Cat. # RR651A

For Research Use

TaKaRa

PrimeDirect™ Probe RT-qPCR Mix, with UNG

Product Manual

v201906Da

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I. Description

PrimeDirect Probe RT-qPCR Mix, with UNG is designed for one-step real-time RT-PCR via probe detection (5'-nuclease method). This product is supplied as a 2X premix to facilitate easy preparation of reaction mixtures, requiring only the addition of your primer, probe, and sample. The RT-qPCR can be performed by simply adding your sample directly to the reaction mixture without intervening nucleic acid purification steps. The protocol for performing all steps in a single tube, from extraction of the nucleic acid to reverse transcription and qPCR, greatly reduces hands-on time and risk of contamination. The continuous reaction from nucleic acid extraction (90℃) through reverse transcription (55 to 65℃) is also suitable for the detection of RNA viruses with complex higher-order structures. High-efficiency PCR after cDNA synthesis detects DNA amplification in real time by increasing fluorescence from the probe. In addition, PrimeDirect Probe RT-qPCR Mix, with UNG contains Uracil N-Glycosylase (UNG), which can avoid false positives from carryover contamination by degrading previously amplified PCR products with this kit. Because this product is highly resistant to various PCR inhibitory substances such as heparin (blood) and humic acid (soil), it can be used to directly detect viruses and bacteria in various biological specimens and to directly analyze gene expression in cells.

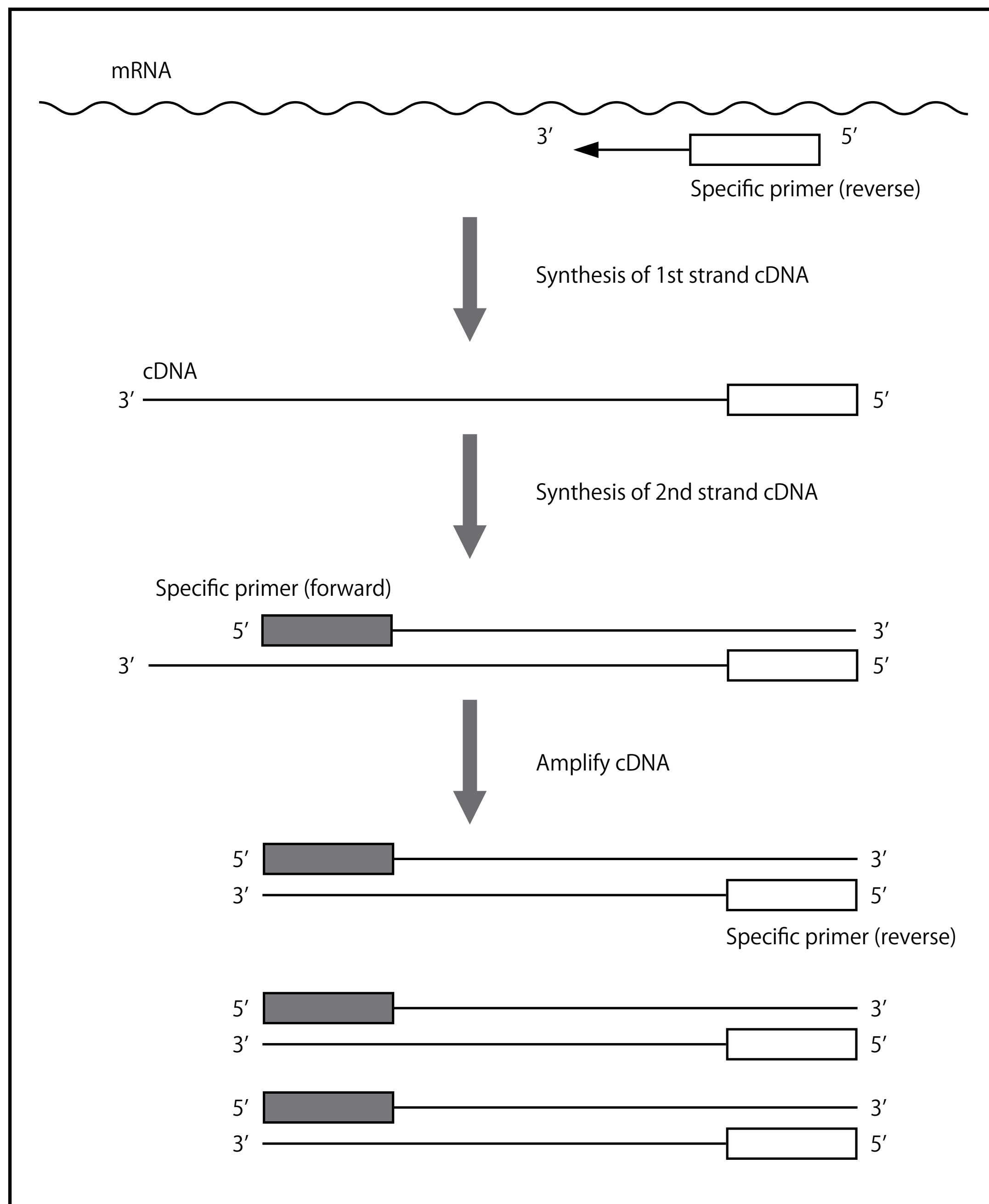
II. Principle

With PrimeDirect Probe RT-qPCR Mix, with UNG, extraction of nucleic acid from biological specimens, cDNA synthesis with reverse transcription, and PCR amplification are continuously performed in a single tube. The PCR amplification is monitored in real time by fluorescent probes.

1. RT-PCR

RNA cannot serve as a direct template for PCR, but it is possible to apply PCR to RNA analyses by synthesizing cDNA from RNA through reverse transcription. This is what is known as RT-PCR and it is the most sensitive method of RNA detection. With this product, one-step RT-PCR is performed. The principle is shown on the following page.

In one-step RT-PCR, reverse transcription is performed using a specific primer (reverse). cDNA is synthesized, and PCR amplification by a specific primer pair (forward and reverse) is performed in a single tube using the cDNA as template.



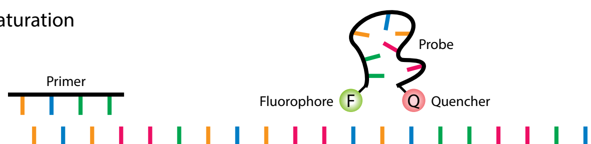
Principle of the one-step RT-PCR method

2. Fluorescence detection

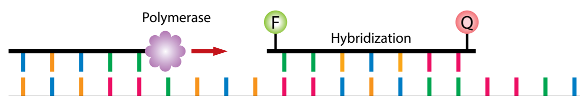
Oligonucleotide probes labeled with a 5' fluorophore (e.g., FAM) and a 3' quencher (e.g., TAMRA, BHQ1, etc.) are added to the reaction together with the PCR primers. Fluorescence of the fluorophore is suppressed by the quencher.

Under annealing conditions, the primers and probe hybridize in a sequence-specific manner to the template DNA. During the extension reaction, the 5′ → 3′ exonuclease activity of the DNA polymerase degrades the hybridized probe, releasing quencher suppression and allowing fluorescence.

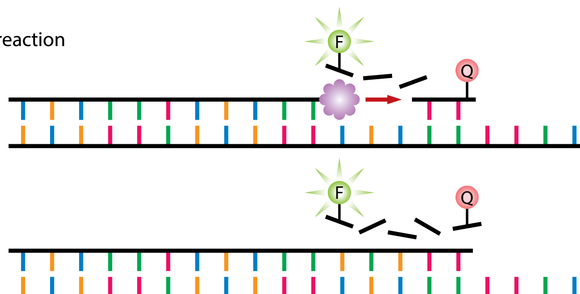
1) Heat denaturation



2) Primer annealing/probe hybridization



3) Extension reaction



III. Components

1.	PrimeDirect Probe RT-qPCR Mix, with UNG (2X)	625 μl x 4
2.	RNase Free H_2O	1.25 ml x 2
3.	ROX Reference Dye (50X conc.)*	100 μl
4.	ROX Reference Dye II (50X conc.)*	100 μl

* When using a device that corrects the fluorescence signal between wells, such as Applied Biosystems' real-time PCR devices, add at 0.5 times the final concentration.

IV. Materials required but not provided

1. Gene amplification system for real-time PCR
   Systems that are compatible with this kit:
   - Thermal Cycler Dice™ Real Time System III (Cat. # TP950/TP970/TP980/TP990)
   - Thermal Cycler Dice Real Time System *Lite* (Cat. # TP700/TP760)
   - Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
   - Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
   - LightCycler 96 System (Roche Diagnostics)
   - LightCycler 480 System (Roche Diagnostics)
   - CFX96 Real-Time PCR Detection System (Bio-Rad)
   - CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad)
2. Reaction tubes or plates designed specifically for the qPCR instrument used
3. PCR primers
4. Probe for detection
5. Micropipettes and tips (sterile, with filter)

V. Storage

-20℃

VI. Features

1. 2X for probe-based one-step RT-qPCR
2. Direct nucleic acid extraction from biological specimens in the reaction mix
3. High-temperature reverse transcription (55 - 65℃)
4. Highly resistant to various PCR inhibitors
5. Inclusion of UNG avoids false positives due to carryover contamination.

VII. Precautions Before Use

Read these precautions before use and follow them when using this product.

1. After thawing the PrimeDirect Probe RT-qPCR Mix, with UNG (2X) at room temperature, mix gently and spin down before use. After use, immediately store it at -20℃.
2. When dispensing, be sure to use new disposable filter tips to prevent between-sample contamination.
3. We recommend preparing a large master mix (PrimeDirect Probe RT-qPCR Mix, with UNG (2X), RNase-Free H_2O, and primer/probe) and aliquoting when setting up PCR reactions to minimize inter-experimental variability.
4. You must use a specific primer for reverse transcription with this kit; it is not possible to use random or oligo dT primers.

VIII. Protocol

* Follow the instructions in the user manual for each instrument.

1. Preparation of the reaction mixture

   For each reaction, prepare a reaction mixture as shown below at room temperature.

When using an instrument that does NOT require a ROX Reference Dye[*6]

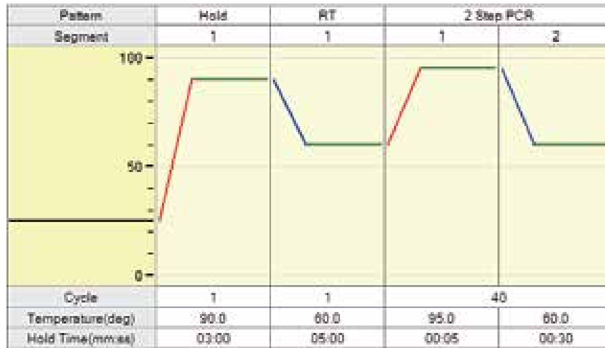
Reagent	Amount	Final conc.
PrimeDirect Probe RT-qPCR Mix, with UNG (2X)[*1]	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM [*2]
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM [*2]
Probe (10 μM)	0.5 μl	0.2 μM [*3]
Sample	≤ 2.5 μl [*4]	
RNase Free Water	X μl	
Total	25 μl [*5]	

When using an instrument that DOES require a ROX Reference Dye[*6]

Reagent	Amount	Final conc.
PrimeDirect Probe RT-qPCR Mix, with UNG (2X)[*1]	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM [*2]
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM [*2]
Probe (10 μM)	0.5 μl	0.2 μM [*3]
ROX Reference Dye or Dye II (50X)[*6]	0.25 μl	0.5X
Sample	≤ 2.5 μl [*4]	
RNase Free Water	X μl	
Total	25 μl [*5]	

2. Real-time RT-qPCR reaction

   After spinning down the reaction tube or plate, set it in the real-time PCR device and start the reaction under the following conditions.
   It is recommended that the reaction first be performed according to the standard protocol shown below, and then optimize as necessary (refer to VIII-4. "RT-qPCR reaction conditions" on page 9).



Stage 1: Reverse transcription
(37℃ 10 min)[*7]
90℃ 3 min (for biological specimens, virus solutions, etc.)
or 95℃, 30 sec (for purified nucleic acid)[*8]
60℃ 5 min

Stage 2: PCR reaction
Number of cycles: 40
95℃ 5 sec
60℃ 30 sec[*9]

3. After the reaction is complete, check the amplification curves and plot a standard curve if absolute quantification will be performed. Refer to the instrument's instruction manual for specific analysis methods.

*** 1 to 9: Refer to VIII-4. "RT-qPCR reaction conditions" on page 9.**

4. RT-qPCR reaction conditions

First, test the standard protocol described on page 8.

Heat extraction/denaturation of nucleic acid

Step	Temp.	Time	Detection	Comment
Denaturation (Purified nucleic acid)	95 - 98℃	30 sec	OFF	Optimal denaturation temperature differs depending on device type and target. If reactivity is poor at 95℃, consider using 96 - 98℃.

Reverse transcription

Step	Temp.	Time	Detection	Comment
Reverse transcription	55 - 65℃	2 - 5 min	OFF	Improvement may be observed if the temperature and/or time is adjusted according to target.

PCR reaction, 30 to 45 cycles

Step	Temp.	Time	Detection	Comment
Denaturation	95 - 98℃	3 - 5 sec	OFF	Optimal denaturation temperature differs depending on the device type and target. If reactivity is poor at 95℃, consider using 96 - 98℃. Since the amplicon size is generally 300 bp or less in real-time PCR, a denaturation time of 3 to 5 sec should be sufficient.
Annealing/Extension	55 - 65℃	15 - 30 sec	ON	When performing optimization, consider using 55 - 65℃. If reactivity is poor, an improvement may result from adjusting the time of this step.

* 1 Thaw the reagent at room temperature and prepare the reaction mixture. Precipitate may be observed if it is thawed on ice.

* 2 A final primer concentration of 0.2 μM works well in most cases. However, should further optimization be required, try adjusting primer concentrations in the range of 0.1 to 1.0 μM.

* 3 The probe concentration will differ depending on the type of real-time PCR device used and the specific probe fluorophore. Refer to the data sheet attached to the probe when considering how much to add. When using the Thermal Cycler Dice Real Time System, a final concentration in the range of 0.1 to 0.5 μM is generally optimal.

* 4 It is recommended to use less than 10% of the PCR reaction mixture volume. When purified RNA or DNA is used, use within the range of 10 pg to 1 μg. If sample concentration is low, you may use more than 10% of the PCR mixture, but caution is warranted as this may inhibit the RT-qPCR reaction.

* 5 Adjust the reaction volume according to the recommendations for the real-time PCR instrument used.

* 6 Refer to the following table for ROX Reference Dye.

Devices that use ROX Reference Dye (50X)
◆ Add ROX Reference Dye (final concentration x 0.5) • ABI StepOnePlus ◆ Add ROX Reference Dye II (final concentration x 0.5) • ABI 7500 Fast
Devices that do not use ROX Reference Dye
• Takara Thermal Cycler Dice Real Time System series • Bio-Rad CFX series • Roche LightCycler series

* 7 When contamination from the previous reaction with this kit is suspected, add a step that incubates the mixture at 37℃ for 10 minutes. PCR products that were carried over from the previous experiment are degrated by the UNG activity.

* 8 This step is essential, so be sure to perform denaturation at 90℃ for 3 minutes or at 95℃ for 30 sec.

* 9 Depending on the real-time PCR instrument used, it may not be possible to set the detection step within 30 sec. Set the detection step to 31 sec or more for the Applied Biosystems 7300 Real-Time PCR System and 34 sec or more for the Applied Biosystems 7500 Real-Time PCR System.

IX. Appendix: preparation of RNA samples

This kit synthesizes cDNA from RNA to perform PCR amplification. In order to perform cDNA synthesis successfully, it is important to inhibit RNase activity in the sample as much as possible and avoid RNase contamination. When preparing RNA, set up a lab table dedicated to RNA preparation, wear clean, disposable gloves and, whenever possible, use disposable plastic labware. Use only dedicated solutions and nuclease-free water in RNA experiments.

X. Related products

RNase-free Water (Cat. # 9012)
Thermal Cycler Dice™ Real Time System III (Cat. # TP950)

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