Cat. # RR393S
RR393A

For Research Use

TaKaRa

Probe qPCR Mix MultiPlus

Product Manual

v202406Da

Table of Contents

I. Description

Probe qPCR Mix MultiPlus is a real-time PCR (qPCR) reagent specifically designed for targeted, probe-mediated nucleic acid detection by the 5'-nuclease method. The reagent pairs an antibody-mediated hot-start PCR enzyme with a real-time PCR buffer that have together been optimized for fast and highly specific amplification with a broad dynamic range. It can also accommodate multiplex reactions for simultaneous detection of multiple targets with high accuracy and reproducibility.

The 2X concentrated premixed reagent contains heat-resistant RNase H (Tli RNaseH) and Uracil-*N*-Glycosylase (UNG) as additives. Tli RNaseH minimizes reaction interference from residual mRNA when cDNA is used as the PCR template. UNG reduces the presence of false positives through faciliating the degradation of carryover contamination from previous amplification or misprimed, nonspecific products.

Models that are compatible with this product:

- Thermal Cycler Dice™ Real Time System IV (Cat. #TP1000/TP1010/TP1030)*
- Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
- CronoSTAR™ 96 Real-Time PCR System (Cat. #640231/640232)*
- QuantStudio 5 Real-Time PCR System (96-well, 0.1/0.2 mL block, Thermo Fisher Scientific)
- Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
- StepOnePlus Real-Time PCR System (Thermo Fisher Scientific)
- CFX96 Real-Time PCR Detection System (Bio-Rad)

* Not available in all geographic regions. Please check for availability in your area.

II. Principle

This product contains a heat-resistant DNA polymerase that is used in combination with a user-provided probe to perform real-time PCR amplification.

1. PCR

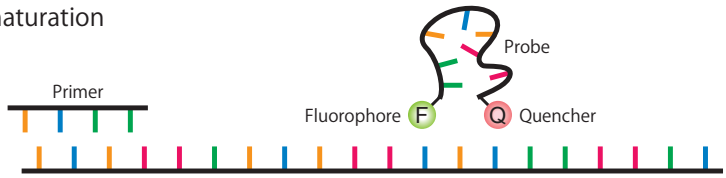
Polymerase chain reaction (PCR) uses primers to amplify a desired gene fragment from minute amounts of DNA. The reaction involves three steps: thermal denaturation of DNA, primer/probe annealing, and elongation/extension by DNA polymerase. By cycling through these three steps, it is possible to rapidly amplify the desired gene fragment one-million fold.

2. Fluorometric detection

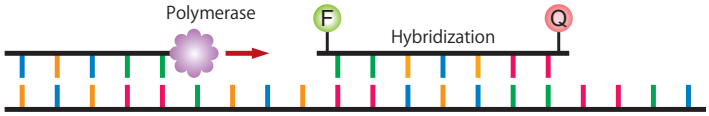
An oligonucleotide probe modified with a fluorescent substrate (FAM, etc.) at the 5′ end and a quencher substance (TAMRA, etc.) at the 3′ end is added to the PCR reaction.

Under annealing conditions, the probe specifically hybridizes to the template DNA, and fluorescence is inhibited by the quencher. During DNA extension, the probe that has hybridized to the template is degraded by the 5′ → 3′ exonuclease activity of the polymerase. The fluorescent substrate is released from inhibition by the quencher and can now be detected.

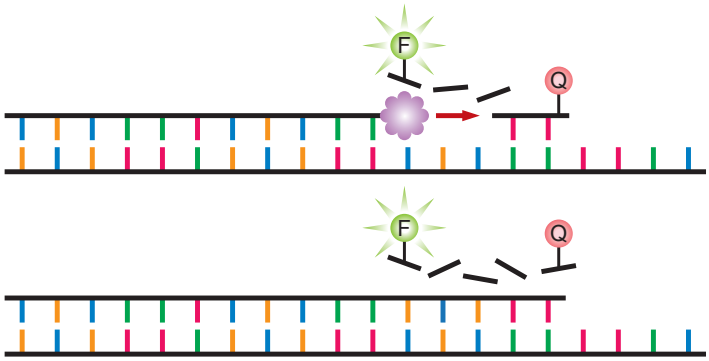
1) Heat denaturation



2) Primer annealing/probe hybridization



3) Extension



III. Components [80 reactions (RR393S) / 400 reactions (RR393A), 25 μl volume per reaction]

	Cat. #RR393S	RR393A
Probe qPCR Mix MultiPlus (2X)*1	1 ml	1 ml x 5
ROX Reference Dye (50X)*2	200 μl	200 μl
ROX Reference Dye II for RR393 (100X)*2	200 μl	200 μl

*1 Contains PCR enzyme, dNTP Mixture, Mg^{2+}, Tli RNaseH, and UNG.

*2 ROX reference dyes are used to normalize the variation in flourescence signal between wells and on certain real-time PCR instruments.

- Use ROX Reference Dye (50X) with the following instruments (Please use at a final concentration of 1X):
  - StepOnePlus Real-Time PCR System
- Use ROX Reference Dye II for RR393 (100X) with the following instruments (Please use at a final concentration of 1X):
  - QuantStudio 5 Real-Time PCR System
  - Applied Biosystems 7500 Fast Real-Time PCR System
- Instruments that do not require a ROX reference dye:
  - Thermal Cycler Dice Real Time System series (Cat. #TP1000/TP950/TP900, etc.)
  - CronoSTAR 96 Real-Time PCR System (Cat. #640231/640232)
  - CFX96 Real-Time PCR Detection System

IV. Materials Required but Not Provided

1. Real-time PCR instrument (see "I. Description" for recommended instruments)
2. Dedicated reaction tubes or plates
3. PCR primer
4. Detection probe (TaKaRa qPCR Probe, etc.)
5. Sterile purified water
6. Micropipettes and tips (autoclaved)

V. Storage -20℃

VI. Features

1. 2X premixed qPCR reagent for probe detection
2. High-speed reactions facilitated by a hot-start PCR enzyme and improved PCR buffer
3. Accommodates multiplex reactions
4. Includes Tli RNaseH and UNG to avoid PCR reaction interference and false positives

VII. Precautions for Use

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

1. Before use, mix by gently inverting the tube, taking care not to create bubbles, to ensure that the reagent is evenly mixed. Sufficient reactivity may not be obtained if the reagent composition is uneven. DO NOT mix with a vortex mixer.
   During storage of Probe qPCR Mix MultiPlus, a precipitate may form. To dissolve the precipitate, warm the product briefly at room temperature and then mix by inverting the tube gently. It is important to make sure the precipitate is completely dissolved and mixed before use to ensure that the reagent composition is even.
2. Immediately place the thawed reagent on ice.
3. This product does not include probe(s). Please prepare one separately.
4. Be sure to use a new disposable tip when preparing and dispensing the reaction solution. Take every precaution to prevent sample-to-sample contamination.
5. If the sample or primer is degraded by contamination with a nucleolytic enzyme (e.g., nuclease), accurate detection will not be possible. Wear disposable gloves and a mask and exercise caution during each step of the PCR reaction setup as contamination with nucleases from perspiration or saliva can occur.
6. With this product, there is no need to analyze the amplification product by electrophoresis, etc., after the reaction ends. Please do not remove the amplification product from the tube as this could cause nucleic acid contamination within your workspace.
7. Please follow the instruction manual when setting up the analytical parameters for your real-time PCR instrument. Inappropriate correction functions in the analytical software can lead to erroneous interpretation of the results.

VIII. Note

If contamination by PCR products is suspected, add a step at 25℃ for 2 min before the reaction before the reaction detailed on page 8. PCR products will be decomposed by the action of UNG. If there is no contamination, this step is usually unnecessary.

The DNA polymerase used in this product is a hot-start PCR enzyme with anti-*Taq* antibodies that inhibit polymerase activity. DO NOT perform an activation step at 95℃ for (5 -)15 min, as is necessary with other companies' chemically modified hot-start PCR enzymes.
Excessive heat treatment tends to cause a decrease in enzyme activity, adversely affecting amplification efficiency and quantitation accuracy. Even if initial denaturation of the template is performed before the PCR reaction, the usual 95℃ for 20 sec will be sufficient.

IX. Protocol

Note: Please operate all equipment in accordance with the instruction manual.

1. Preparation of the PCR reaction solution

<Per reaction>

Reagents	Volume	Final conc.
Probe qPCR Mix MultiPlus	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
Probe*2	1.0 μl	
Template	2.0 μl*3	
Sterile purified water	8.5 μl	
Total	25.0 μl	

【When ROX Reference Dye is used】

<Per reaction>

Reagents	Volume	Final conc.
Probe qPCR Mix MultiPlus	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
Probe*2	1.0 μl	
ROX Reference Dye (50X)	0.5 μl	1X
Template	2.0 μl*3	
Sterile purified water	8.0 μl	
Total	25.0 μl	

【When ROX Reference Dye II for RR393 is used】

<Per reaction>

Reagents	Volume	Final conc.
Probe qPCR Mix MultiPlus	12.5 μl	1X
PCR Forward Primer (10 μM)	0.5 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.5 μl	0.2 μM*1
Probe*2	1.0 μl	
ROX Reference Dye II for RR393 (100X)	0.25 μl	1X
Template	2.0 μl*3	
Sterile purified water	8.25 μl	
Total	25.0 μl	

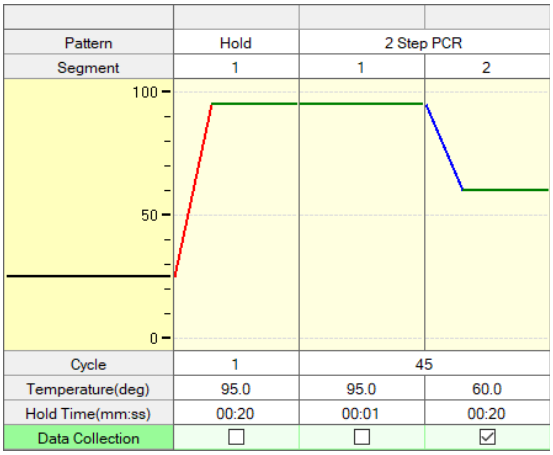
*1 In many cases, good results can be obtained with a final primer concentration of 0.2 μM. If you are having problems with amplification, it may be useful to try primer concentrations within the range of 0.1 - 1.0 μM. When two or more primer sets are used, the final concentration of each primer should be adjusted to 0.2 μM for initial testing.

*2 The probe concentration will differ depending on the real-time PCR instrument used and the fluorescence labeling agent of the probe. Please refer to the instruction manual for your instrument and/or the data sheet of your probe for concentration recommendations. When using two or more probes, check to make sure that there is no crosstalk with other filters or cross-reaction between probes. For the Thermal Cycler Dice Real Time System III, we recommend a final probe concentration of 0.1 - 0.5 μM.

*3 The template concentration will differ depending on the number of copies of the target that are present in the template solution, and serial dilutions can be performed to determine optimal concentration. For DNA templates, a concentration of no more than 100 ng is recommended. For cDNA templates (using the RT reaction solution), the added amount should not exceed 10% of the volume of the PCR reaction solution.

2. Start of reaction

It is recommended that you perform the PCR reaction according to the following two-step PCR standard protocol. Try this protocol first, and then optimize your cycling conditions as necessary (refer to "PCR condition considerations" on page 10).



Two-step PCR standard protocol

Hold (initial denaturation)*4
Cycle: 1
95℃ 20 sec
Two-Step PCR
Cycles: 45
95℃ 1 sec
60℃ 20 sec*5

*4 If contamination by PCR products (including dUTP) is suspected, perform a step at 25℃ for 2 min before the initial denaturation. PCR products carried over from the previous experiment will be decomposed by the action of UNG.

*5 Depending on the PCR instrument, it may not be possible to set the extension time to 20 sec if detection is performed using multiple wavelengths. If this is the case, use the shortest extention time possible.

3. End of reaction

Check the amplification curve and prepare a calibration curve if quantitation is to be performed.

Optimizing PCR conditions

If good amplification cannot be obtained with the recommended conditions (Two-step PCR standard protocol), consider the primer concentration and PCR conditions described below.

When optimizing PCR conditions, take into account both the reaction specificity and amplification efficiency. In an experimental system with a good balance of the two, it is possible to perform accurate quantitation over a wide range of concentrations.

○ PCR optimized for high specificity

- The Ct values obtained from a low-concentration template are reproducible.
- There is little decrease in the fluorescence value in detection of a low-concentration template.

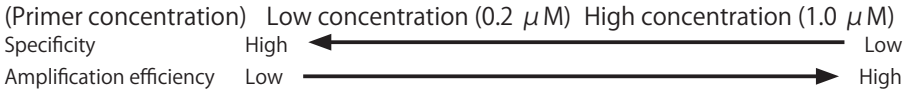
○ PCR optimized for high amplification efficiency

- Amplification products are detected in an earlier cycle (Ct value is small).
- PCR amplification efficiency is high (close to the theoretical value of 100%).

[Primer concentration considerations]

The following type of relationship exists between the primer concentration and the reaction specificity and amplification efficiency:

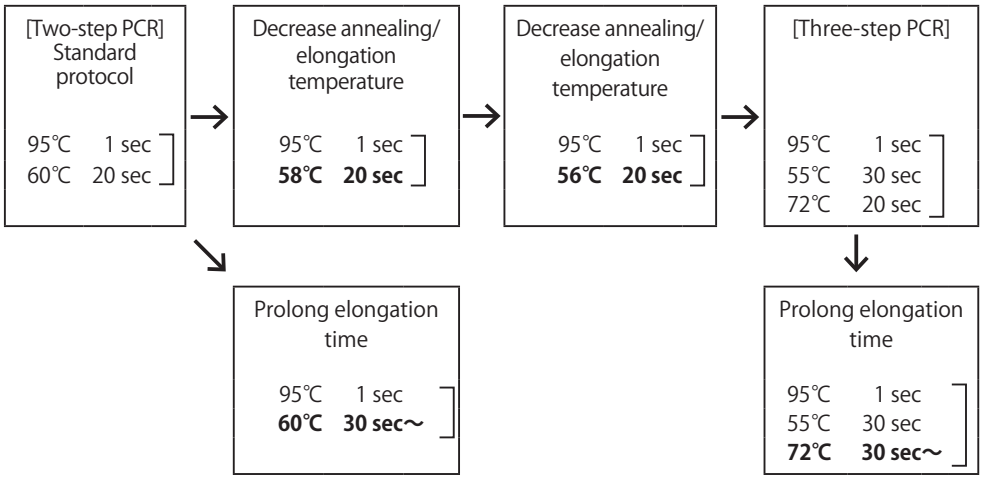
- To increase the reaction specificity, decrease the primer concentration.
- To increase the amplification efficiency, increase the primer concentration.



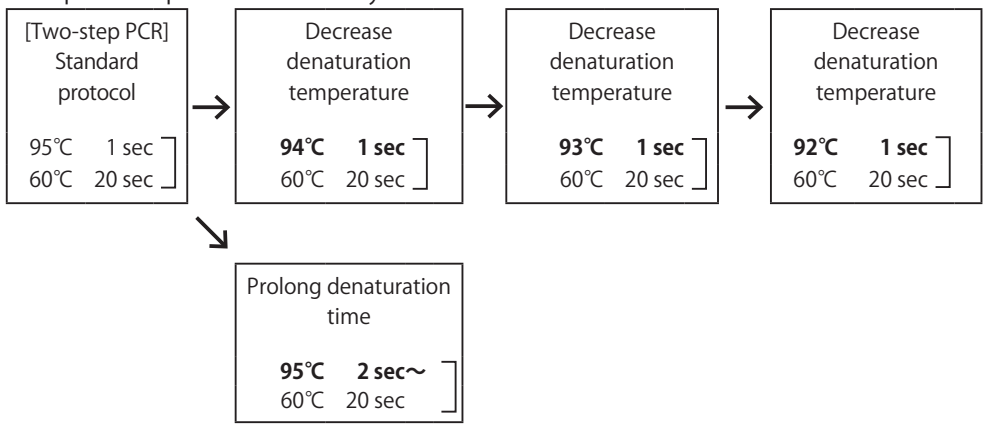
【 PCR conditions considerations 】

○ To increase the amplification efficiency:

(1) Decreasing the annealing/elongation temperature, switching to three-step PCR, or prolonging the elongation time may improve the amplification efficiency.

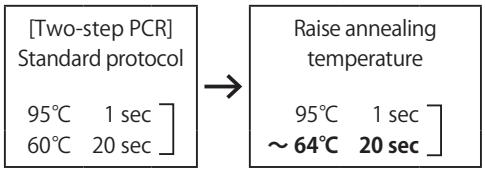


(2) Decreasing the denaturation temperature from 95℃ to 92℃ in 1℃ increments may improve amplification efficiency.



○ To increase specificity:

Raising the annealing temperature may improve the reaction specificity, however, this may affect amplification efficiency.



○ Initial denaturation

For initial denaturation, 20 sec at 95℃ is usually sufficient, even with a template that is difficult to denature, such as circular plasmid or genomic DNA. Depending on the condition of the template, it may be possible to extend the initial denaturation to 1 - 2 min at 95℃. If the initial denaturation is extended for too long it may cause deactivation of the enzyme. Therefore, an initial denaturation cycle of >2 min is not recommended.

X. Appendix: Primer and Probe Design

In order to perform probe-based real-time PCR efficiently, it is important to design primers and probes with good specificity. For multiplex reactions in particular, the presence of multiple primers and probes in the reaction solution presents a risk for nonspecific amplification. Even if correct amplification product is achieved, crosstalk where the fluorescence of the probe is detected by an adjacent fluorescence filter can occur. For these reasons, pay careful attention to the following when designing primers/probes and determining primer/probe concentrations to be used in the PCR reaction solution.

1. Design primers so that the Tm values are approximately the same across primer sets.
2. Design primers so that dimers do not occur between primer sets.
3. Choose combinations of probes so that there is little overlap between the fluorescence wavelengths of the fluorescent pigments (fluorochromes).

XI. Related Products

PrimeScript™ RT Reagent Kit (Perfect Real Time) (Cat. #RR037A/B)
PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Cat. #RR047A/B)
PrimeScript™ RT Master Mix (Perfect Real Time) (Cat. #RR036A/B)
PrimeScript™ FAST RT reagent Kit with gDNA Eraser (Cat. #RR092S/A/B)
Probe qPCR Mix (Cat. #RR391S/A/B)
Probe qPCR Mix, with UNG (Cat. #RR392S/A/B)
One Step PrimeScript™ III RT-qPCR Mix, with UNG (Cat. #RR601A/B)
Thermal Cycler Dice™ Real Time System IV (Cat. #TP1000/TP1010/TP1030)*
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*
CronoSTAR™ 96 Real-Time PCR System (Cat. #640231/640232)*

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