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THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix (Lyo-Ready Mix)

QRX-101 250 reactions

Store at −20 °C, protected from light

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CAUTION

All reagents in this kit are intended for research purposes only. This kit is not intended for use with diagnostic, clinical, food or cosmetic purposes. Please see the SDS and observe general laboratory precautions and safety procedures while using this kit.

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[1] Introduction

Description

THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix is a one-step real-time reverse-transcription polymerase chain reaction (RT-PCR) mix that uses the highly efficient reverse transcriptase "ReverTra Ace™" and Hot Start TTx DNA Polymerase. The combination of these two enzymes and our optimized buffer system enables the quickly and highly sensitively detection and quantification of small quantities of RNA.

This mix does not contain glycerol and contains a lyophilization stabilizer; therefore, there should be no need to add any further excipients to assist lyophilization.

After freeze-drying, it can be stored stably at room temperature and transported without the need for ice packs. In addition, real-time PCR reactions can be performed immediately by simply adding a sample containing a template and water. Freeze-drying does not impact the performance of this PCR mix.

Features

-Rapid and highly sensitive

THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix can rapidly quantify a small amount of RNA with high sensitivity via a one-step qRT-PCR method with probes. This mix is suitable for quantifying RNA viruses or mRNA expressed at low levels.

-Tolerant of target sequence diversity

This mix can detect various types of RNA efficiently because of its high tolerance for diverse target sequences.

-Tolerant of PCR inhibitors

This mix can reduce the inhibition by PCR inhibitors such as hematin.

-Utilization of dUTP

This 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 product. We recommend that you use Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101, 201).

-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.

-Enables lyophilization

This mix can be lyophilized.

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[2] Components

Product name/contents	Store	QRX-101 (reactions)
THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix	−20 °C	1.25 mL
50×ROX reference dye	−20 °C	100 µL

This product includes the following components for 250 reactions with a 20 µL total reaction volume. All reagents should be stored at −20 °C.

Notes:
This mix contains a lyophilization stabilizer; hence, it can be used to prepare reagents for lyophilization without excipient optimization.

[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 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

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.

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[4] Template RNA

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 (5 µl in a 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 that 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 [5] Protocol. If this does not improve the result, the primers and/or TaqMan™ probe should be redesigned.

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 spin column method) can be used.

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[5] Protocol

Example of qRT-PCR reaction

1. Reaction setup

Before preparing the reaction mixture, this product should be completely thawed.

Components	Volume		Final Concentration
PCR-grade water	X µL	X µL	
THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix	5 µL	12.5 µL	1×
10 µM Primer #1	10 pmol	25 pmol	0.5 µM
10 µM Primer #2	10 pmol	25 pmol	0.5 µM
TaqMan™ Probe (10 µM)	4 pmol	10 pmol	0.2 µM
50×ROX reference dye[*1]	0.4/0.04 µL	1/0.1 µL	1×/0.1×
(Uracil N-glycosylase) [optional][*2]	0.4 unit	1 unit	
Template RNA or DNA (Sample)	Y µL	Y µL	
Total reaction volume	20 µL	50 µL	

Notes:

-Both DNA and RNA can be amplified using the above reaction mixture.

-The recommended final concentration for primers is 0.2–0.5 µM, and that for the TaqMan™ probe is 0.2–0.4 µM. If the amplification efficiency is poor, performance can be improved by increasing the concentration of the probes and/or primers. Please note that excessive probe and/or primer concentrations can cause non-specific reactions, which subsequently decrease detection sensitivity.

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

[*1] 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.

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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, Bio-Rad, and Qiagen cyclers, etc.	Not required

*2 Heat-labile uracil-N-glycosylase (UNG) should be used. UNG is not supplied with this product. We recommend that you use Uracil-DNA Glycosylase (UNG), Heat-labile (Code No. UNG-101, 201).

2. Cycling conditions

These following cycling conditions are provided as an example. Please optimize the cycling conditions to suit your needs.

Standard cycling mode

Step	Temperature	Time	
(UNG treatment)*1	(20~25 °C)	(10 min)	
Reverse transcription	50 °C	10 min	
Initial Denaturation	95 °C	1 min	
Denaturation	95 °C	15 s	⌉ 40–45 cycles
Annealing/extension	60 °C	45 s	

Fast cycling mode

Step	Temperature	Time	
(UNG treatment)*1	(20~25 °C)	(10 min)	
Reverse transcription	50 °C	10 min	
Initial Denaturation	95 °C	30 s	
Denaturation	95 °C	5 s	⌉ 40–45 cycles
Annealing/extension	60 °C	10 s	

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

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[6] Lyophilization guidelines

Example for preparation of lyophilized RT-PCR reagents

The following cycle has been provided as an example. The conditions may vary depending on the amount of reagent and lyophilization system. Please optimize the conditions for user requirements and systems.

Step	Temperature	Time	Pa	Description
Freezing	–45 °C	240 min		Hold
Primary drying I	−45 °C to −40 °C	+5.0 °C/60 min	20.0	Ramp
Primary drying II	−40 °C	600 min	20.0	Hold
Secondary drying I	−40 °C to 20 °C	+1.0 °C/min	2.0	Ramp
Secondary drying II	20 °C	360 min	2.0	Hold

Notes:

-Lyophilized reagents easily absorb moisture. Please handle under conditions of <5% humidity and store with desiccant.

-When using lyophilized reagents, dissolve the cake in a 20 µL solution containing template DNA and RNA, mix well, and set the solution in a thermal cycler. As for the reaction cycle, please refer to the previous examples.

-5-12mer random primers are not recommended.

[7] Application data

Experimental example: Detection of RNA before and after freeze-drying

<Method>

Using THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix, we attempted to detect influenza A before and after freeze-drying. After preparing 20 µL of reaction solution, lyophilization was performed, and the cake was prepared after lyophilization. Furthermore, we prepared a reaction solution before lyophilization as a comparison to that after lyophilization. Influenza A RNA was added as a 4× extract (5 steps) to a 20 µL reaction system at a concentration of 1.5×10^3 to 5.9 copies/5 µL.

<Reaction Buffer>

	20 µL/rxn	Final conc.
RNase free water	X µL	
THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix	5 µL	1×
Forward Primer	10 pmol	0.5 µM
Reverse Primer	10 pmol	0.5 µM
TaqMan™ probe	4 pmol	0.2 µM
Total	20 µL	

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<Primers and probes>

Influenza A	Sequence
Forward primer	CTTCTAACCGAGGTCGAAACGTA
Reverse primer	GGTGACAGGATTGGTCTTGTCTTTA
TaqMan™ probe	[FITC(6FAM)]- TCAGGCCCCCTCAAAGCCGAG -[BHQ1]

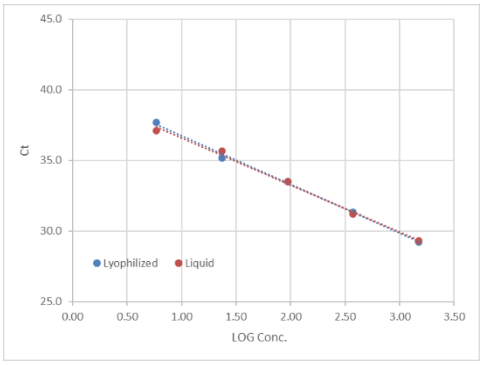
< Lyophilization >

Step	Temperature	Time	Pa	Description
Freezing	–45 °C	240 min		Hold
Primary drying I	−40 °C	+5.0 °C/60min	20.0	Ramp
Primary drying II	−40 °C	600 min	20.0	Hold
Secondary drying I	20 °C	+1.0 °C/min	2.0	Ramp
Secondary drying II	20 °C	360 min	2.0	Hold

<Cycling conditions>

Step	Temperature	Time	
Reverse transcription	50 °C	10 min	
Initial Denaturation	95 °C	30 s	
Denaturation	95 °C	5 s	40–45 cycles
Annealing/extension	60 °C	10 s	

<Results>
As a result, good amplification was obtained before and after freeze-drying, even at 1.5×10^3 to 5.9 copies/test. This indicates that freeze-drying had no effect.



	Lyophilized	Liquid
PCR Eff.	95.0%	99.4%
R^2	0.99	0.99

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[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 denaturation, extension (annealing) temperature or time	Denaturation, extension (annealing) temperature and time should be optimized according to the instructions.
	Inappropriate concentration of ROX reference dye	The ROX reference dye concentration should be determined depending on the real-time cycler (see [5] Protocol).
	Adding an incorrect quantity of THUNDERBIRD™ Next Probe One-step qRT-PCR 4×Mix	Pipettes can be very sticky, so be careful when handling it after it is at room temperature. If it is still difficult to handle, please dilute it 2× before use.
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 its amplification. In such cases, primers and/or probes should be redesigned according to [3] Primer/probe design. Alternatively, DNase I treatment of RNA is also an effective means of ameliorating 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.
	Dispersion of reaction volume	Insufficient reaction volumes tend to reduce the sensitivity or reproducibility. The reaction volume should be determined according to the instruction manual of each real-time cycler.
Detection of signal in a negative control	Contamination of positive samples or PCR products	If a 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	The TaqMan™ probe itself may generate a non-specific signal by forming unexpected structures. In such cases, the TaqMan™ probe should be redesigned.

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[9] Related products

Product name	Storage	Code No.
Uracil-DNA Glycosylase (UNG), Heat-labile	−20 °C	UNG-101
Uracil-DNA Glycosylase (UNG), Heat-labile ＜Glycerol-Free＞	−20 °C	UNG-201
THUNDERBIRD® Probe One-step qRT-PCR Kit	−20 °C	QRZ-101
MagExtractor™ -Viral RNA-High Efficiency Viral RNA Purification Kit	4 °C	NPK-401F
MagExtractor™ -RNA-High Efficiency Total RNA Purification Kit	4 °C	NPK-201F
Magical Trapper™ Magnetic Stand	Room temperature	MGS-101

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