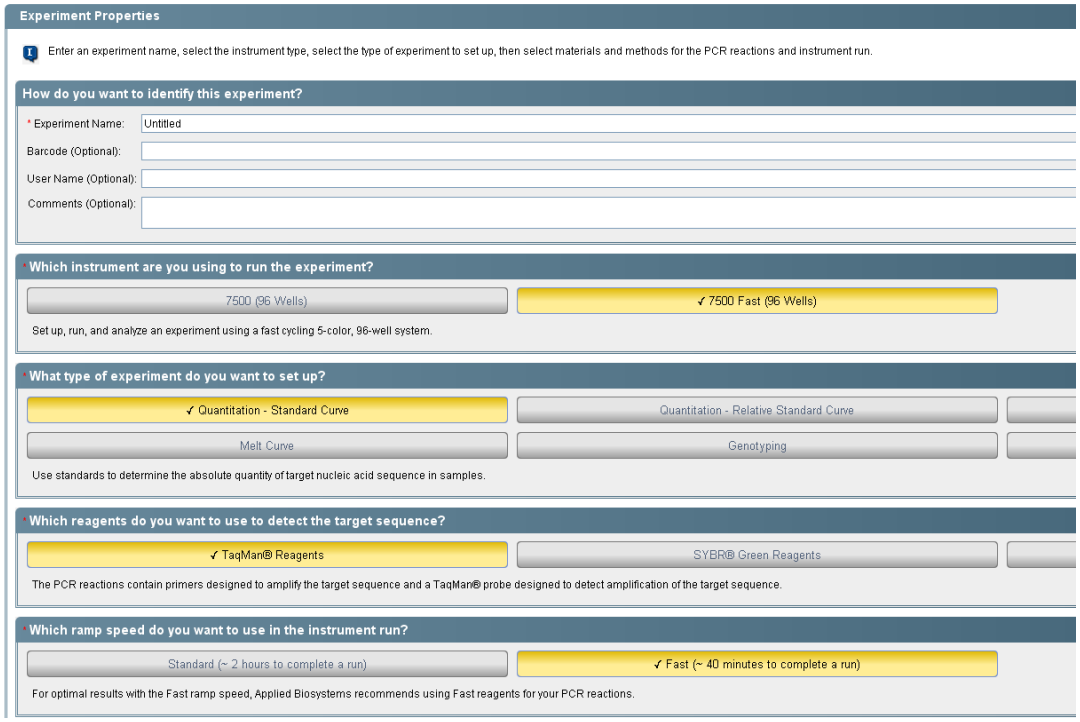
Example of absolute quantification

Perform the GI reactions and GII reactions, respectively, with the following samples:Samples for generating a standard curve: 1 copy - 1 x 10^7 copies (n = 3)
Experimental samples and negative control (n = 2)

(1) Experiment parameters setup

Instrument＊1: 7500 Fast (96 Wells)
Type of Experiment: Quantification-Standard Curve
Reagent: TaqMan Reagents
Ramp Speed＊2: Select "Fast" (run time approx. 40 min)

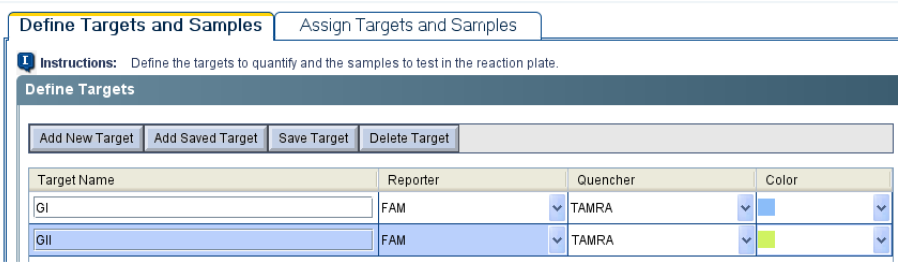
＊1 When using StepOnePlus, select StepOnePlus Instrument (96 Wells).
＊2 You can also select "Standard" (run time approx. 2 hours).



(2) Plate setup

1) Select the "Define Targets and Samples" tab and enter the target name, reporter, and quencher.

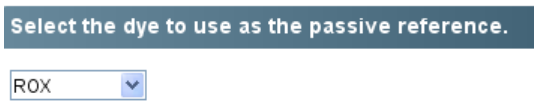
1. Click "Add New Target" to add a row.
2. Enter information.
   Target Name: Enter GI and GII.
   Reporter: Select "FAM" (the default setting).
   Quencher: Select "TAMRA" from the drop-down menu.
   Color: The color may be changed from the drop-down menu.



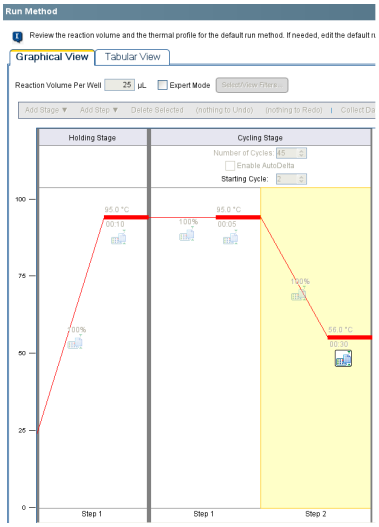
3. Define the samples.
   Click "Add New Sample" to add additional rows for the appropriate number of samples.
   Enter the sample name.
   If necessary, the color may be changed.

2) Select the "Assign Targets and Sample" tab and set "Plate Layout" and "Passive Reference".

1. Select wells on the "View Plate Layout" screen and configure wells by clicking "Assign ☑" for targets.
2. On the "Task" screen, select "Sample", "Standard" and "Negative Control".
   U [Unknown]: Sample
   S [Standard]: Standard curve
   N [Negative Control]: Negative control
3. For sample wells, select sample name(s) from "Assign Sample(s)".
4. For standard-curve wells, enter the concentration either directly under "Quantity" or under "Define and Set Up Standards".
5. Select ROX as the passive reference (the default setting).



(3) Run method setup



Hold
Number of cycles: 1
95°C 10 sec

2-step PCR
Number of cycles: 45
95°C 5 sec
56°C 30 sec (detection)

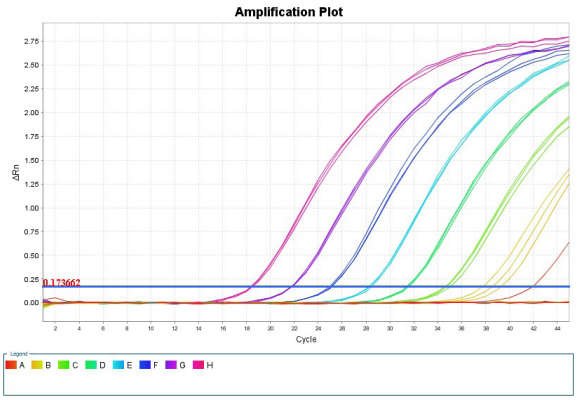
Reaction volume: 25 μl

(4) Click the "Start" button to start the reactions.

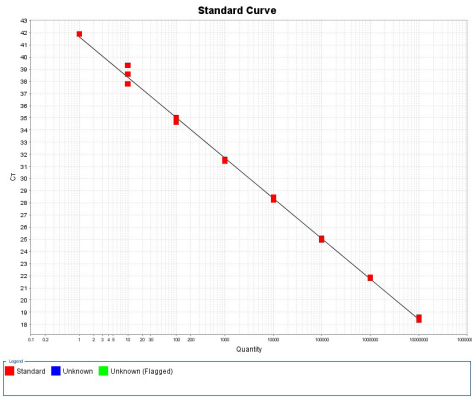
(5) After the reactions are complete, check the amplification plots of GI and GII, respectively, and generate standard curves.
Samples for generating a standard curve range in quantity from 1 copy - 1 x 10^7 copies (n = 3)

GI reaction

Amplification Plot

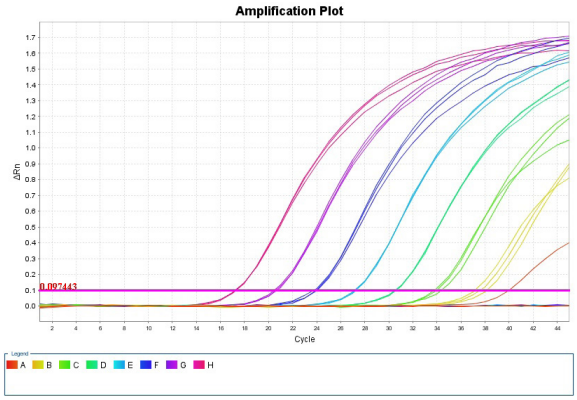


Standard Curve

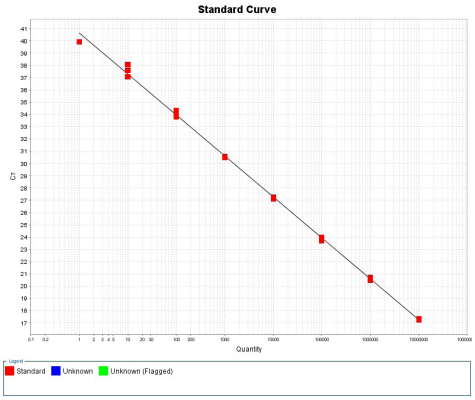


GII reaction

Amplification Plot



Standard Curve



Note: The amplification curve for the single-copy standard sample may not exceed the threshold.

The Ct value and the copy number in the experimental samples may be verified on the "View Well Table" tab.

Example: Standard curve and samples in GI reaction

View Plate Layout | View Well Table

Select Wells With: - Select Item - - Select Item -

Show in Table ▼ Group By ▼

#	Well	Omit	Flag	Sample Na...	Target Name	Task	Dyes	Ct	Ct Mean	Ct SD	Quantity	Quantity ...	Quantity ...	HI(
67	H11													
68	H12													
69	A3				GI	NTC	FAM-TAMRA	Undetermi...						
70	B3				GI	NTC	FAM-TAMRA	Undetermi...						
71	A4		⚠		GI	STANDARD	FAM-TAMRA	41.636	41.221	0.588	1			
72	A5		⚠		GI	STANDARD	FAM-TAMRA	Undetermi...	41.221	0.588	1			
73	A6		⚠		GI	STANDARD	FAM-TAMRA	40.805	41.221	0.588	1			
74	B4				GI	STANDARD	FAM-TAMRA	39.205	39.115	0.129	10			
75	B5				GI	STANDARD	FAM-TAMRA	39.173	39.115	0.129	10			
76	B6				GI	STANDARD	FAM-TAMRA	38.968	39.115	0.129	10			
77	C4				GI	STANDARD	FAM-TAMRA	35.615	35.58	0.171	100			
78	C5				GI	STANDARD	FAM-TAMRA	35.732	35.58	0.171	100			
79	C6				GI	STANDARD	FAM-TAMRA	35.394	35.58	0.171	100			
80	D4				GI	STANDARD	FAM-TAMRA	32.078	32.113	0.095	1,000			
81	D5				GI	STANDARD	FAM-TAMRA	32.041	32.113	0.095	1,000			
82	D6				GI	STANDARD	FAM-TAMRA	32.220	32.113	0.095	1,000			
83	E4				GI	STANDARD	FAM-TAMRA	28.629	28.706	0.067	10,000			
84	E5				GI	STANDARD	FAM-TAMRA	28.739	28.706	0.067	10,000			
85	E6				GI	STANDARD	FAM-TAMRA	28.750	28.706	0.067	10,000			
86	F4				GI	STANDARD	FAM-TAMRA	25.446	25.496	0.061	100,000			
87	F5				GI	STANDARD	FAM-TAMRA	25.563	25.496	0.061	100,000			
88	F6				GI	STANDARD	FAM-TAMRA	25.478	25.496	0.061	100,000			
89	G4				GI	STANDARD	FAM-TAMRA	22.173	22.218	0.046	1,000,000			
90	G5				GI	STANDARD	FAM-TAMRA	22.265	22.218	0.046	1,000,000			
91	G6				GI	STANDARD	FAM-TAMRA	22.217	22.218	0.046	1,000,000			
92	H4				GI	STANDARD	FAM-TAMRA	18.838	18.92	0.072	10,000,000			
93	H5				GI	STANDARD	FAM-TAMRA	18.951	18.92	0.072	10,000,000			
94	H6				GI	STANDARD	FAM-TAMRA	18.971	18.92	0.072	10,000,000			
95	F7			Sample 1	GI	UNKNOWN	FAM-TAMRA	24.932	24.934	0.003	151,414.531	151,211.266	287.461	
96	F8			Sample 1	GI	UNKNOWN	FAM-TAMRA	24.936	24.934	0.003	151,008	151,211.266	287.461	

In this example of GI detection, detections were successful in 2 reaction series for the single-copy standard curve in triplicate (n = 3).

A standard curve based on the Ct values of all standard samples was used to determine the copy number in each experimental sample. The mean Ct (CP) in Sample 1 (n = 2) was 24.93, equivalent to a quantitative mean (CP) of 1.51×10^5 copies.

IX. Precautions for Interpretation

- If the FAM filter/amplification curve (primary curve) showed amplification occurring in the negative control reactions:
  → Contamination may have occurred. Decontaminate the laboratory area used to prepare the reaction mixtures as well as the apparatuses and instruments used. Then perform the reaction again.
- If the standard reactions (other than the single-copy reactions) produced no amplification curves:
  → The PCR reaction or probe detection failed to work properly. Perform the reactions again.
- If no amplification occurred for the experimental sample(s):
  → The samples may contain NoV at a concentration below the limit of detection or may contain a reaction inhibitor. Dilute the samples and perform the reverse transcription again. Alternatively, prepare the samples again and then perform the reactions.

X. References

1) Kapikian A Z, *et al*. *Virol*. (1972)**10**:1075-1081.
2) Siebenga J J, *et al*. *Emerg Infect Dis.* (2007)**13**:144-146.
3) Oogane T, *et al*. *Jpn J Infect Dis*. (2008)**61**:423-424.
4) Kojima S, *et al*. *J Virol. Methods*. (2002)**100**:107-114.
5) Director of Inspection and Safety Division, Department of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare: Norovirus Detection Method. November 5, 2003, Notification No. 1105001 of ISD-DFS; latest version, Notification 1022 No. 1 of ISD-DFS, October 22, 2013.
6) Director of Department of Food Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare: Management Manual of Mass Cooking Facilities. Appendix to March 24, 1997-dated JFH Notification No. 85, June 18, 2008-dated DFS No. 0618005 (2008).
7) Fujii K, *et al*. *Jpn J Food Microbiol*. (2011)**28:**139-142.

XI. Related Products

Thermal Cycler Dice™ Real Time System lll with PC (Cat. #TP970)*
0.1 ml 8-strip tube, individual Flat Caps (Cat. #NJ902)
0.2 ml 8-strip tube, individual Flat Caps (Cat. #NJ600)*
EASY Dilution (for Real Time PCR) (Cat. #9160)
RNase-OFF® (Cat. #9037)

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