THUNDERBIRD™ Next SYBR® qPCR Mix

QPX-201T 1 mL x 1
QPX-201 1.67 mL x 3

Store at -20°C, protected from light

Contents

CAUTION

All reagents in this kit are intended for research purposes. Do not use for diagnosis or clinical purposes. Please observe general laboratory precaution and utilize safety while using this kit.

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FOR RESEARCH USE ONLY. NOT FOR HUMAN OR DIAGNOSTIC USE.

[1] Introduction

Description

THUNDERBIRD™ Next SYBR® qPCR Mix is a highly efficient 2× Master Mix for real-time PCR using SYBR® Green I. The master mix contains all required components, except primers. The master mix facilitates reaction setup, and improves the reproducibility of experiments.
This product is an improved version of THUNDERBIRD™ SYBR® qPCR Mix (Code No. QPS-201). In particular, the reaction specificity and PCR efficiency is enhanced.

Features

-High specificity
The specificity for the detection of low-copy targets is improved.
-Homogeneous amplification
The dispersion of PCR efficiency between targets is reduced by a new PCR enhancer.
-Broad dynamic range
High specificity and effective amplification enable the detection of a broad dynamic range.
-Compatibility for various real-time cyclers.
The reagent is applicable to most real-time cyclers (i.e. Block type and glass capillary type). Because the passive reference dye is included with this kit, the kit can be applied to real-time cyclers that require a passive reference dye.
-Fast PCR
This reagent enables amplification using fast cycle condition.
-Hot start PCR
The master mix contains anti- DNA polymerase antibodies for hot start technology. The antibodies are easily inactivated in the first denaturation step, thereby activating the DNA polymerase.
-Utilization of dUTP
This master mix contains dUTP instead of dTTP. Therefore, the rate of false-positive detection can be reduced by adding uracil-N-glycosylase (UNG). *UNG is not supplied with this kit. Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101) can be used.
-Utilization of a visible tracking dye
This master mix contains visible tracking dye. This dye helps to eliminate pipetting errors, and does not spectrally overlap with fluorescent dyes used for qPCR and will not interfere with real-time detection.

About the SYBR® Green I detection system

The SYBR® Green I assay system utilizes fluorescent emission when SYBR® Green is intercalated into double-stranded DNA. The signal depends on the amount of amplified DNA. However, this system cannot distinguish between target and non-specific amplicons. Therefore, melting curve analysis is necessary after amplification.

[2] Components

This kit includes the following components for 100 reactions (QPX-201T) and 500 reactions (QPX-201), with a total of 20 µL per reaction. All reagents should be stored at -20 °C.

<QPX-201T>
THUNDERBIRD™ Next SYBR® qPCR Mix 1 mL

<QPX-201>
THUNDERBIRD™ Next SYBR® qPCR Mix 1.67 mL x 3

Notes:
-THUNDERBIRD™ Next SYBR® qPCR Mix can be stored, protected from light, at 2-8°C for up to 3 months. For longer storage, this reagent should be kept at -20°C and protected from light. No negative effect was detected by 10 freeze-thaw cycles of THUNDERBRID™ Next SYBR® qPCR Mix.

[3] Primer design

1. Primer conditions

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

-Primer length: 20-30 mer
-GC content of primer: 40-60%
-Target length: ≤ 500 bp (optimally, 80-200 bp)
-Melting temperature (Tm) of primers: 60-65°C
-Purification grade of primers: Cartridge (OPC) grade or HPLC grade

Notes:
-Longer targets (>500 bp) reduce efficiency and specificity of amplification.
-Tm of the primers can be flexible, because the Tm value depends on the calculation formula.

[4] Template DNA

The following DNA samples can be used as templates.

1. cDNA

Non-purified cDNA, generated by reverse transcription reactions, can be used directly for real-time PCR using THUNDERBIRD™ Next SYBR® qPCR Mix. Up to 10% of the volume of a cDNA solution can be used for a real-time PCR reaction. However, excess volume of the cDNA may inhibit the PCR. Up to 20% (v/v) of the cDNA solution from the ReverTra Ace™ qPCR RT Kit (Code No. FSQ-101), the ReverTra Ace™ qPCR RT Master Mix (Code No. FSQ-201) and the ReverTra Ace™ qPCR RT Master Mix with gDNA remover (Code No. FSQ-301) can be used for real-time PCR.

2. Genomic DNA, Viral DNA

Genomic DNA and viral DNA can be used at up to 200 ng in 50 µL reactions.

3. Plasmid DNA

Although super-coiled plasmids can be used, linearized plasmid DNA produces more accurate assays. The copy number of the plasmid DNA can be calculated by the following formula.

Copy number of 1µg of plasmid DNA = 9.1×10^{11} / Size of plasmid DNA (kb)

[5] Protocol

1. Standard reaction set up

	Reaction volume		Final
Reagent	50µL	20µL	Concentration
Sterilized water	X µL	X µL	
THUNDERBIRD™ Next SYBR® qPCR Mix	25 µL	10 µL	1x
Forward Primer	15 pmol	6 pmol	0.3 µM*1
Reverse Primer	15 pmol	6 pmol	0.3 µM*1
(Uracil-N-glycosylase) [optional]	1 unit*2	0.4 units*2	
DNA solution	Y µL	Y µL	
Total	50 µL	20 µL	

Notes:

*1 Higher primer concentration tends to improve the amplification efficiency, and lower primer concentration tends to reduce the non-specific amplification. The primer concentration should be set between 0.2-0.6 µM.

*2 Heat-labile uracil-N-glycosylase (UNG) should be used. UNG is not supplied with this kit. It is recommended that you use Uracil-DNA Glycosylase (UNG), Heat-labile (UNG-101) optionally.

2. Cycling condition

The following table shows the recommended thermal conditions using primers designed according to the recommended primer described in [3]. Almost all targets can also be amplified using the ongoing conditions with other real-time PCR reagents.
When satisfactory results are not obtained or the Tm value of the primers is lower than 60°C, a 3-step cycle should be used. The detailed conditions are described in [5] 2-4.

Fast cycling mode

<2-step cycle>	Temperature	Time	Ramp	
(UNG treatment)	(20–25°C[*1])	(10 min[*1])	(Maximum)	
Pre-denaturation	95°C	30 sec[*2]	Maximum	
Denaturation:	95°C	5 sec[*3]	Maximum	↰ 40 cycles
Extension:	60°C[*4]	10 sec[*5]	Maximum	
	(data collection should be set at the extension step)			
Melting / Dissociation Curve Analysis[*6]				

Standard cycling mode

<2-step cycle>	Temperature	Time	Ramp	
(UNG treatment)	(20–25°C[*1])	(10 min[*1])	(Maximum)	
Pre-denaturation	95°C	30 sec[*2]	Maximum	
Denaturation:	95°C	5 sec[*3]	Maximum	↰ 40 cycles
Extension:	60°C[*4]	30 sec[*5]	Maximum	
	(data collection should be set at the extension step)			
Melting / Dissociation Curve Analysis[*6]				

[*1] [Optional] 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. Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101) can be used.

[*2] Due to the anti- DNA polymerase antibody hot start PCR system, the pre-denaturation can be completed within 60 sec. The pre-denaturation time should be determined according to the recommendations of each real-time cycler. If the optimal pre-denaturation time cannot be determined, the time should be set at 30 sec.

[*3] If the optimal denaturation time cannot be determined, the time should be set at 5 sec.

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*4 Insufficient amplification may be improved by decreasing the extension temperature, and non-specific amplification (e.g. abnormal shapes of the amplification curve at low template concentrations) may be reduced by increasing the extension temperature. The extension temperature should be set at 56-64°C.

*5 If the target size is smaller than 300 bp, the extension time can be set at 10 sec on almost all real-time cyclers. Instability of the amplification curve or variation of data from each well may be improved by setting the extension time at 30-60 sec. Some real-time cyclers or software need over 25 sec for the extension step. In these cases, the time should be set according to each instruction manual (e.g. Applied Biosystems® QuantStudio™ 5, 96-well, 0.2 mL: ≥ 25 sec; Applied Biosystems® 7000/73000: ≥ 31 sec; Applied Biosystems® 7500: ≥ 35 sec.).

*6 The melting curve analysis should be performed according to the recommendations of each real-time cycler.

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2-1. Real-time PCR conditions using Applied Biosystems® StepOnePlus™

(Normal block type, software version 2.3)

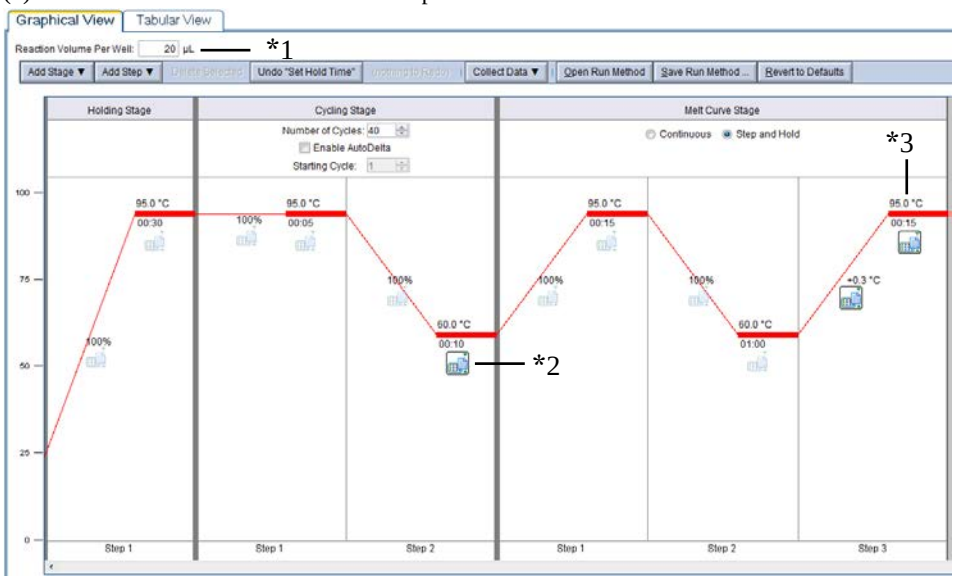
The following is an example of a SYBR® Green I assay using Applied Biosystems® StepOnePlus™. These conditions can also be useful for cyclers such as the Applied Biosystems® 7500 Fast Real-Time PCR System and QuantStudio™ Real-Time PCR System.

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

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

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

(3) Select “Run Methods” and set the temperature conditions as follows:



*1 Input of actual reaction volume is important to achieve a successful analysis.

*2 Set the data collection at the extension step. Some real-time cyclers or software need over 25 sec for the extension step. In these cases, the time should be set according to each instruction manual (e.g. Applied Biosystems® QuantStudio™ 5, 96-well, 0.2mL:
≥ 25 sec; Applied Biosystems® 7000/73000: ≥ 31 sec; Applied Biosystems® 7500: ≥ 35 sec.).

*3 Add the condition for melting curve analysis.

(4) Insert the PCR tubes or plate.

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(5) Start the program.

2-2. LightCycler® 96

(software version 1.1)

The following is an example of a SYBR® Green I assay using the Roche LightCycler® 96.

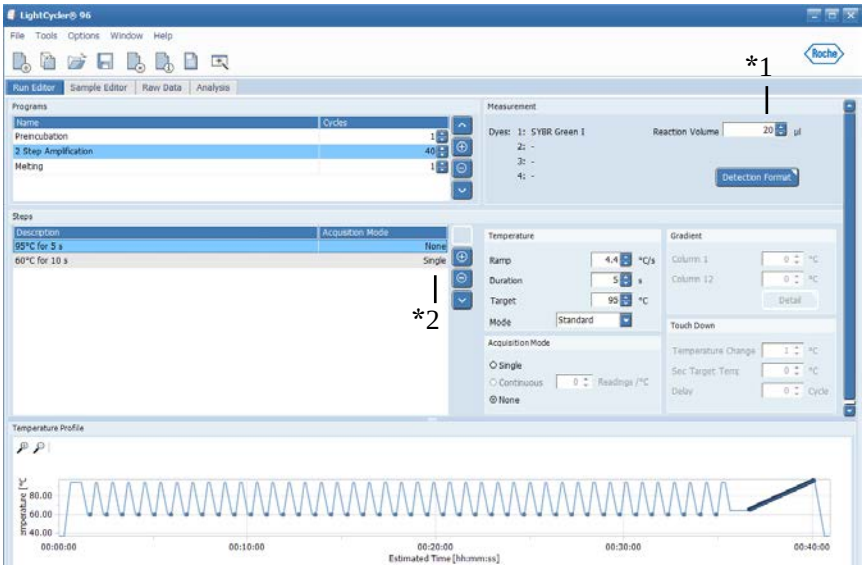
(1) Select “Create New Experiment”.

(2) Input reaction volume.

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

(4) Select the first “Preincubation” and input 95 °C as “Target” and 30 s as “Duration”.

(5) Select the second “2 Step Amplification” and input 95°C as “Target” and 5 s as “Duration” followed by 60°C as “Target” and 10 s as “Duration”.



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

(6) Insert the PCR tubes or plate.

(7) Start the program.

2-3. CFX96 Touch™ Deep Well

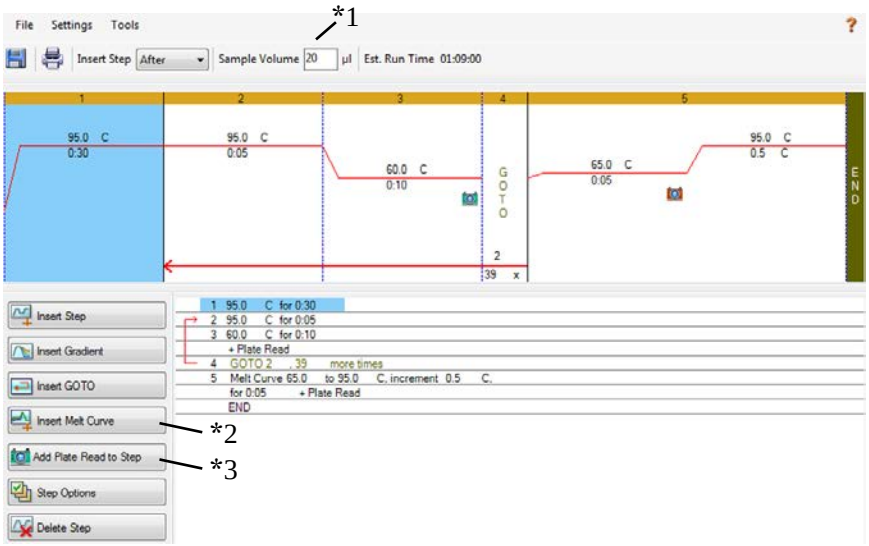
(software version 3.1)

The following is an example of a SYBR® Green I assay using the Bio-Rad CFX96 Touch™ Deep Well.

(1) Select “User-defined”.

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

(3) Input the following conditions: 95°C, 30 s, 95°C, 5 s, 60°C, 10 s, 40 cycles.



*1 Input of the actual reaction volume is important for a successful analysis.
*2 Select “Insert Melt Curve” and add the cycle for the melting curve.
*3 Select “Add Plate Read to Step” and set the data collection point at the extension step.

(4) Insert the PCR tubes or plate.

(5) Start the program.

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2-4. 3-step cycle

In the event of the 2-step cycle failing, the following 3-step cycle may improve results. In the following cases, the 3-step cycle conditions may improve the result efficiently.

-The Tm of the primer is lower than 60°C.
-The target is longer than 500 bp.
-PCR efficiency is low.

<3-step cycle>	Temperature	Time	Ramp	
Pre-denaturation	95°C	20-60 sec[*1]	Maximum	
Denaturation:	95°C	1-15 sec[*1]	Maximum	40 cycles
Annealing	55-65°C[*2]	5-30 sec[*3]	Maximum	
Extension:	72°C	10-60 sec[*4]	Maximum	
	(data collection should be set at the extension step)			
Melting / Dissociation Curve Analysis[*5]				

[*1] The denaturation step should be determined according to [5] 2.

[*2] The annealing temperature should be set at primer's Tm-5°C. A higher annealing temperature may improve non-specific amplification.

[*3] The annealing time should be set at 5 sec (Fast cycler), 15 sec (Normal cycler). Shorter annealing times may reduce non-specific amplification. Longer annealing times (up to 30 sec) may increase the PCR efficiency when the efficiency is low.

[*4] Shorter targets (≤300 bp) require shorter extension times (≤10 sec). However, certain cyclers require >10 sec detection time at the extension step. An unstable signal may be improved by prolonging the extension time up to 30-60 sec. Also note that some cyclers cannot have an extension time of 25 sec (e.g. Applied Biosystems® QuantStudio™ 5, 96-well, 0.2 mL: ≥ 25 sec.; Applied Biosystems® 7000/73000: ≥ 31 sec.; Applied Biosystems® 7500: ≥ 35 sec.).

[*5] The melting curve analysis should be performed according to each cycler's recommendations.

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[6] Related Protocol

cDNA synthesis

cDNA synthesized by various cDNA synthesis reagents can be used with THUNDERBIRD™ Next SYBR® qPCR Mix. However, cDNA synthesized by a reagent specialized for real-time PCR can increase sensitivity.
ReverTra Ace™ qPCR RT Kit (Code No. FSQ-101), ReverTra Ace™ qPCR RT Master Mix (Code No. FSQ-201) and ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Code No. FSQ-301) are cDNA synthesis kits suitable for real-time PCR. Here, the protocol with ReverTra Ace™ qPCR RT Master Mix and ReverTra Ace™ qPCR RT Master Mix with gDNA Remover is described. However, for the detailed protocol, please refer to the instruction manual of the kit.

ReverTra Ace™ qPCR RT Master Mix (Code No. FSQ-201)

(1) Denaturation of RNA [optional]
Incubate the RNA solution at 65°C for 5 min, and then keep on ice.

Notes:
-This step increases the efficiency of reverse transcription of RNA templates that form secondary structures.
-This step should be performed before adding 5x RT Master Mix.

(2) Preparation of the reaction solution

Prepare the following reagents on ice.

5x RT Master Mix	2 µL
RNA template	1 pg – 1 µg
Nuclease-free Water	X µL
Total Volume	10 µL

(3) Reverse transcription reaction

-Incubate at 37°C for 15 min.
-Incubate at 50°C for 5 min. [optional]
-Heat to 98°C for 5 min.

-Store the reacted solution* at 4°C or – 20°C
*This solution can be used in the real-time PCR reaction directly or after dilution.

Notes:
-ReverTra Ace™ excels at high reaction temperatures (up to 50°C). This step may increase the efficiency of the reverse transcription.

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ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Code No. FSQ-301)

(1) Preparation of the "4x DN Master Mix" and "gDNA Remover" mixture.
Prior to use, a 1 in 50 volume of gDNA remover should be added to 4x DN Master Mix (*e.g.* 4x DN Master Mix : gDNA remover = 440 μL : 8.8 μL).

Notes
4x DN Master Mix with gDNA remover can be stored at -20°C for at least for 3 months. The mixture can be prepared in a smaller volume [*e.g.* 4x DN Master Mix : gDNA remover = 220 μL : 4.4 μL].

(2) Denaturation of RNA [optional]
Incubate the RNA solution at 65°C for 5 min, and then keep on ice.

Notes
-This step increases the efficiency of reverse transcription of RNA templates that form secondary structures.
-This step should be performed before adding 4x DN Master Mix.

(3) Preparation of the DNase I reaction solution:
Prepare the following reagents on ice.

4x DN Master Mix	2 μL
RNA template	0.5 pg – 0.5 μg
Nuclease-free Water	X μL
Total Volume	8 μL

(4) Incubate at 37°C for 5 min.

(5) Preparation of the for reverse transcription solution;

Prepare the following reagents on ice.

Reacted solution from (4)	8 μL
5x RT Master Mix II	2 μL
Total Volume	10 μL

(6) Reverse transcription reaction

-Incubate at 37°C for 15 min.
-Incubate at 50°C for 5 min. [optional]
-Heat to 98°C for 5 min.

-Store the reacted solution* at 4°C or – 20°C
*This solution can be used in the real-time PCR reaction directly or after dilution.

Notes:

-ReverTra Ace™ excels at high reaction temperatures (up to 50°C). This step may increase the efficiency of the reverse transcription.

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[7] Troubleshooting

Symptom	Cause	Solution
Loss of linearity in the high cDNA/DNA concentration region.	Intercalation of SYBR® Green I into the template DNA.	Because SYBR® Green I is also intercalated into the template DNA, the base line tends to be higher when high concentration DNA samples are used. Diluted template should be used to obtain a correct Ct value.
Loss of linearity or lower signal in the low DNA/cDNA concentration region.	The template DNA is insufficient.	When the DNA/cDNA copy number is lower than 10 copies per reaction, the linearity of the reaction tends to be lost. The template concentration should be increased.
	Adsorption of the DNA to the tube wall.	The diluted DNA templates tend to be absorbed onto the tube wall. Dilution should be performed just prior to experiments.
	Competition with primer dimer formation.	Dimer formation may reduce the amplification efficiency of the target, especially for reactions at low template concentration. The reaction condition should be optimized or the primer sequences should be changed.
Loss of linearity of the amplification curves.	Competition with non-specific amplification.	Non-specific amplification may reduce the amplification efficiency of the target. The reaction conditions should be optimized or the primer sequences should be changed.
The PCR efficiency is lower than 80% (slope: <-3.95).	Inappropriate cycling conditions.	Optimize the cycling conditions according to [5].
	Degradation of the primers.	Fresh primer solution should be prepared.
	The calculation of the PCR efficiency is inappropriate.	The Ct value on the linear region should be used to calculate PCR efficiency.
The PCR efficiency is higher than 110% (slope: >-3.1).	The calculation of the PCR efficiency is inappropriate.	The Ct value on the linear region should be used to calculate PCR efficiency.
Reproducibility is not good.	Poor purification of the template DNA	Low-purity DNA may contain PCR inhibitors. Re-purify the DNA samples.
	Absorption of the template DNA to the tube wall.	Diluted DNA templates tend to be absorbed onto the tube wall. Dilution of the template DNA/cDNA should be performed just prior to experiments.
	Plasmid DNA or PCR product is used as a template.	In general, plasmid DNA or PCR product is used at low concentration. Diluted DNA templates tend to be absorbed onto the tube wall. Dilution of the template DNA/cDNA should be performed just prior to experiments. Dilution with a carrier nucleic acid solution (Yeast RNA) is also effective in improving linearity.
	Inappropriate thermal conditions.	Optimize the thermal conditions according to [5].
	Low purity of the primers	Different lots of primers may show different results. When the lot is changed, prior testing of the primer should be performed.

Symptom	Cause	Solution
Amplification from the non-template control (NTC).	Formation of primer dimer.	On the melting curve analysis, a peak at a temperature lower than that of the target peak suggests a primer dimer. The PCR cycle should be optimized according to [5] (2). If the result is not improved, the following should be performed: change the primer sequence and/or change the purification grade of the primer (HPLC grade)
	Contamination or carry over of the PCR products.	When the no-template control generates a peak at the same melting temperature as the target on the melting curve analysis, the amplification is caused by a carry-over or contamination. Use fresh reagents.
Low amplification curve signal / Unstable amplification curve signal.	Inappropriate settings of fluorescence measurement	Settings should be confirmed according to the instruction manual of each detector.
	Insufficient reaction volume.	Low reaction volume may cause an unstable signal. Increase the reaction volume.
Detection of multiple peaks on the melting curve analysis	Non-specific amplification.	Optimize the reaction conditions. If the result is not improved, the primer sequence should be changed.
	Formation of primer dimer.	On the melting curve analysis, a peak at a temperature lower than that of the target peak suggests a primer dimer. The PCR cycle should be optimized according to [5] (2). If the result is not improved, the following action should be performed: change the primer sequence and/or change the purification grade of the primer (HPLC grade)

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[8] Related products

Product name	Package	Code No.
High efficient real-time PCR master mix **THUNDERBIRD™ Probe qPCR Mix**	1mL × 1 (100 rxns) 1.67mL × 3 (500 rxns)	QPS-101T QPS-101
High efficient cDNA synthesis kit for real-time PCR **ReverTra Ace™ qPCR RT Kit**	200 rxns	FSQ-101
High efficient cDNA synthesis master mix for real-time PCR **ReverTra Ace™ qPCR RT Master Mix**	200 rxns	FSQ-201
High 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*™ Realtime PCR Master Mix**	0.5mL × 5 (250 rxns)	QRT-101
One-step Real-time PCR master mix for SYBR® Green assay ***RNA-direct*™ SYBR® Realtime PCR Master Mix**	0.5mL × 5 (250 rxns)	QRT-201
One-step Real-time PCR master mix for probe assay **THUNDERBIRD™ Probe One-step qRT-PCR Kit**	250 rxns	QRZ-101
Heat-labile Uracil-DNA Glycosylase **Uracil-DNA Glycosylase (UNG), Heat-labile**	200 U	UNG-101

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