THUNDERBIRD™ Probe qPCR Mix

QPS-101T 1 mL x 1
QPS-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 diagnosis 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 qPCR Mix is a highly efficient 2x 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 Realtime PCR Master Mix (Code No. QPK-101). In particular, 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*. (*Patent pending)

-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 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.

-Hot start PCR

The master mix contains anti-Taq DNA polymerase antibodies for hot start technology. The antibodies are easily inactivated in the first denaturation step, thereby activating the DNA polymerase.

About the fluorescent probe detection system

The TaqMan® probe system utilizes fluorescence emission from the probes. The probes hybridize to the target amplicons and then emit fluorescence upon degradation by the 5'-3' exonuclease activity of Taq DNA polymerase. This type of detection system can achieve higher specificity in real-time PCR assays than the SYBR® Green I detection system.

[2] Components

This kit includes the following components for 40 reactions (QPS-101T) and 200 reactions (QPS-101), with 50 µl per reaction. All reagents should be stored at -20°C.

<QPS-101T>

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

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<QPS-101>

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

Notes:

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

0.1x solution

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

For real-time cyclers that do not require a passive reference dye, THUNDERBIRD™ 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™ 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 ReverTra Ace® qPCR RT Kit (Code No. FSQ-101) can be used for real-time PCR (see [6]).

2. Genomic DNA, Viral RNA

Genomic DNA and viral RNA 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. Reaction mixture setup

Reagent	Reaction volume 50 µl	20 µl	Final Concentration
DW	X µl	X µl	
THUNDERBIRD™ 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[*1]
50x ROX reference dye	1µl / 0.1 µl	0.4µl / 0.04µl	1x / 0.1x[*2]
DNA solution	Y µl	Y µl	
Total	50 µl	20 µl	

Notes:

[*1] Primer / probe concentration should be determined according to the manufacturer's instructions.
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] 50x 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 etc.	1x (50:1)
Applied Biosystems 7500, 7500Fast, Stratagene cyclers (Optional) etc.	0.1x (500:1)
Roche' cyclers, Bio-Rad cyclers, BioFlux cyclers etc.	Not required

Notes:

The ROX dye in Realtime PCR Master Mix (Code No. QPK-101) corresponds to 1x concentration.

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2. PCR cycling conditions

The following table shows the recommended thermal conditions using primers designed according to the recommended primer and probe conditions described in [3]. Almost all targets can also be amplified using the ongoing conditions with other real-time PCR reagents.

<3-step cycle>	Temperature	Time	Ramp	
Pre-denaturation:	95°C	20-60 sec[*1]	Maximum	
Denaturation:	95°C	1-15 sec[*2]	Maximum	40 cycles
Extension:	60°C[*3]	30-60 sec[*4]	Maximum	
	(data collection should be set at the extension step)			

[*1] Due to the anti-Taq 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 60 sec.

Table 2 The recommended pre-denaturation time for each real-time cycler

Real-time cycler	Pre-denaturation time
High speed cycler (e.g. Applied Biosystems 7500Fast)	20 sec
Capillary cycler (e.g. Roche LightCycler™ 1.x, 2.0)	30 sec
General real-time cyclers (Applied Biosystems 7700, 7500, 7900HT (normal block), Stratagene cyclers, BioFlux cyclers	60 sec

[*2] The following table shows the optimal denaturation times for each real-time cycler. If the optimal denaturation time cannot be determined, the time should be set at 15 sec.

Table 3 The recommended denaturation time for each real-time cycler

Real-time cycler	denaturation time
High speed cycler (e.g. Applied Biosystems 7500Fast)	3 sec
Capillary cycler (e.g. Roche LightCycler™ 1.x, 2.0)	5 sec
General real-time cyclers (Applied Biosystems 7700, 7500, 7900HT (normal block), Stratagene cyclers, BioFlux cyclers	15 sec

[*3] 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.

[*4] If the target size is smaller than 300 bp, the extension time can be set at 30 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 45-60 sec. Some real-time cyclers or software need over 30 sec for the extension step. In these cases, the time should be set according to each instruction manual (e.g. Applied Biosystems 7000/73000: ≥ 31 sec; Applied Biosystems 7500: ≥ 35 sec.).

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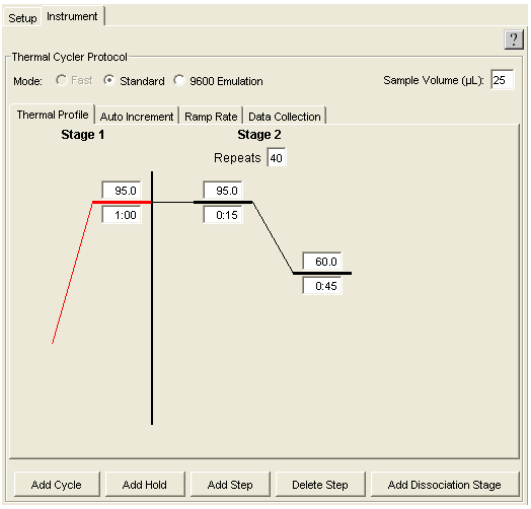
2-1. Real-time PCR conditions using Applied Biosystems 7900HT

(Normal block type, software version 2.2.2)

The following is an example of a TaqMan® assay using Applied Biosystems 7900HT.

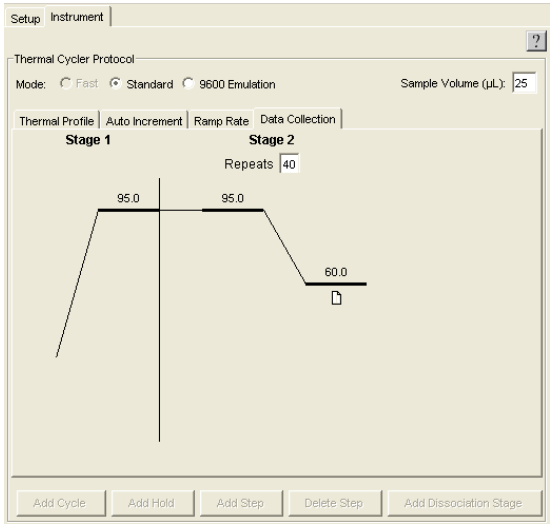
(1) The cycling parameters should be set according to the following "Thermal Cycler Protocol" window under the "Instrument" tab.



Notes:

- Appropriate sample volumes should be set.
- ≥ 45 sec is necessary for the extension step.

(2) Click the "Data collection" tab.



(3) Insert the PCR plate

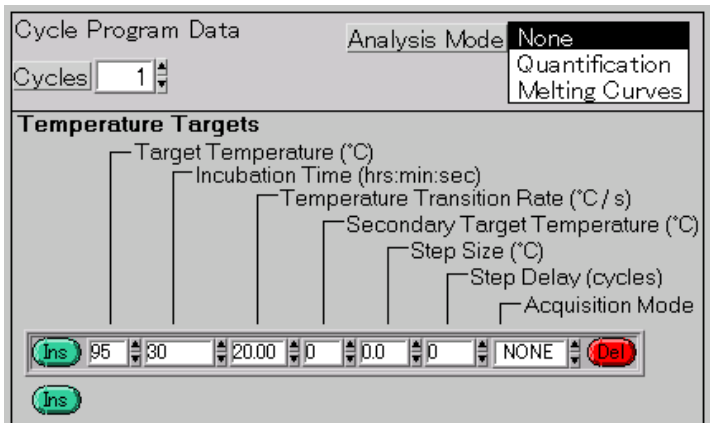
(4) Start the program

2-2. Real-time PCR conditions using Roche LightCycler 1.1

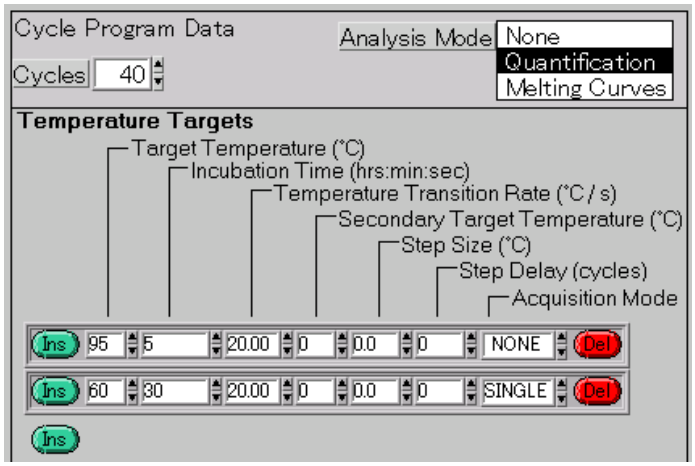
(Software version 3.5)

The following is an example of a TaqMan® probe assay using Roche LightCycler 1.1.

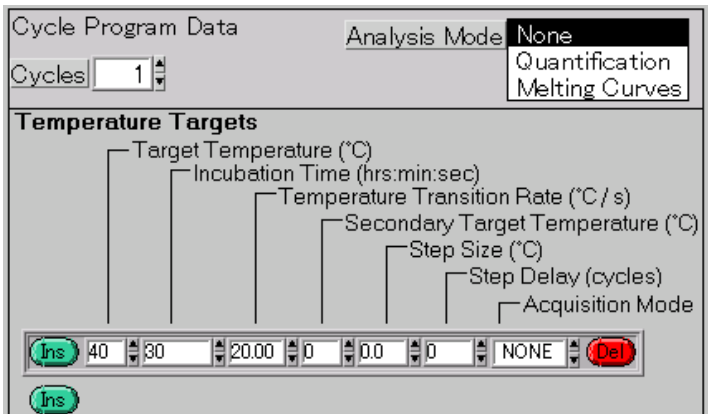
(1) The cycling parameters should be set according to the following window. Analysis and Acquisition mode of the initial denaturation step must be set at “None”.



(2) The cycling parameters should be set according to the following window. Analysis mode of the PCR step must be set at “Quantification”. Acquisition modes of 95°C and 60°C must be set at “None” and “Single”, respectively.



(3) The cycling parameters should be set according to the following window. Analysis and Acquisition mode of the cooling step must be set at “Non”.



(4) Insert the capillaries in the carousel, and start the cycling program.

[6] Related Protocol

1. cDNA synthesis

cDNA synthesized by various cDNA synthesis reagents can be used with THUNDERBIRD™ 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) is a cDNA synthesis kit suitable for real-time PCR. Here, the protocol with ReverTra Ace® qPCR RT Kit is described. However, for the detailed protocol, please refer to the instruction manual of the kit.

(1) Denaturation of RNA

Incubate the RNA solution at 65°C for 5 min and then chill on ice.

Notes:

-This step can be omitted. But this step may increase the efficiency of the reverse transcription of RNA, which forms secondary structures.

-Do not add 5x RT Buffer and/or enzyme solution at this step.

(2) Preparation of the reaction solution

Reagent	Volume (amount)
Nuclease-free Water	X µl
5x RT Buffer	2 µl
Primer Mix	0.5 µl
Enzyme Mix	0.5 µl
RNA solution	0.5 pg-1 µg
Total	10 µl

(3) Reverse transcription reaction

-Incubate at 37°C for 15 min. <Reverse transcription>

-Heat at 98°C for 2 min. <Inactivation of the reverse transcriptase>

-Store at 4°C or -20°C.*

*This solution can be used in the real-time PCR reaction directly or after dilution.

Notes:

The above temperature conditions are optimized for ReverTra Ace® qPCR RT Kit.

[7] 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%.	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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[8] Related products

Product name	Package	Code No.
High efficiency real-time PCR master mix **THUNDERBIRD™ SYBR® qPCR Mix**	200 reactions	QPS-201
High efficiency cDNA synthesis kit for real-time PCR **ReverTra Ace® qPCR RT Kit**	200 reactions	FSQ-101
One-step Real-time PCR master mix for probe assay ***RNA-direct*™ Realtime PCR Master Mix**	0.5 mL x 2 0.5 mL x 5	QRT-101T QRT-101
One-step Real-time PCR master mix for SYBR® Green assay ***RNA-direct*™ SYBR® Realtime PCR Master Mix**	0.5 mL x 2 0.5 mL x 5	QRT-201T QRT-201

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