TOYOBO

Instruction manual for THUNDERBIRD Next Probe qPCR Mix F2143K

THUNDERBIRD™ Next Probe qPCR Mix

QPX-101T 1 mL x 1
QPX-101 1.67 mL x 3

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™ Next Probe qPCR Mix is a highly efficient 2× Master Mix for real-time PCR using TaqMan® probes. The master mix contains all required components, except for ROX reference dye, probe and primers (50x ROX reference dye is individually supplied with this kit). The master mix facilitates reaction setup, and improves the reproducibility of experiments. This product is an improved version of THUNDERBIRD™ Probe qPCR Mix (Code No. QPS-101). This kit is optimized for multiplexing and crude samples containing PCR inhibitors.

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.

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

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

-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.

-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 50x 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 (QPX-101T) and 500 reactions (QPX-101), with 20 µl per reaction. All reagents should be stored at -20°C.

<QPX-101T>

THUNDERBIRD™ Next Probe qPCR Mix	1 ml x 1
50x ROX reference dye	50 µl x 1

<QPX-101>

THUNDERBIRD™ Next Probe qPCR Mix	1.67 ml x 3
50x ROX reference dye	250 µl x 1

Notes:

-THUNDERBIRD™ Next Probe 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 Probe qPCR Mix. This reagent does not contain the ROX reference dye.

-50x ROX reference dye can be stored, protected from light, at 2-8°C or -20°C. For real-time cyclers that require a passive reference dye, this reagent must be added to the reaction mixture at a concentration of 1x or 0.1x. The master mix solution with the ROX reference dye 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. The pre-mixed reagents can be prepared according to the following ratios. [5] Table 1 shows the optimal concentration of the ROX dye.

1x solution

THUNDERBIRD™ Next Probe qPCR Mix : 50x ROX reference dye= 1.67 ml : 66.8 µl
THUNDERBIRD™ Next Probe qPCR Mix : 50x ROX reference dye= 1 ml : 40 µl

0.1x solution

THUNDERBIRD™ Next Probe qPCR Mix : 50x ROX reference dye = 1.67 ml : 6.7 µl
THUNDERBIRD™ Next Probe qPCR Mix : 50x ROX reference dye = 1 ml : 4 µl

For real-time cyclers that do not require a passive reference dye, THUNDERBIRD™ Next Probe qPCR Mix without the ROX reference dye can be used.

[3] Primer/probe 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: ≤ 200 bp (optimally, 80-150 bp)
-Melting temperature (Tm) of primers: 60-65°C

-Purification grade of primers: Cartridge (OPC) grade or HPLC grade

Notes:

-Longer targets (>200 bp) reduce efficiency and specificity of amplification.

-Tm of the primers can be flexible, because the Tm value depends on the calculation formula.

2. Fluorescent probe

The probes should be designed according to the guidelines of each probe system. Because insufficiently purified probes may inhibit the reaction, HPLC-grade probes should be used.

[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 Probe 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)

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[5] Protocol

1. Standard reaction set up

Reagent	Reaction volume 50 µl	20 µl	Final Concentration
Sterilized water	X µl	X µl	
THUNDERBIRD™ Next Probe 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]
TaqMan® Probe	10 pmol	4 pmol	0.2 µM[*2]
50x ROX reference dye	1µl / 0.1 µl	0.4µl / 0.04µl	1x / 0.1x[*3]
(Uracil-N-glycosylase) [optional]	1 unit[*4]	0.4 units[*4]	
DNA solution	Y µl	Y µl	
Total	50 µl	20 µl	

Notes:

*1 If the expected results are not obtained at 0.3 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, QuantStudio™ 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. UNG is not supplied with this kit. It is recommended that you use Uracil-DNA Glycosylase (UNG), Heat-labile (UNG-101) optionally.

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.

Fast cycling mode

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

Standard cycling mode

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

*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 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 and QuantStudio™ Real-Time PCR System.

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

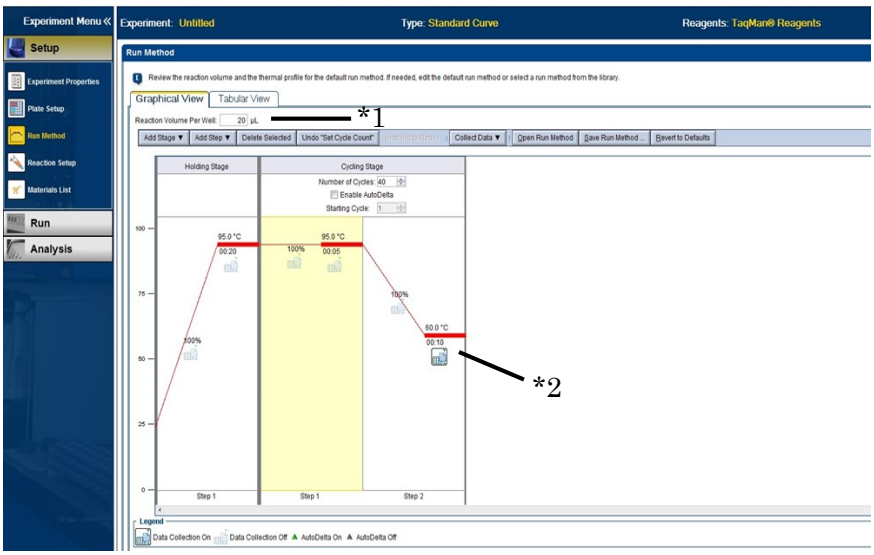
(2) ” Select “TaqMan® Reagents” as reagents in the following tabs.

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 “Holding Step” and input 95°C, 20 s.

(5) Select “Cycling Stage” and input 95°C, 5 s, 60°C, 10 s, 40 cycles.



*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.).

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

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2-2. LightCycler® 96

(software version 1.1)

The following is an example of a TaqMan® assay using the Roche LightCycler® 96.

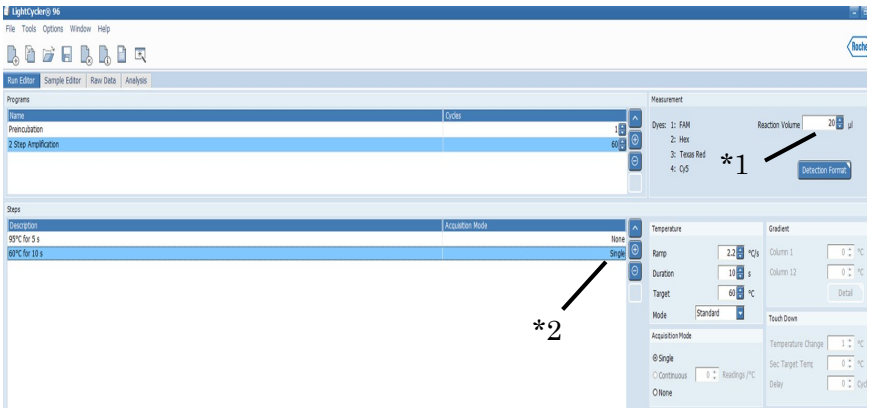
(1) Select “Create New Experiment”

(2) Input reaction volume.

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

(4) Select the first “Preincubation” and input 95°C as “Target” and 60 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.

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2-3. CFX96 Touch™ Deep Well

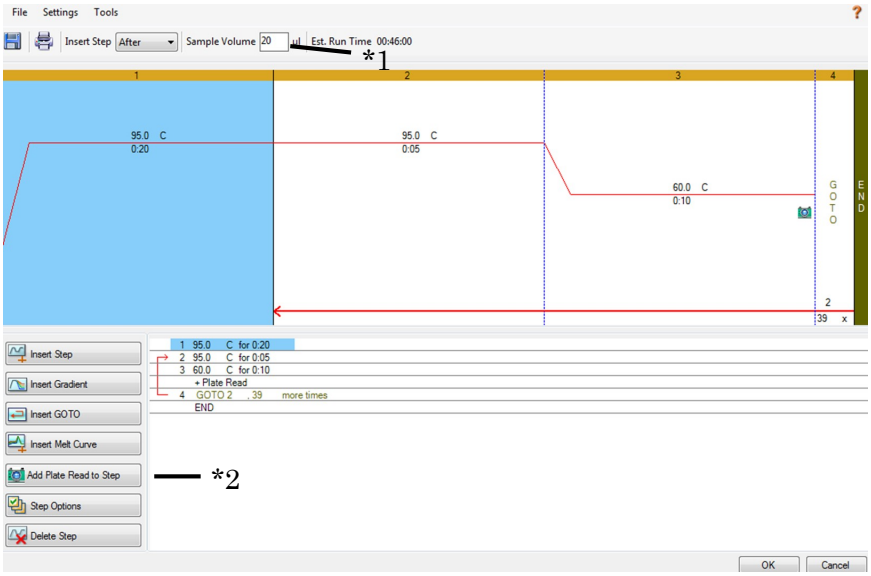
(software version 3.1)

The following is an example of a TaqMan® 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, 20 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 “Add Plate Read to Step” and set the data collection point at the extension step.

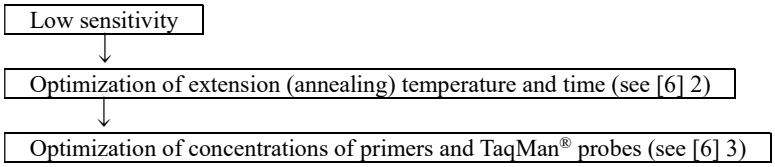
(4) Insert the PCR tubes or plate.

(5) Start the program.

[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.

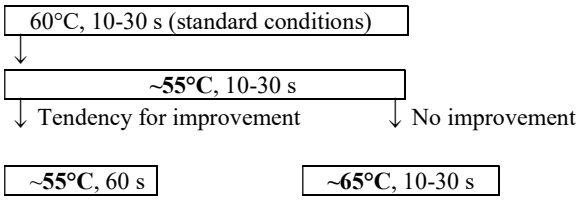


2. Optimization of extension (annealing) temperature and time

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>



Notes

The number of cycles can be increased up to 45.

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 0.3 and 0.5 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 0.3 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.

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[7] Related Protocol

cDNA synthesis

cDNA synthesized by various cDNA synthesis reagents can be used with THUNDERBIRD™ Next Probe 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.

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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[8] Troubleshooting

Symptom	Cause	Solution
Loss of linearity in the high cDNA/DNA concentration region.	Inhibition by the components in the cDNA/DNA solution.	-DNA: The DNA sample may contain PCR inhibitors. The DNA samples should be repurified. -cDNA: The components in the cDNA synthesis reagent may inhibit the PCR reaction. The cDNA sample should be used after dilution.
Lost 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.	In the probe assay, primer dimers are not detected. However, dimer formation may reduce the amplification efficiency of the target, especially for reactions at low template concentration. The reaction conditions should be optimized or the primer sequences should be changed.
Loss of linearity of the amplification carves.	Competition with non-specific amplification.	In the probe assay, non-specific amplification is not detected. However, 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 90% (slope: <-3.6)	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 or probes.	Different lots of primers or probes may show different results. When the lot is changed, prior testing of the primer or probe should be performed.

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Symptom	Cause	Solution
Amplification from the non-template control (NTC).	Contamination or carry over of the PCR products.	Change the contaminated reagent.
	Inappropriate settings of fluorescence measurement, such as in the case of multiplex PCR.	In multiplex experiments, inappropriate setting of fluorescence measurement may cause the detection of noise by the cross talk of fluorescent dyes. Settings should be reconfirmed.
Low amplification curve signal / Unstable amplification curve signal.	Excessive amount of ROX reference dye.	Excessive amount of ROX reference dye may cause low signal. 50x ROX reference dye should be used according to [5] Table 1.
	Inappropriate settings of fluorescence measurement.	Settings should be confirmed according to the instruction manual of each detector.
	Low purity of fluorescent probes.	Low purity of the probe may increase the base line. HPLC grade probes should be used.
	Excessive intensity of the quencher Dye.	Certain quenchers (e.g. TAMRA) may cause a higher baseline because of its fluorescence. Use of a non-fluorescent quencher may improve the high baseline.
	Degradation of the probe.	Store the probes according to the manufacture's recommendations.
	Insufficient fluorescence measurement time.	Certain detection systems require a longer time to detect the fluorescent signal. Longer extension (measurement) time (45-60 sec) may improve the unstable signal.
	Insufficient reaction volume.	Low reaction volume may cause an unstable signal. Increase the reaction volume.

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[9] Related products

Product name	Package	Code No.
Highly efficient real-time PCR master mix **THUNDERBIRD™ Next SYBR® qPCR Mix**	1mL × 1 (100 rxns) 1.67mL × 3 (500 rxns)	QPX-201T QPX-201
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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