Takara Bio USA

5X One Step PrimeScript™ III RT-qPCR Kit, ROX plus, GPR User Manual

Cat. Nos. 638336, XU0001
(071322)

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I. Introduction

5X One Step PrimeScript III RT-qPCR Kit, ROX plus, GPR is a dedicated reagent for one-step real-time, probe-based RT-qPCR using the 5′ nuclease method. The 5X premix does not freeze at its storage temperature –20°C, enabling a quick reaction setup by adding the template RNA sample, primers, and a probe for detecting the desired target. In addition, reverse transcription (RT) and qPCR reactions can be performed in the same tube, making the protocol quick and easy. This is highly concentrated, allowing for flexible sample preparation such as working with lower concentration primer sets and larger sample volumes to improve detection limits.

The RT uses the PrimeScript III RTase, which displays increased heat tolerance (up to 55°C) while maintaining the specificity and extensibility of PrimeScript RTase. The higher heat tolerance allows cDNA synthesis from RNA with more complex secondary structures. This product can be used for various applications such as gene expression studies, RNA virus detection, etc.

II. Principle

The 5X One Step PrimeScript III RT-qPCR Kit allows reverse transcription using PrimeScript III RTase and qPCR using TaKaRa Taq™ HS in the same tube, reducing the risk of contamination. A user-provided probe monitors PCR amplification products in real-time.

A. One-Step RT-PCR

In a one-step RT-PCR, a reverse transcription reaction is performed on the RNA template using a specific reverse primer to generate cDNA. Then the synthesized cDNA is amplified using specific forward and reverse primers in the same tube. Although RNA inherently does not serve as a direct template for PCR, this method allows PCR to be used for RNA analysis. See Figure 1 for the one-step RT-PCR workflow of this product.

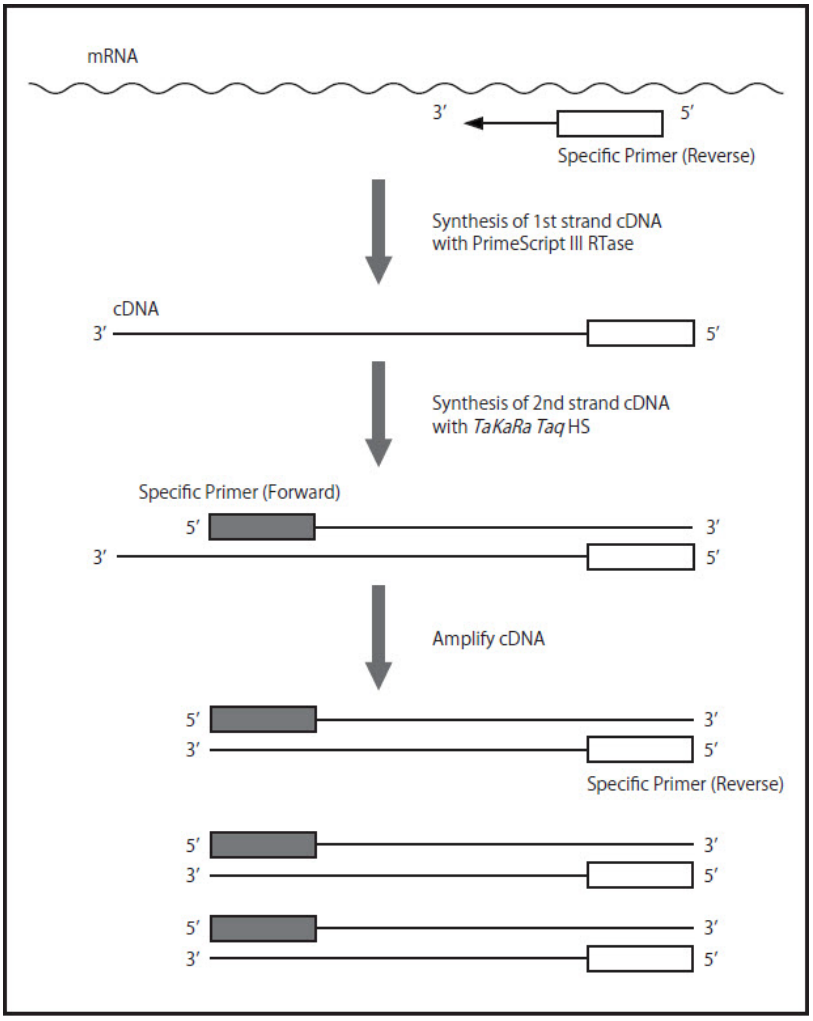


Figure 1. Principle of one-step RT-PCR method.

B. Fluorescence Detection

5X One Step PrimeScript III RT-qPCR Kit is compatible with oligonucleotide detection probes (provided by the user) whose 5' and 3' ends are each modified with a fluorescent substance (FAM, etc.) and a quencher substance (TAMRA, BHQ1, etc.), respectively. Under the annealing conditions, the probe specifically hybridizes to template DNA, but the fluorescence is suppressed by the quencher. During extension, the probe is degraded by the 5'→3' exonuclease activity of Taq DNA polymerase, such that the fluorescence is no longer suppressed. The real-time PCR machine detects fluorescence produced in this process, enabling sample quantitation in real-time. Figure 2 explains the principle of quantitative RT-PCR (RT-qPCR).

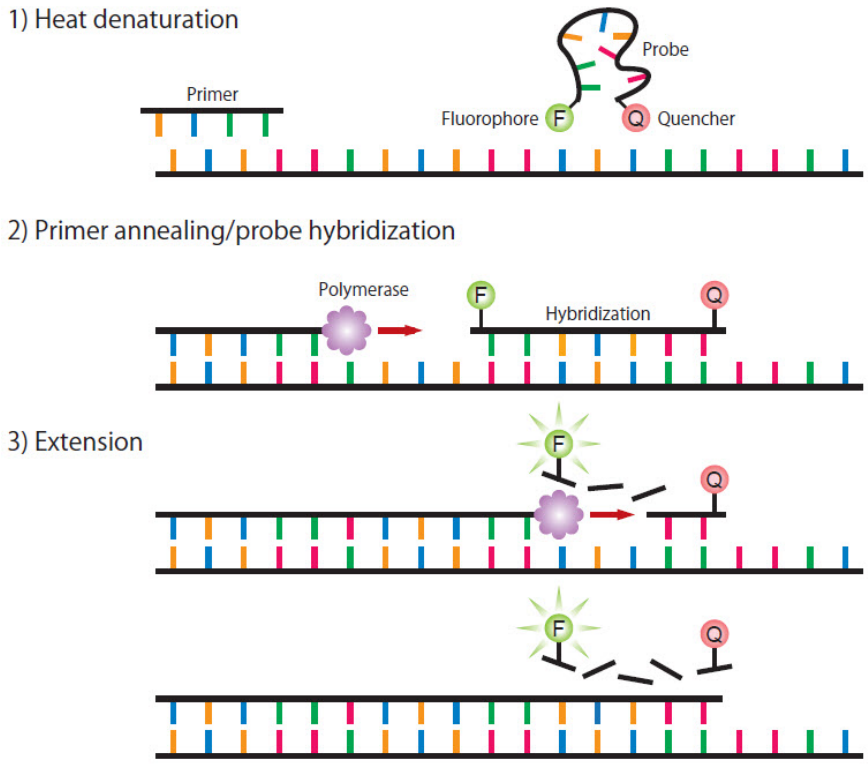


Figure 2. Schematic diagram explaining the principle of quantitative PCR (qPCR).

III. List of Components

- Cat. No. XU0001 contains enough materials for 200 x 25 µl reactions
- Cat. No. 638336 contains five boxes of Cat. No. XU0001, enough for 1,000 x 25 µl reactions

Table 1. Components of 5X One Step PrimeScript III RT-qPCR Kit, ROX plus, GPR (Cat. No. XU0001)

5X One Step PrimeScript III RT-qPCR Kit, ROX plus, GPR (Store at –20°C)	XU0001 (200 rxns)
5X One Step PrimeScript III RT-qPCR Mix, ROX plus, GPR	1 ml x 1
RNase Free H_20	1.5 ml x 2

IV. Additional Required Materials

- Primers and probes
- Micropipettes and tips (with hydrophobic filters)
- Vortex mixer
- Benchtop centrifuge for tubes or plates
- 1.5 ml Eppendorf tubes, 200 µl PCR tubes, or 200 µl PCR plates for sample preparation
- Tubes or plates for real-time PCR
- A real-time PCR machine compatible with **low ROX** (e.g., QuantStudio 3 (or later), ABI 7500, ABI 7500 Fast, ViiA 7, Stratagene MX4000P, MX3000P, MX3005P, etc.)

V. Precautions

Carefully read and follow these precautions before using this product.

- The reverse transcription reaction performed with this kit uses specific template-specific primers. Random or Oligo dT primers cannot be used.
- Always use new disposable tips and tubes to dispense reagents and avoid contamination between samples.
- Mix the 5X One Step PrimeScript III RT-PCR mix gently and thoroughly by inverting the tube and spinning it down briefly before use. Immediately store at –20°C after use. If the product is frozen, it may be thawed and used without any loss of quality. If the product appears cloudy, it can still be used after mixing evenly, followed by a brief centrifuge.
- We recommended preparing an appropriate amount of PCR reaction mix (a mixture of 5X One Step PrimeScript III RT-qPCR Mix, RNase Free H_2O, and primer/probe or RNA sample) to reduce experimental variability.

VI. Protocol

NOTE: Follow the instructions in the user manual of the qPCR machine. For information regarding the RNA preparation method, refer to Appendix B: RNA Sample Preparation.

CAUTION: The TaKaRa Taq HS used in this product is a hot-start enzyme utilizing an anti-Taq antibody that suppresses polymerase activity. **Do not** perform the 5–15 min activation step at 95°C before the PCR reaction, required for other 'companies' chemically-modified hot-start PCR enzymes. Unnecessary heat treatment decreases enzyme activity and can affect amplification efficiency and quantitation accuracy. Generally, 95°C for 10 sec is sufficient for heat inactivation of reverse transcriptase before the PCR reaction.

A. Protocol when Using the QuantStudio 3 (or later) Real-Time PCR System

1. On ice, prepare the RT-qPCR reaction mix for all reactions of the RNA sample, plus 10% of the total reaction mix volume for overage, using the recipe in Table 1 (next page).

   NOTES:

   – Although the recipe lists the RNA sample, it should not be added to the reaction mix; it will be added during Step 3.

   – If 10% of the total number of reactions results in a fraction, round up to the nearest whole reaction number when determining how much mix to create.

   Example:

   For 16 rxns, calculated overage would be: 16 rxns * 10% = 1.6 rxns

   Round 1.6 rxns up to 2 rxns (overage) and create an RT-qPCR reaction mix for 18 rxns (16 + 2).

   Before adding the 5X One Step PrimeScript III RT-qPCR Mix, mix the contents of the tube by inverting several times and then spin down; the mix may appear cloudy after storage, but this does not affect its performance.

   Use the reaction mix immediately.

Table 2. Preparation of RT-qPCR reaction mix for singleplex or multiplex reaction

RT-qPCR reaction mix (per 25 µl reaction)

Reagent	Final concentration	Singleplex	Multiplex (*N** targets)
5X One Step PrimeScript III RT-qPCR Mix, ROX plus, GPR	1X	5 µl	5 µl
PCR Forward Primer (10 µM)	0.2 µM†	0.4 µl	*N** x 0.4 µl
PCR Reverse Primer (10 µM)	0.2 µM†	0.4 µl	*N** x 0.4 µl
Probe (10 µM)	0.2 µM‡	0.4 µl	*N** x 0.4 µl
RNA sample	—	2 µl§	2 µl§
RNase Free H_2O	—	Up to 25 µl**	Up to 25 µl**
Total volume per reaction		**25 µl**	**25 µl**

*Where *N* represents the total number of targets of interest. The total reaction volume will still be 25 µl, while 0.4 µl of each primer and probe will be added per target.

†A final primer concentration of 0.2 µM works well in most cases. However, if further optimization is required, try adjusting primer concentrations in the range of 0.1 to 1.0 µM.

‡The probe concentration varies depending on the model of the real-time PCR instrument used and the fluorescent labeling dye of the probe. Refer to the instrument manual and the probe datasheet to determine the appropriate concentration.

§The preferred sample concentration is between 10 pg and 1 µg of RNA in 1/10 or less of the reaction volume. Although more than 1/10 reaction volume can be used with low target RNA concentration, this may inhibit the RT-qPCR reaction in some cases.

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

2. Add [25 µl – Volume of RNA Sample] of the reaction mixture into a PCR tube or a 96-well PCR plate.
3. Dispense the volume of RNA sample into the PCR tube or plate well.
4. Place the tube or plate in the real-time PCR machine and start the reaction using the conditions below. We recommended using the standard protocol for PCR described below first and then optimizing the PCR conditions as necessary. Refer to Section VI.B, "Determining RT-qPCR reaction conditions".

52°C	5 min
95°C	10 sec
40 cycles:	
95°C	5 sec
60°C	30 sec

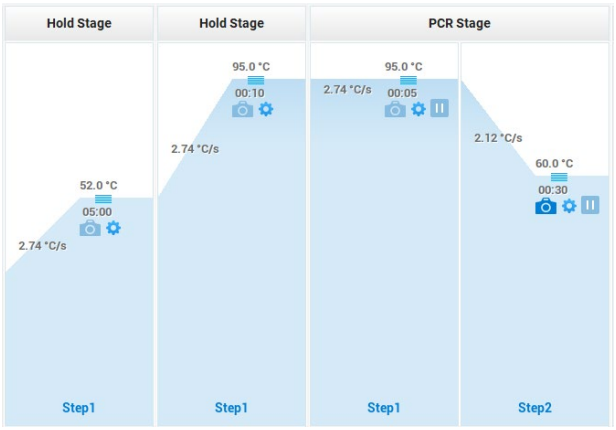


Figure 3. PCR cycle curve.

1, 2: Reverse transcription

52°C	5 min
95°C	10 sec

3, 4, 5: PCR reaction (40 cycles)

95°C	5 sec
60°C	30 sec*

GO TO 3, 39 cycles

*Depending on the real-time PCR instrument used, it may not be possible to set the detection step within 30 sec. In such cases, use a time suitable for the instrument (31 or 34 sec, etc.).

5. After the reaction is complete, assess the amplification curve and create a standard curve if a quantitative determination will be performed. For analytical methods, refer to the manual for the real-time PCR instrument used.

B. Determining RT-qPCR Reaction Conditions

Use the tables below for suggestions about optimizing the RT-qPCR reaction conditions to suit your sample.

Table 3. Optimization of RT-qPCR conditions

Reverse transcription reaction

Step	Temp.	Time	Detection	Comment
Reverse transcription	42–55°C	5 min	Off	Improvement can be seen in some cases when the temperature is adjusted depending on the target.
Denaturation	95°C	10 sec	Off	Generally, 95°C for 10 sec is enough for heat inactivation of the reverse transcriptase.

PCR reaction (30–45 cycles)

Step	Temp.	Time	Detection	Comment
Denaturation	95°C	1–5 sec	Off	Denaturation at 95°C for 1–5 sec is usually sufficient since the amplified product size for RT-PCR does not typically exceed 300 bp.
Annealing/ Extension	56–64°C	20–30 sec*	On	When optimizing reaction conditions, evaluate results using a 56–64°C annealing/extension temperature range. Increasing incubation time for this step may improve performance.

*Depending on the real-time PCR instrument used, it may not be possible to set the detection step within 30 sec. In such cases, use a time suitable for the instrument (31 or 34 sec, etc.).

Appendix A. Experimental Example

Reproducibility data for Human *GAPDH* gene expression (using the QuantStudio 3 Real-Time PCR Detection System).

1. Method: *GAPDH* gene expression analysis was performed using the 5X One Step PrimeScript III RT-qPCR Kit, ROX plus, GPR using 1 pg to 1 µg of qPCR Human Reference Total RNA (Takara Bio, Cat. No. 636690) as a template, and standard curves were prepared.
2. Results: example amplification curve (Figure 4) and a standard curve (Figure 5) are shown below. Each standard value indicated high linearity (R2: >0.99) and stable amplification efficiency (Eff. >95.1%).

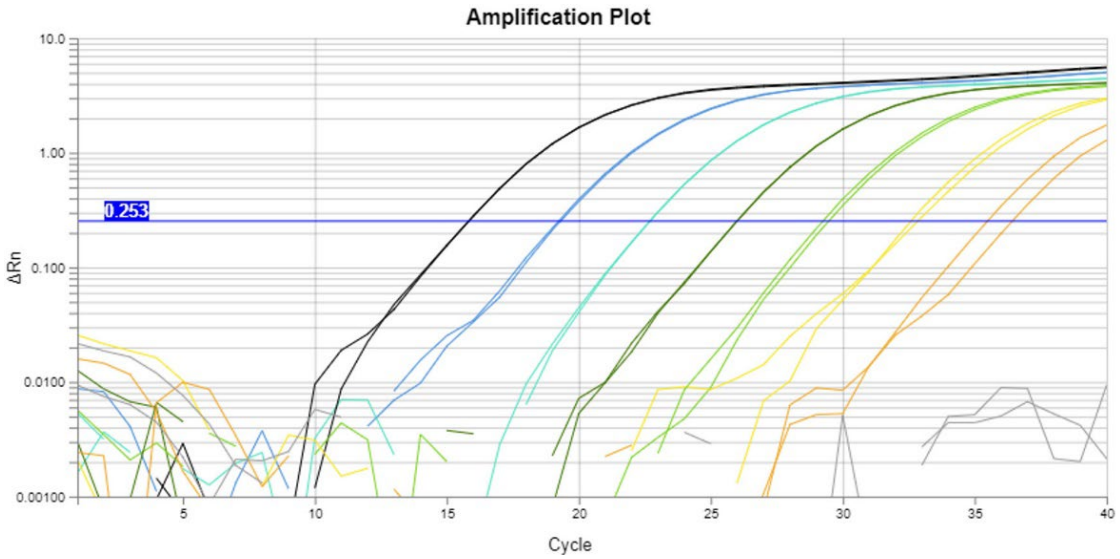
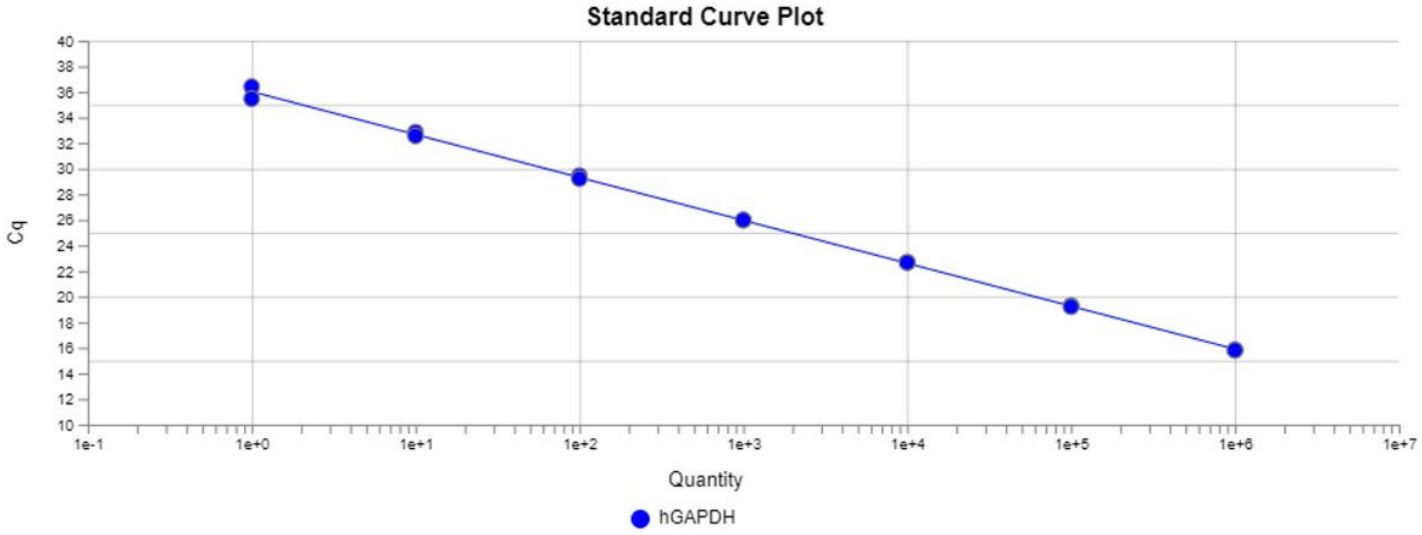


Figure 4. Amplification curve for RT-qPCR of the *GADPH* gene using Human Reference Total RNA.



Target: hGAPDH Slop: -3.354 R²: 0.999 Y-Inter: 36.024 Eff%: 98.686 Error: 0.029

	Standard Curve		
	Day 1	**Day 2**	**Day 3**
R^2	0.999	0.999	0.998
Eff.	98.69%	98.21%	99.04%

Figure 5. Standard curve for RT-qPCR of the *GADPH* gene using Human Reference Total RNA.

3. Data obtained on three different days showed high reproducibility for a wide range of RNA sample dilutions over an order of magnitude of 10^7 (1 pg to 1 µg of total RNA). The greatest difference in Cq values between calibration curves for each RNA amount was 0.51 for 100 pg of RNA (Table 4).

Table 4. Cq values between calibration curves for each RNA amount

Sample RNA (pg)	Log	Standard curve			Highest difference
		Day 1	Day 2	Day 3	
1,000,000	6	15.83	16.19	16.03	0.36
100,000	5	19.26	19.56	19.40	0.30
10,000	4	22.68	22.98	22.81	0.30
1,000	3	25.98	26.39	26.26	0.41
100	2	29.34	29.85	29.68	0.51
10	1	32.71	33.09	33.11	0.40
1	0	35.94	36.29	35.82	0.47
0	-	ND	ND	ND	-

Appendix B. RNA Sample Preparation

This product is for performing cDNA synthesis and PCR amplification from RNA samples. In order to synthesize cDNA successfully, it is essential to inhibit RNase activity in samples and avoid RNase contamination during processing.

- Use clean disposable gloves and work in a designated area used exclusively for RNA preparation.
- Use disposable plastic equipment whenever possible.
- All reagents and purified water should be RNase-free and used exclusively for RNA experiments.
- **RNA preparation method:** this product is optimized to be highly resistant to a wide variety of inhibitors commonly present in PCR reactions with RNA samples obtained by using a simple nucleic acid extraction method. However, use of highly pure RNA is recommended when highly reproducible results are desired. NucleoSpin RNA (Takara Bio, Cat. No. 740955.50 or 740955.250) provides a convenient spin-column method for obtaining high-purity total RNA from cultured cells and tissue samples.

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