THUNDERBIRD® Probe One-step qRT-PCR Kit

QRZ-101 100 reactions

Store at −20°C, protected from light

Contents

CAUTION

All reagents in this kit are intended for research purposes only. Do not use for diagnostic or clinical purposes. Please observe general laboratory precautions and observe safety procedures while using this kit.

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[1] Introduction

Description

THUNDERBIRD® Probe One-step qRT-PCR Kit is a one-step real-time reverse-transcription polymerase chain reaction (RT-PCR) kit using the highly efficient reverse transcriptase "ReverTra Ace®" and Tth DNA polymerase as a PCR enzyme. This product can be used mainly in TaqMan® probe assays. The one-step system is suitable for high-throughput analysis because of its simple reaction setup. In addition, this system can reduce the risk of cross-contamination.
The combination of the two enzymes and optimized buffer system enable the effective detection and quantification of a small amount of RNA. This kit can also detect various kinds of RNA with different sequences because it is tolerant of target sequence diversity.

Features

-Rapid and highly sensitive
THUNDERBIRD® Probe One-step qRT-PCR Kit can achieve the rapid and highly sensitive quantification of a small amount of RNA by a one-step qRT-PCR method with probes. This kit is suitable for the quantification of RNA viruses or mRNA expressed at a low level.

-Tolerant of target sequence diversity
This kit can detect various types of RNA efficiently because of its tolerance of the diversity of target sequences.

-Tolerant of PCR inhibitors
This kit can reduce the inhibition by PCR inhibitors such as hematin.

-Utilization of dUTP
This kit contains dUTP instead of dTTP in 2× Reaction Buffer. Therefore, the rate of false-positive detection can be reduced by adding uracil-N-glycosylase (UNG).
*UNG is not supplied with this kit.

-Multiplex detection
Multiple targets can be detected using TaqMan® probes labeled with different fluorescent dyes in one reaction.

-Hot start system using antibodies
Hot start technology, using anti-DNA polymerase antibodies, allows highly specific and reproducible amplification. The antibodies are easily inactivated in the first denaturation step, minimizing the damage to DNA polymerase.

-Compatibility with various real-time cyclers
The reagent is applicable to most real-time cyclers (*i.e.*, block type and glass capillary type). Because the 50× ROX Reference dye is individually supplied with this kit, the kit can be applied to real-time cyclers that require a passive reference dye.

[2] Components

This kit includes the following components for 100 reactions, with 50 µl per reaction. All reagents should be stored at −20°C.

2× Reaction Buffer	2 × 1.25 ml
DNA Polymerase	125 µl
RT Enzyme Mix	125 µl
50× ROX Reference dye	100 µl
RNase free water	2 × 1.25 ml

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Notes:

-2× Reaction Buffer contains essential components for the reaction (buffer, salts, dATP, dCTP, dGTP, and dUTP, etc.).

-DNA Polymerase is a mixture of Tth DNA polymerase and hot start antibodies.

-RT Enzyme Mix contains ReverTra Ace® as a highly efficient reverse transcriptase and RNase inhibitor.

-50× ROX Reference dye can be used as a passive reference in real-time cyclers from Applied Biosystems® and Agilent Technologies to compensate for the variation of reaction volumes between tubes. The final concentration of ROX dye should be changed according to the real-time cycler. In the case of cyclers that do not require compensation, ROX dye does not need to be added (see [5] Protocol).

-RNase free water should be used for preparation of the reaction mixture.

[3] Primer/probe design

1. Design of primers

Highly sensitive and quantitative data depend on the primer design. The primer should be designed according to the following suggestions:

-Primer length: 18–25-mer
-GC content of primer: 40–60%
-Melting temperature (T_m) of primer: 60–65°C
-Purification grade of primer: Cartridge (OPC) grade or high-performance liquid chromatography (HPLC) grade
-Target length: 70–200 bp

Notes:

-The reverse primer can also work as a primer for reverse transcriptase in this kit. Random or oligo dT primer is not required.
-Larger targets (>200 bp) tend to reduce the efficiency and specificity of amplification.
-Amplification from genomic DNA can be avoided by designing the primers on different exons or exon junctions.

2. Design of TaqMan® probes

Because insufficiently purified probes may inhibit the reaction, HPLC-grade probes should be used. TaqMan® probe should be designed according to the following suggestions:

-Probe length: 20–30-mer
-GC content of probe: 40–60%
-T_m of probe: 65–70°C
-Purification grade of primers: HPLC grade

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Notes:

-Detection of an amplicon from genomic DNA can be avoided by designing the TaqMan® probe on an exon junction.

The T_m of primers and probes should be calculated using the nearest neighbor method. The T_m values in this manual were calculated using this method with the following parameters: Na^+ concentration, 50 mM; and oligonucleotide concentration, 0.5 µM.

3. Checking the performance of primers and TaqMan® probes

The performance of primers and TaqMan probes can be checked using the following experiments.

(1) Prepare a dilution series with three or more dilutions of template RNA (*e.g.*, 0.2 ng/µl, 2 ng/µl, 20 ng/µl).

(2) Perform qRT-PCR assay using the diluted RNA (5µl in 20-µl reaction) with the newly designed primers and TaqMan® probe, and draw a standard curve according to [5] Protocol.

(3) Confirm that the PCR efficiency is between 85% and 115% and R^2 is equal to or greater than 0.98.

If the PCR efficiency and/or R^2 are outside of these ranges, the reaction conditions should be optimized according to [6] Optimization of conditions. If this does not improve the result, the primers and/or TaqMan® probe should be redesigned.

[4] Template RNA

The following RNAs are appropriate for highly efficient detection and quantification.

(1) Total RNA

Total RNA (*e.g.*, the acid-guanidium-phenol-chloroform method or the spin column method) can be used directly as a template with this kit. Total RNA usually contains 1–2% mRNA.

(2) Poly(A)$^+$ RNA (mRNA)

Poly(A)$^+$ RNA is useful to detect low-abundance mRNA. However, poly(A)$^+$ RNA should be treated carefully because it is more sensitive to RNase than total RNA.

(3) Viral RNA

Viral RNA purified by various methods (*e.g.*, the acid-guanidium-phenol-chloroform method or the spin column method) can be used.

[5] Protocol

1. Reaction mixture setup

	Reaction volume		Final
	50-µl reacti	20-µl reaction	Concentration
RNase free water	X µl	X µl	
2× Reaction buffer	25 µl	10 µl	1×
DNA Polymerase	1.25 µl	0.5 µl	
RT Enzyme Mix	1.25 µl	0.5 µl	
Forward primer	25 pmol	10 pmol	0.5 µM[*1]
Reverse primer	25 pmol	10 pmol	0.5 µM[*1]
TaqMan® probe	10 pmol	4 pmol	0.2 µM[*2]
50× ROX Reference dye	1µl / 0.1 µl	0.4 µl / 0.04 µl	1× / 0.1×[*3]
(Uracil-N-glycosylase)[*4] [optional]	1 unit	0.4 units	1 unit / 50 µl
RNA solution	Y µl[*5]	Y µl[*5]	
	50 µl	20 µl	

*1 If the expected results are not obtained at 0.5 µM, [6] Optimization of conditions can be consulted. The same conditions can be applied in the case of multiplex detection.

*2 If the expected results are not obtained at 0.2 µM, [6] Optimization of conditions can be consulted. The same conditions can be applied in the case of multiplex detection.

*3 50× ROX Reference dye must be added when using real-time cyclers that require a passive reference dye, according to Table 1. Table 1 shows the optimum concentration of the ROX Reference dye. This dye is not necessary for real-time cyclers that do not require a passive reference dye.

Table 1 Recommended ROX dye concentration

Real-time cycler	Optimal dye concentration (dilution ratio)
Applied Biosystems® 7000, 7300, 7700, 7900HT StepOne™, StepOnePlus™, etc.	1× (50:1)
Applied Biosystems® 7500, 7500Fast, Agilent cyclers (Optional), etc.	0.1× (500:1)
Roche cyclers, Bio-Rad cyclers, Qiagen cyclers, etc.	Not required

*4 Heat-labile uracil-N-glycosylase (UNG) should be used. The amount of UNG can be changed according to the recommendations from each supplier. UNG is not supplied with this kit.

*5 The concentration of total RNA in a reaction can be increased up to 25 ng/µl. An excessive amount of RNA inhibits the reaction.

2. Cycling conditions

The following table shows the recommended thermal conditions using primers designed according to the recommended primer and probe conditions described in [3] Primer/probe design. These conditions can be applied to multiplex assays.

	Temperature	Time	Ramp	
(UNG treatment)	(20–25°C[*1])	(10 min[*1])	(Maximum)	
Reverse transcription	50°C	10 min	Maximum	
Pre-denaturation	95°C	60 s	Maximum	
Denaturation	95°C	15 s	Maximum	40 cycles[*2]
Extension (annealing)	60°C	45 s	Maximum	
	(Data collection should be performed at the extension step.)			

*1 [Optional] The uracil-N-glycosylase (UNG) treatment step should be added before the cDNA synthesis step. The indicated temperature and time are typical conditions for UNG. The conditions can be optimized according to the particular instruction manual from the supplier of UNG.

*2 The number of cycles can be increased up to 45.

2-1. Real-time PCR conditions using Applied Biosystems® StepOnePlus™
(normal block type, software version 2.3)

The following is an example of a TaqMan® assay using Applied Biosystems® StepOnePlus™. These conditions can also be useful for cyclers such as the Applied Biosystems® 7500 Fast Real-Time PCR System.

(1) Select “Design Wizard”, “Advanced Setup” or “QuickStart” after starting the software.

(2) ” Select “TaqMan® Reagents” as reagents in the following tabs.

Design Wizard	Methods & Materials
Advance Setup	Setup → Experiment Properties
QuickStart	Experiment Properties

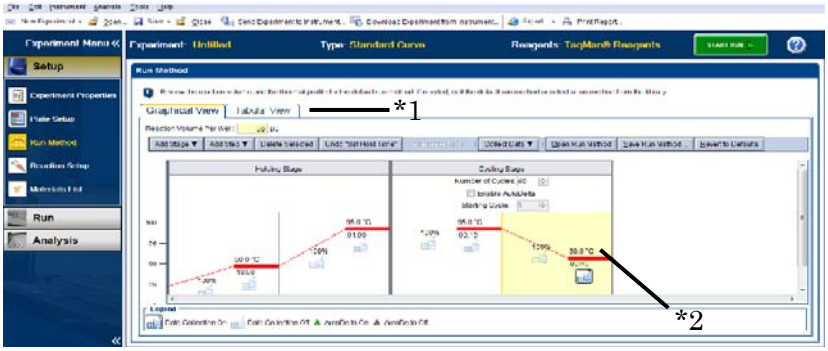
(3) Select “Run Methods” and input the reaction volume at “Reaction Volume Per Well”.

(4) Select “Add Step” and input 50°C, 10 min.

(5) Select “Holding Step” and input 95°C, 60 s.

(6) Select “Cycling Stage” and input 95°C, 15 s, 60°C, 45 s, 40 cycles.

(7) Start the program after setting the plate or tubes.



*1 Input of the actual reaction volume is important for a successful analysis.
*2 Set the data collection at the extension step.

2-2. LightCycler® 96

(software version 1.1)

(1) Select “Create New Experiment”

(2) Input reaction volume.

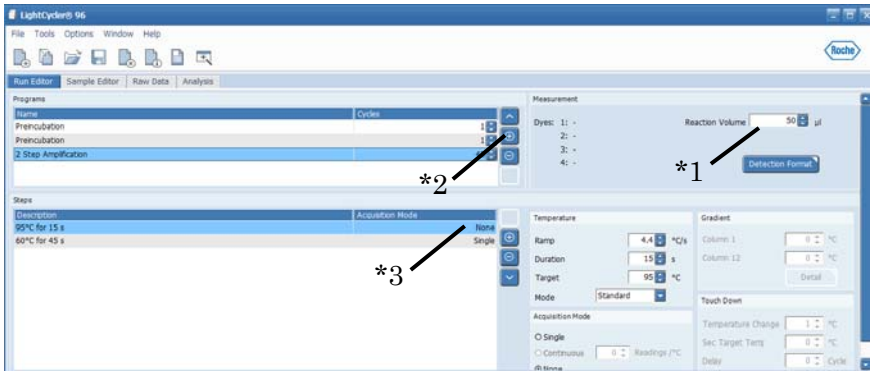
(3) Select Preincubation → Preincubation → Two-Step Amplification at “Predefined Program” in the “Run Editor” tab.

(4) Select the first “Preincubation” and input 50°C as “Target” and 600 s as “Duration”.

(5) Select the second “Preincubation” and input 95°C as “Target” and 60 s as “Duration”.

(6) Select the second “2 Step Amplification” and input 95°C as “Target” and 15 s as “Duration” followed by 60°C as “Target” and 45 s as “Duration”

(7) Start the program after setting the plate or tubes.



*1 Input of the actual reaction volume is important for a successful analysis.
*2 The input screen consists of initial denaturation and two subsequent steps by default. The cDNA synthesis step should be added by selecting “+”.
*3 Set the data collection at the extension (annealing) step.

2-3. CFX96 Touch™ Deep Well

(software version 3.1)

(1) Select “User-defined”.

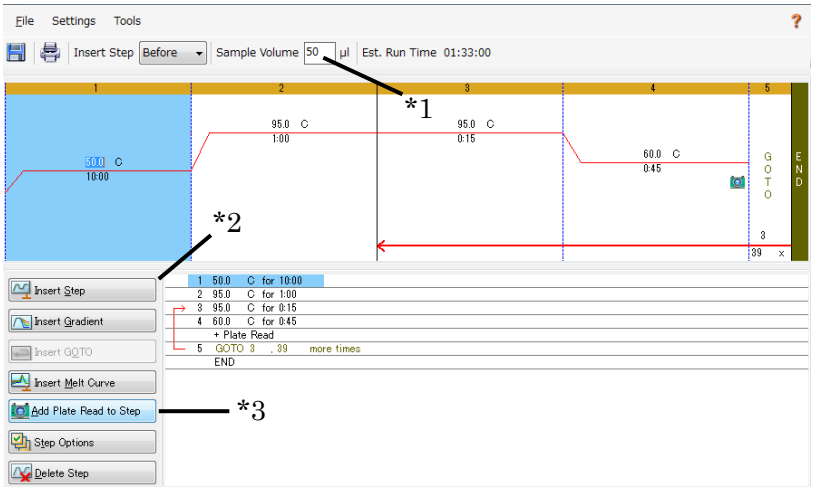
(2) Select “Create New …” and input reaction volume.

(3) Select “Insert Step” and input 50°C and 10 min.

(4) Start the program after setting the plate or tubes.

(5) Input the following conditions: 95°C, 60 s, 95°C, 15 s, 60°C, 45 s, 40 cycles.

(6) Start the program after setting the plate or tubes.



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*1 Input of the actual reaction volume is important for a successful analysis.
*2 The input screen consists of initial denaturation and two subsequent steps by default. The cDNA synthesis step should be added by selecting “Insert Step”.
*3 Set the data collection at the extension (annealing) step.

[6] Optimization of conditions

1. Optimization procedure

If the expected performance is not obtained using the standard conditions ([5] Protocol), the reaction conditions should be optimized. The low sensitivity is caused by poor PCR efficiency or failure of detection with TaqMan® probe. After confirming that the primers and TaqMan® probes have been designed appropriately, the extension (annealing) temperature and time should be optimized according to the following flow chart. If the designs of primers and TaqMan® probes seem to be inappropriate, they should be redesigned based on [3] Primer/probe design.

Low sensitivity
↓
Optimization of extension (annealing) temperature and time (see [6] 2)
↓
Optimization of concentrations of primers and TaqMan® probes (see [6] 3)

2. Optimization of extension (annealing) temperature and time

Decrease or prolonging of extension (annealing) temperature or time can improve the PCR efficiency in cases in which the annealing of the primer or TaqMan® probe is insufficient. In cases in which a decrease of extension (annealing) temperature does not improve the sensitivity, nonspecific amplification such as primer–dimer formation may reduce the PCR efficiency. In such cases, an increase of the extension (annealing) temperature may increase the efficiency.

In cases in which changes of the extension (annealing) temperature and time do not improve the results, the concentration of primers or TaqMan® probes should be optimized according to [6] 3.

<Flow of optimization of extension (annealing) step>

60°C, 45 s (standard conditions)
↓
~55°C, 45 s
↓ Tendency for improvement　　↓ No improvement

~55°C, 60 s　　**~65°C**, 45 s

*1 The number of cycles can be increased up to 45.
*2. VeriFlex™ of Applied Biosystems® StepOnePlus™ or Temperature gradient of Bio-Rad cyclers facilitates optimization of the extension (annealing) temperature.

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3. Optimization of concentrations of primers and TaqMan® probes

(1) Increase primer and/or TaqMan® probe concentration

Increasing the primer concentration can improve the efficiency in cases in which the annealing of primers is insufficient. Similarly, increasing the TaqMan® probe concentration can increase the sensitivity in cases in which the annealing of TaqMan® probes is insufficient.
First, the primer concentration should be optimized between 0.5 and 0.8 µM with the TaqMan® probe concentration at 0.2 µM. Then, the concentration of TaqMan® probe should be increased up to 0.4 µM in cases in which the increase of primers has not improved the results.

(2) Decrease primer concentration

Decreasing the primer concentration may improve the PCR efficiency in cases with primer–dimer formation. In such cases, the primer concentration should be decreased between 0.2 and 0.5 µM with the TaqMan® probe concentration at 0.2 µM.
Please note that a low concentration of TaqMan® probe of less than 0.2 µM decreases the sensitivity because of low fluorescence intensity.

[7] Application data

1. Detection of respiratory syncytial (RS) virus in clinical specimens

RNA of RS virus type A was detected from throat swab specimens using THUNDERBIRD® Probe One-step qRT-PCR Kit with Applied Biosystems® StepOnePlus™. The RNA (6.5×10^4 copies) purified by the QIAmp Virus RNA Kit (QIAGEN) was used in the assay after serial dilution from 10^0 to 10^{-5}.

<Cycling conditions>

Step		Temperature	Time
Reverse transcription		50°C	10 min
Predenaturation		95°C	60 s
PCR	Denaturation	95°C	15 s
(45 cycles)	Extension (annealing)	60°C	45 s

<Primer and probes>

	Sequence	Length	GC	Tm
Forward primer	GCTCTTAGCAAAGTCAAGTTGAATGA	26-mer	38.5%	65.4°C
Reverse primer	TGCTCCGTTGGATGGTGTATT	21-mer	47.6%	66.3°C
TaqMan® probe	[FAM]ACACTCAACAAAGATCA ACTTCTGTCATCCAGC[TAMRA]	33-mer	42.4%	73.9°C

<Results>
The RS virus was successfully detected from the RNA purified from clinical specimens.

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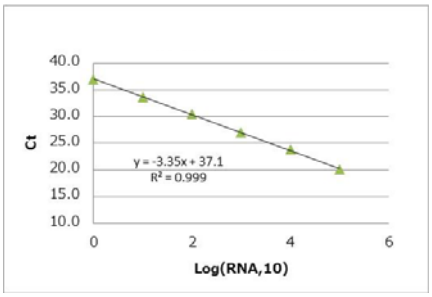
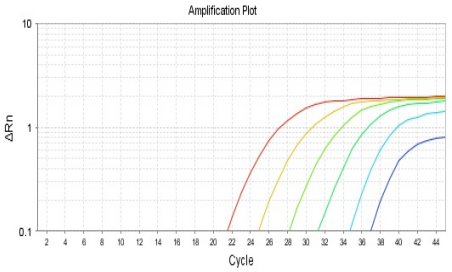




2. Simultaneous quantification of multiple targets

HeLa S3 cells (4×10^5 cells in a six-well plate) were cultured for 20 h with or without 100 nM phorbol 12-myristate 13-acetate. Then, IL-1β, TNF-α, and GAPDH genes were detected from the HeLa total RNA (1–100 pg) using THUNDERBIRD® Probe One-step qRT-PCR Kit. The genes were analyzed using singleplex and multiplex detection systems with LightCycler® 96.

<Cycling conditions>

Step		Temperature	Time
Reverse transcription		50°C	10 min
Predenaturation		95°C	60 s
PCR	Denaturation	95°C	15 s
(45 cycles)	Extension (annealing)	60°C	45 s

<Primers and probes>

IL-β	Sequence	Length	GC	T_m
Forward primer	ACAGATGAAGTGCTCCTTCCA	21-mer	47.6%	63.7°C
Reverse primer	GTCGGAGATTCGTAGCTGGAT	21-mer	52.4%	64.3°C
TaqMan® probe	[FAM]CTCTGCCCTCTGGATGGCGG [TAMRA]	20-mer	70.0%	73.5°C

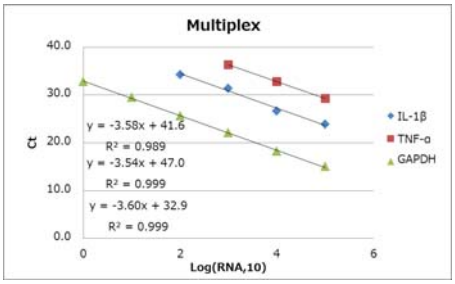
TNF-α	Sequence	Length	GC	Tm
Forward primer	CCCAGGGACCTCTCTCTAATC	21-mer	57.1%	62.8°C
Reverse primer	ATGGGCTACAGGCTTGTCACT	21-mer	52.4%	64.8°C
TaqMan® probe	[Cy5]TGGCCCCAGGCAGTCAGATCATC [BHQ2]	23-mer	60.9%	75.3°C

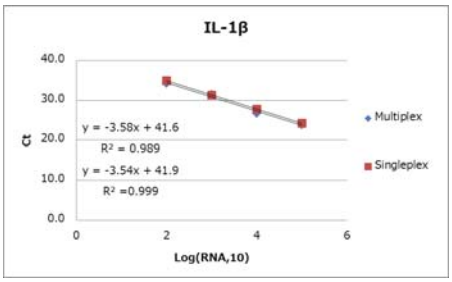
GAPDH: human GAPD (GAPDH) endogenous control (VIC[R]/MGB probe, primer limited) (Applied Biosystems, 4326317E)

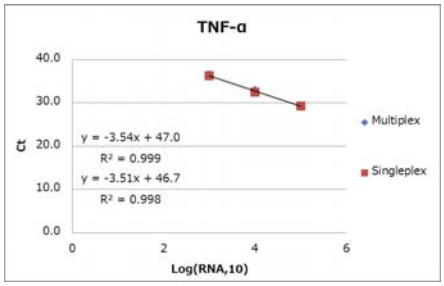
<Results>

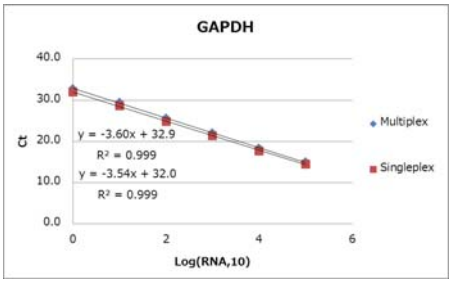
The singleplex and multiplex detection systems showed similar results of PCR efficiency and correlation coefficient (R^2). The results indicated that this system facilitates analysis of the expression of multiple genes.

	IL-β		TNF-α		GAPDH	
	Efficiency	R^2	Efficiency	R^2	Efficiency	R^2
Multiplex	90.2%	0.989	91.8%	0.999	89.6%	0.999
Singleplex	91.6%	0.999	92.9%	0.998	91.6%	0.999









[8] Troubleshooting

Symptom	Cause	Solution
Low PCR efficiency, low sensitivity, divergence of results	Incorrect setting of detector mode for the fluorescence dye	Confirm the detector setting.
	Incorrect setting for data collection	Confirm the data collection setting.
	Incorrect setting for sample position	Reposition the sample tubes.
	Degradation of RNA	RNA should be purified again. RNA purification method, reagents, and equipment should also be reconfirmed simultaneously.
	Inappropriate extension (annealing) temperature or time	Extension (annealing) temperature and time should be optimized according to the instructions (see [6] Optimization of conditions).
	Inappropriate concentration of primers or probes	If the results are not improved by optimizing the extension (annealing) temperature and time, primer or probe concentration should be optimized according to the instructions (see [6] Optimization of conditions).
	Inappropriate design of primers or probes	If the results are not improved by changing primer and probe concentrations, the primer or probe design should be reconfirmed (see [6] Optimization of conditions).
	Inappropriate concentration of ROX Reference dye	The concentration of ROX Reference dye should be determined depending on the real-time cycler (see [5] Protocol).
Dispersion of quantification	Failure or malfunction of device	Check the device.
	Low quality of sample DNA	Purify the RNA sample again by an appropriate method. Contaminated genomic DNA may also cause divergence of data by it being amplified. In such cases, primers and/or probes should be redesigned according to [3] Primer/probe design. Alternatively, DNase I treatment of RNA is also effective to improve this problem.
	Absorption of RNA	A small amount of RNA tends to be absorbed by microtubes. Low-absorption tubes should be used.
	Excessive or insufficient template RNA	Quantification should be performed in the linear range of the standard curve.
	Inappropriate extension (annealing) temperature or time	Extension (annealing) temperature and time should be optimized according to the instructions (see [6] Optimization of conditions).
	Inappropriate concentration of primers or probes	If the results are not improved by optimizing the extension (annealing) temperature and time, primer or probe concentration should be optimized according to the instructions (see [6] Optimization of conditions).
	Inappropriate design of primers or probes	If the results are not improved by changing primer and probe concentrations, the primer or probe design should be reconfirmed (see [6] Optimization of conditions).
	Dispersion of reaction volume	Insufficient reaction volume tends to reduce the sensitivity or reproducibility. The reaction volume should be determined according to the instruction manual of each real-time cycler.

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Symptom	Cause	Solution
Detection of signal in a negative control	Contamination of positive samples or PCR products	If the signal is detectable even when a fresh negative control is used, all reagents should be checked and prepared again.
	Design of TaqMan® probe is not appropriate	TaqMan® probe itself may generate a nonspecific signal by forming unexpected structures. In such cases, the TaqMan® probe should be redesigned.

[9] Related products

Product name	Package	Code No.
Highly efficient real-time PCR master mix **THUNDERBIRD® SYBR® qPCR Mix**	200 rxns	QPS-201T QPS-201
Highly efficient cDNA synthesis kit for real-time PCR **ReverTra Ace® qPCR RT Kit**	200 rxns	FSQ-101
Highly efficient cDNA synthesis master mix for real-time PCR **ReverTra Ace® qPCR RT Master Mix**	200 rxns	FSQ-201
Highly efficient cDNA synthesis master mix for real-time PCR with genomic DNA remover **ReverTra Ace® qPCR RT Master Mix with gDNA remover**	200 rxns	FSQ-301
One-step real-time PCR master mix for probe assay ***RNA-direct*™ Real-time PCR Master Mix**	0.5 mL × 5	QRT-101
One-step real-time PCR master mix for SYBR® Green assay ***RNA-direct*™ SYBR® Green Real-time PCR Master Mix**	0.5 mL × 5	QRT-201

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