Cat. # RR601A

For Research Use

TaKaRa

One Step PrimeScript ™ III RT-qPCR Mix, with UNG

Product Manual

v202111Da

Table of Contents

I. Description 3
II. Principle 3
III. Components 6
IV Storage 6
V. Features 6
VI. Precautions before use 7
VII. Precautions during use 7
VIII. Protocol 8
IX. Experimental example 13
X. Appendix: RNA sample preparation 15
XI. Related products 15

I. Description

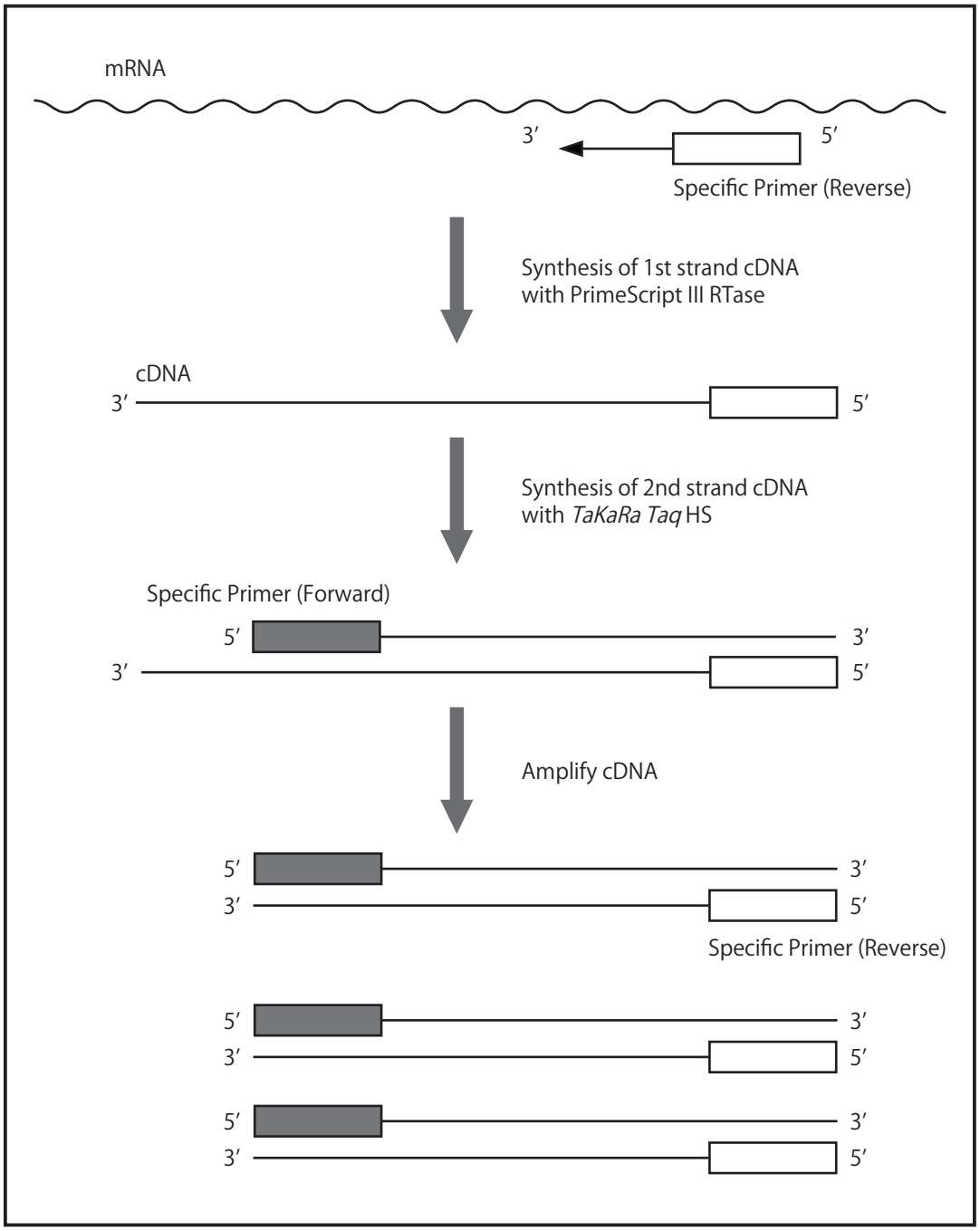
One Step PrimeScript III RT-qPCR Mix, with UNG is a dedicated reagent for one-step real-time, probe-based RT-qPCR (using the 5' nuclease method). This 2X premix does not freeze at its storage temperature of -20℃, so a reaction can be started simply by adding the template sample, primer, and a probe for detecting the desired target. The quick and simple protocol allows the reverse transcription and qPCR reactions to be performed in the same tube. Because the premix contains Uracil N-Glycosylase (UNG), which degrades amplification products from the previous reaction, it can prevent carryover contamination even when a specific target is repeatedly tested for the purpose of inspection, etc. The reverse transcription reaction uses the novel PrimeScript III RTase, which displays increased heat tolerance (up to 55℃) while maintaining the specificity and extensibility of PrimeScript RTase. This allows cDNA synthesis from RNA with a more complex secondary structure. After cDNA synthesis, *TaKaRa Taq*™ HS performs highly specific and efficient PCR amplification, while the fluorescence emitted by the probe is detected in real-time. One Step PrimeScript III RT-qPCR Mix, with UNG is also highly resistant to a wide variety of inhibitory substances such as heparin (blood) and humic acid (soil), allowing stable one-step real-time RT-qPCR to be performed on a wide range of samples. This product can be used for various applications such as gene expression, RNA virus detection, etc.

II. Principle

One Step PrimeScript III RT-qPCR Mix, with UNG allows reverse transcription using PrimeScript III RTase and qPCR using *TaKaRa Taq* HS to be performed in the same tube. PCR amplification products are monitored by a probe in real-time.

1. RT-PCR

Although RNA does not serve as a direct template for PCR, synthesizing cDNA from RNA using reverse transcriptase allows PCR to be used for RNA analysis. This highly sensitive RNA detection method is known as RT-PCR. This product performs one step RT-PCR, as shown in the figure on the following page.
In one step RT-PCR, a reverse transcription reaction is performed using a specific primer (reverse) for PCR and then PCR amplification is performed by specific primers (forward, reverse) using synthesized cDNA as a template. Both steps are performed in the same tube.

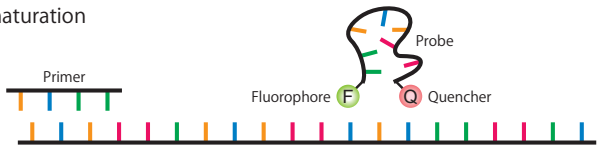


Principle of One Step RT-PCR method

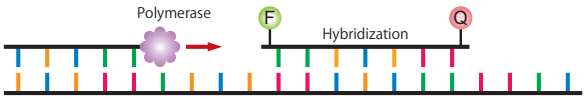
2. Fluorescence detection

This reagent uses a detection probe that is an oligonucleotide whose 5′ and 3′ ends are modified with a fluorescent substance (FAM, etc.) and a quencher substance (TAMRA, BHQ1, etc.), respectively. Under the annealing conditions, the probe specifically hybridizes to a template DNA, but the fluorescence is suppressed by the quencher. At the time of the extension reaction, however, the probe hybridizing to the template is degraded by the 5′ → 3′ exonuclease activity of *Taq* DNA polymerase and the suppression by the quencher disappears. Fluorescence produced in this process is detected by a real-time PCR machine. A method combining the above principles enables sample quantitation at real-time, therefore it is called One Step RT-q (quantitative) PCR.

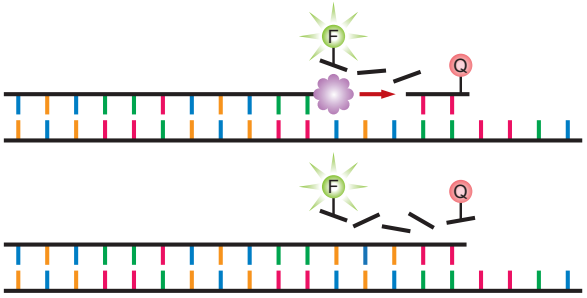
1) Heat denaturation



2) Primer annealing/probe hybridization



3) Extension



III. Components (200 reactions, 25 μl reaction volume)

1.	One Step PrimeScript III 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.)*1	100 μl
4.	ROX Reference Dye II (50X conc.)*1	100 μl

*1 It is added when a machine that performs fluorescence signal correction between wells such as a real-time PCR machine made by Applied Biosystems, etc. is used.

- ◆ An example of a machine where ROX Reference Dye is added
  - Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific)
- ◆ An example of a machine where ROX Reference Dye II is added
  - Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific)
- ◆ Machines not requiring addition
  - Thermal Cycler Dice™ Real Time System series (Cat. #TP950/TP970/TP980/TP990 etc.)*2
  - LightCycler series (Roche Diagnostics)
  - CFX series (Bio-Rad)

Materials required but not provided

1. Gene amplification system for real-time PCR

   [Thermal cyclers compatible with this product]
   - Thermal Cycler Dice Real Time System III (Cat. #TP950/TP970/TP980/TP990)*2
   - Thermal Cycler Dice Real Time System *Lite* (Cat. #TP700/TP760: discontinued)
   - Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific)
   - LightCycler 96 System (Roche Diagnostics)
   - CFX96 Real-Time PCR Detection System (Bio-Rad)
2. Dedicated reaction tube or plate
3. Primers for PCR
4. Probe for detection
5. Micropipette and tips

*2 Not available in all geographic locations. Check for availability in your area.

IV. Storage -20℃

V. Features

1. One-step RT-qPCR reagent for probe detection
2. Simple and quick protocol using a 2X premix that does not freeze at its storage temperature of -20℃
3. Carryover contamination is prevented by UNG activity.
4. Reverse transcription at high temperatures (up to 55℃) is enabled by using a novel heat-resistant reverse transcriptase, PrimeScript III RTase.
5. Highly resistant to inhibitory substances
6. High reproducibility

VI. Precautions before use

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

1. Mix gently One Step PrimeScript III RT-qPCR Mix (2X) by inverting the tube and centrifuge quickly the tube to remove the solution attached to the tube lid before use. Immediately store it at -20℃ after use. If the product is frozen, it may be thawed and used without any loss of quality. When white turbidity may be observed during storage, the product can be used after mixed evenly by inverting the tube then centrifuge quickly it to remove the solution attached to the tube lid before use.
2. When reagents are dispensed, always use a new disposable tip and avoid contamination between samples.
3. For the reaction mixture, it is convenient to prepare a required amount+ α of Master Mix (a mixture of One Step PrimeScript III RT-qPCR Mix, with UNG (2X), RNase -free H_2O, and primer/probe or RNA sample). Data dispersion between experiments resulting from reagent preparation can be minimized by dispensing the minimum number of aliquots required from a Master Mix with a uniform composition.
4. The reverse transcription reaction performed with this kit uses specific primers. Random Primer and Oligo dT Primer cannot be used.

VII. Precautions during use

The *TaKaRa Taq* HS that is used in this product is a hot-start PCR enzyme utilizing anti-*Taq* antibody that suppresses polymerase activity. Do not perform the 5 - 15 minute activation step at 95℃ before the PCR reaction that is 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℃ for 10 sec is sufficient for heat inactivation of reverse transcriptase before the PCR reaction.

VIII. Protocol

* Follow the instructions in the user manual for each machine. For information regarding the RNA preparation method, refer to <X. Appendix: RNA sample preparation>.

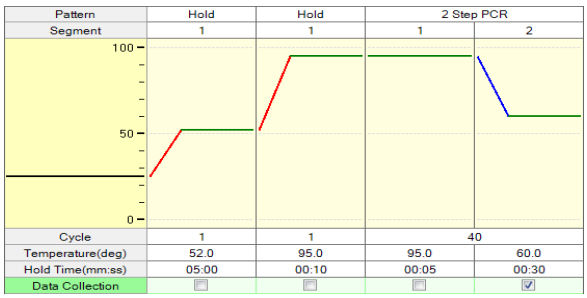
【 Protocol when using the Thermal Cycler Dice Real Time System III 】

1. Preparation of reaction mixture
   Prepare a PCR reaction mixture as indicated below on ice and add 25 μl the reaction mixture in a tube or a well.

   <For 1 reaction>

Reagent	Volume	Final Conc.
One Step PrimeScript III RT-qPCR Mix, with UNG (2X)	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 (10 μM)	0.5 μl	0.2 μM *2
RNA sample*3	≤2.5 μl	
RNase Free H_2O	X μl *4	
Total	25 μl	

2. RT-qPCR reaction
   After gently spin down the reaction tube or plate, place it in the Thermal Cycler Dice Real Time System and start the reaction under the following conditions. The recommended protocol for PCR reactions is the standard protocol described below. Try this protocol first and then optimize the PCR reaction conditions as necessary. (Refer to <Determining RT-qPCR reaction conditions> on page 12.)



Pattern 1: Reverse transcription reaction
(25℃ 10 min)*5
52℃ 5 min
95℃ 10 sec
Pattern 2: PCR reaction
Cycle: 40
95℃ 5 sec
60℃ 30 sec

3. 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.

*** 1 to 5: Check <Determining RT-qPCR reaction conditions> on page 12.**

【 Protocol when using the Applied Biosystems 7500 Fast Real-Time PCR System 】

1. Preparation of reaction mixture

   Prepare a PCR reaction mixture as indicated below on ice and add 25 μl or 50 μl the reaction mixture in a tube or a well.

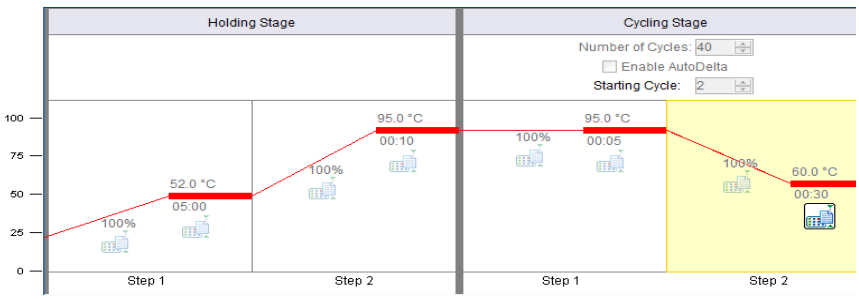
   <For 1 reaction>

Reagent	Volume	Volume	Final Conc.
One Step PrimeScript III RT-qPCR Mix, with UNG (2X)	10 μl	25 μl	1X
PCR Forward Primer (10 μM)	0.4 μl	1 μl	0.2 μM*1
PCR Reverse Primer (10 μM)	0.4 μl	1 μl	0.2 μM*1
Probe (10 μM)	0.4 μl	1 μl	0.2 μM*2
ROX Reference Dye II (50X)*7	0.4 μl	1 μl	
RNA sample*3	≤ 2 μl	≤ 5 μl	
RNase Free H_2O	X μl*4	X μl*4	
Total	20 μl	50 μl	

2. Real-time RT-qPCR reaction

   After gently spin down the reaction tube or plate, place it in the Applied Biosystems 7500 Fast Real-Time PCR System and start the reaction under the following conditions.

   The recommended protocol for PCR reactions is the standard protocol described below. Try this protocol first and then optimize the PCR reaction conditions as necessary. (Refer to <Determining RT-qPCR reaction conditions> on page 12.)



Holding Stage: Reverse transcription reaction
(25℃ 10 min) *5
52℃ 5 min
95℃ 10 sec
Cycling Stage: PCR reaction
Number of Cycles: 40
95℃ 5 sec
60℃ 30 sec *6

3. 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.

*** 1 to 7: Check <Determining RT-qPCR reaction conditions> on page 12.**

【 Protocol when using the LightCycler 96 System 】

1. Preparation of reaction mixture
   Prepare a PCR reaction mixture as indicated below on ice and add 20 μl the reaction mixture in a tube or a well.

   <For 1 reaction>

Reagent	Volume	Final Conc.
One Step PrimeScript III RT-qPCR Mix, with UNG (2X)	10 μl	1X
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM *1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM *1
Probe (10 μM)	0.4 μl	0.2 μM *2
RNA sample*3	≤2 μl	
RNase Free H_2O	X μl *4	
Total	20 μl	

2. Real-time RT-qPCR reaction
   After gently spin down the reaction tube or plate, place it in the LightCycler and start the reaction under the following conditions.
   The recommended protocol for PCR reactions is the standard protocol described below. Try this protocol first and then optimize the PCR reaction conditions as necessary. (Refer to <Determining RT-qPCR reaction conditions> on page 12.)

   Preincubation
   Cycle: 1
   (25℃ 10 min)*5
   ↓
   52℃ 5 min
   ↓
   95℃ 10 sec
   2-Step Amplification
   Cycle: 40
   95℃ 5 sec
   ↓
   60℃ 30 sec *6

3. 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.

*** 1 to 6: Check <Determining RT-qPCR reaction conditions> on page 12.**

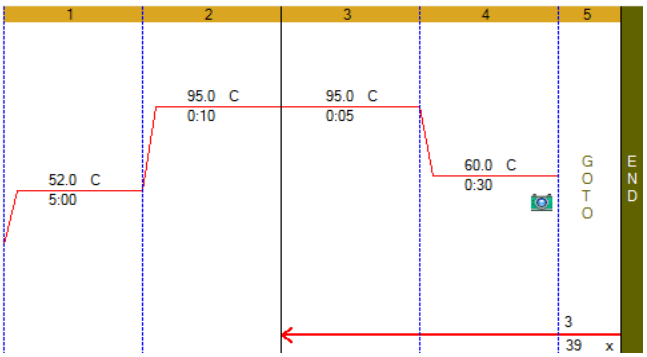
【 Protocol when using the CFX96 real-time PCR detection system 】

1. Preparation of reaction mixture
   Prepare a PCR reaction mixture as indicated below on ice and add 20 μl the reaction mixture in a tube or a well.

   <For 1 reaction>

Reagent	Volume	Final Conc.
One Step PrimeScript III RT-qPCR Mix, with UNG (2X)	10 μl	1X
PCR Forward Primer (10 μM)	0.4 μl	0.2 μM *1
PCR Reverse Primer (10 μM)	0.4 μl	0.2 μM *1
Probe (10 μM)	0.4 μl	0.2 μM *2
RNA sample*3	≤2 μl	
RNase Free H_2O	X μl *4	
Total	20 μl	

2. Real-time RT-qPCR reaction
   After gently spin down the reaction tube or plate, place it in the CFX96 and start the reaction.
   The recommended protocol for PCR reactions is the standard protocol described below. Try this protocol first and then optimize the PCR reaction conditions as necessary. (Refer to <Determining RT-qPCR reaction conditions> on page 12.)



1, 2: Reverse transcription
(25℃ 10 min)*5
52℃ 5 min
95℃ 10 sec
3, 4, 5: PCR reaction (40 cycles)
95℃ 5 sec
60℃ 30 sec *6
GO TO 3, 39 cycles

3. 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.

*** 1 to 6: Check <Determining RT-qPCR reaction conditions> on page 12.**

<Determining RT-qPCR reaction conditions>

Reverse transcription reaction

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

PCR reaction 30-45 cycles

Step	Temp.	Time	Detection	Comment
Denaturation	95℃	3 - 5 sec	OFF	Generally the amplification product size for real-time PCR does not exceed 300 bp. Therefore, 95℃ for about 3 to 5 sec is usually sufficient.
Annealing/ Extension	56 - 64℃	20 - 30 sec*6	ON	When optimizing reaction conditions, evaluate results using an annealing/extension temperature in the range of 56℃ to 64℃. If poor reactivity occurs, increasing incubation time for this step may improve results.

*1 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.

*2 The probe concentration varies depending on the model of real-time PCR instrument used and the fluorescent labeling dye of the probe. Refer to the instrument manual and the probe data sheet to determine the appropriate concentration. When using the Thermal Cycler Dice Real Time System, generally, try a final concentration in the range of 0.1 to 0.5 μM.

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

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

*5 When contamination with PCR products is suspected, add a step that incubates the sample at 25℃ for 10 minutes. PCR products that were carried over from the previous experiment are degraded by the UNG activity.

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

*7 For guidelines regarding the use of ROX Reference Dye, refer to the tables below.

ROX Reference Dye (50X)	ROX Reference Dye II (50X)
StepOne, StepOnePlus ABI 7300/7700/7900HT	ABI 7500, ABI 7500 Fast

Real-time PCR instruments that do not use ROX Reference Dye
Thermal Cycler Dice Real Time System series, CFX96, CFX384, Lightcycler

IX. Experimental example

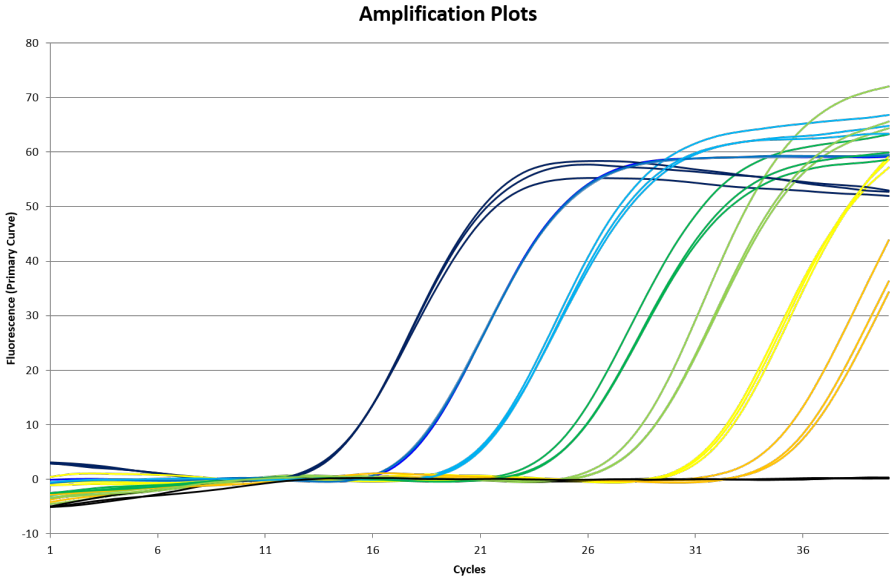
Day-to-day reproducibility of Human ACTB gene expression data (using the Thermal Cycler Dice Real Time System III)

1. Method

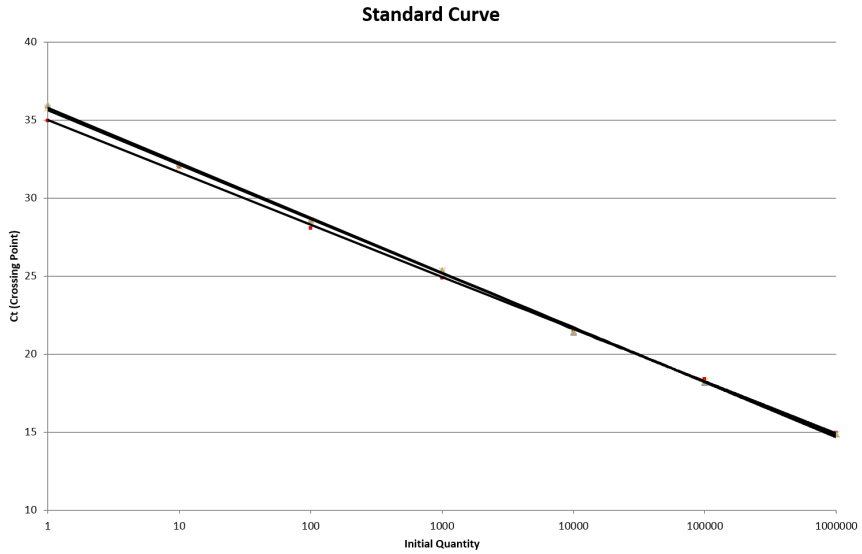
ACTB gene expression analysis was performed on 3 different days with One Step PrimeScript™ III RT-qPCR Mix, with UNG using 1 pg to 1 μg of Human HeLa Cell Total RNA (Cat. #636543) as a template, and standard curves were prepared.

2. Results

Amplification curve



Each standard curve shown below indicated high linearity (Rsq: 1) and stable amplification efficiency (Eff. ≥ 92.5%).



	Day 1's standard curve	Day 2's standard curve	Day 3's standard curve
Rsq	1	1	1
Eff.	98.9%	92.5%	93.8%

In addition, the greatest difference in Ct values between calibration curves for each amount of RNA is shown in the table below. The maximum difference was 1.02 for 1 pg of RNA.

Sample RNA (pg)	Log	Day 1's standard curve	Day 2's standard curve	Day 3's standard curve	Highest difference
1,000,000	6	14.95	14.88	14.83	0.12
100,000	5	18.41	18.18	18.25	0.23
10,000	4	21.41	21.40	21.47	0.07
1,000	3	24.87	25.35	25.32	0.48
100	2	28.05	28.57	28.57	0.52
10	1	32.00	32.22	32.02	0.22
1	0	34.93	35.95	35.76	1.02
0	-	ND	ND	ND	-

*ND = Not Detected

3. Conclusion

Data obtained on 3 different days showed high reproducibility for a wide range of RNA sample dilutions over an order of magnitude of 10^6 (1 pg to 1 μg of total RNA) using One Step PrimeScript III RT-qPCR Mix, with UNG.

X. Appendix: RNA sample preparation

This product is a kit for performing cDNA synthesis and PCR amplification from RNA. In order to synthesize cDNA successfully, it is essential to inhibit RNase activity in samples and avoid RNase contamination of equipment and solutions. Additional precautions should be taken during sample preparation, such as using clean disposable gloves and setting aside a designated area exclusively for RNA preparation.

[Equipment]

Disposable plastic equipment should be used whenever possible.

[Solutions]

All reagents and purified water should be used exclusively for RNA experiments.

[RNA preparation method]

This product is optimized to be highly resistant to a wide variety of inhibitory substances that may be present in PCR reactions using RNA samples obtained by using a simple nucleic acid extraction method. However, use of highly pure RNA is recommended when more highly reproducible results are required. NucleoSpin RNA (Cat. #740955.50/.250) provides a convenient spin column method for obtaining high-purity total RNA from cultured cells and tissue samples.

XI. Related products

RNase-free Water (Cat. #9012)
NucleoSpin RNA (Cat. #740955.50/.250)
NucleoSpin RNA Plus (Cat. #740984.50/.250)
NucleoSpin RNA Virus (Cat. #740956.50/.250)
Thermal Cycler Dice™ Real Time System III (Cat. #TP950/TP970/TP980/TP990)*

* Not available in all geographic locations. Check for availability in your area.