Q&A - RT-PCR (Please see our manuals at 101Bio.com , Click *List by Cat. # to f*ind our products quickly.)

FAQ: Should I use TaqMan probe- or SYBR Green dye-based detection for my RT-PCR assays?

Format	No. Targets	RT Primers	Detection	Fluorescence signal
Dye-based	Single	A pair	SYBR Green	Non-specific due to dimer and false primering
Probe-based	Single	A pair	A probe	nonspecific
	Multiple	Multiple pairs	Multiple probes	Non-specific

FAQ: Which kit contains SYBR-Green or TaqMan probe?

Format	No. Target	Catalog
SYBR Green Dye	Singleplex	RT-PCR 144, RT-PCR 147
TaqMan Probe	Singleplex	RT-PCR 143
	Multiplex	RT-PCR 146, RT-PCR 149

FAQ: What is the difference between probe- and dye-based versions of One-Step RT-PCR kits?

They are different in 1) reaction buffer and 2) polymerase composition. They are included in their instructions.

FAQ: What temperature should I use for reverse transcription?

The reverse transcription can be completed within 5-10 minute at 60-62°C.

FAQ: How can the thermophilic reverse transcriptase be inactivated?

The enzymes can be simply heat-inactivated at ≥90°C within 1 minute.

FAQ: Can I set up my RT-PCR at room temperature?

Yes, you can set up at room temperature because the thermophilic reverse transcriptase virtually

has no observable activity at room temperature. However, for best results, RT-PCR reaction mixtures are preferably kept on ice prior to thermo-cycling.

FAQ: How could I set up a RT-PCR reaction?

As for the volume of each reaction, you can choose 20µL or 10µL.

For SYBR Green dye (Cat 144 and 147): Setting Up a 20µL or 10µL Reaction

Component	Volume per 20µL	Volume per 10µL	Final concentration
2X Master Mix	10µL	5µL	1X
Primers	Variable	Variable	Each 150-900nM
RNA template	Variable	Variable	As low as single digit copies of target RNA to ≤1µg total RNA
H_2O	To 20µL	To 10µL	

For TaqMan probes (Cat 143, 146 and 149): Setting Up a 20µL or 10µL reaction

Component	Volume per 20µL	Volume per 10µL	Final concentration
2X Master Mix	10µL	5µL	1X
Primers	Variable	Variable	Each 150-900nM
TaqMan probes	Variable	Variable	150-250nM
RNA templates	Variable	Variable	As low as single digit copies of target RNA to ≤1µg total RNA
H_2O	To 20µL	To 10µL	

FAQ: How should I design primers for RT-PCR?

For high efficient RT-PCR, primers are designed with Tm ≥60°C, preferably 63-65°C, a probe with Tm 70°C and an amplicon with size 70bp to 150bp.

Longer amplicons may require longer extension times. The proper time will depend on the length of the amplicon and instrument type and should be determined empirically.

It is highly recommended to design primers across known splicing sites (exon-exon junctions) or spanning two neighboring exons in order to prevent amplification from genomic DNA.

In addition, the One-Step RT-PCR Kits are also compatible with primers and probes from many commercially available RT-PCR assays.

FAQ: How much primer should I use with the RT-PCR Kit?

For most targets, with primers and probe designed properly as above,150-200nM of each primer and 150-250nM of a probe will work efficiently under standard thermo-cycling conditions and 400nM will preferable under fast thermo-cycling conditions.

If needed, the final primer concentration can be optimized within 100–900nM.

FAQ: Which thermocycling program should I use primer should I use?

There are three programs are included of each category of RT-PCR 143, 144, 146, 147 and 149:

Table 1. Two-Step Standard Thermo-Cycling Protocol

Stage	Temperature	Period	Number of cycles
I	60°C	10min	1
II	95°C	1min	1
III	95°C	12sec	35-40
	60°C, signal acquisition	60sec	
IV	60°C to 95°C	Various	1

Table 2. Three-Step Thermo-Cycling Protocol

Stage	Temperature	Period	Number of cycles
I	60°C	10min	1

II	95°C	1min	1
III	95°C	10sec	35-40
	60°C	30sec	
	68°C, signal acquisition	30sec	
IV	60°C to 95°C	Various	1

Footnotes of Tables 1 and 2

The three-step thermocycling protocol in Table 2 increases overall polymerase activity by 50%, a more effective protocol than Table 2.

The primer concentration used in Tables 1 and 2 is typically 0.15-0.20uM if the primers are designed properly.

Table 3. Two-Step Fast Thermocycling Protocol

Stage	Temperature	Period	Number of cycles
I	60°C	5-10min	1
II	95°C	1min	1
III	95°C	5sec	35-40
	60°C, signal acquisition	30sec	
IV	60°C to 95°C	Various	1

Footnotes of Table 3

The product size for the fast two-step thermocycling protocol is preferred to be less than 90bp.

The primer concentration used in Table 3 is typically between 0.4uM and 0.9uM.

FAQ: Does your kit contain ROX ?

(FAQ: Do I need to add ROX?)

For each category of RT-PCR 143, 144, 146, 147 and 149, ROX reference dyes has three formats of

1) No ROX, 2) Low ROX and 3) High ROX, which you can choose.

FAQ: Are your RT-PCR kits compatible to my PCR instrument?

PCR Instruments and kits can be run in one of two way: 1) without ROX passive reference dye, and 2) with ROX passive reference dye which is only used in ABI instruments.

Table 1. Compatible instruments to our kits without ROX reference dye added

RT-PCR Instrument	ROX required by instrument	Our Program setup
Bio-Rad® iQ™5, CFX96, CFX384, Opticon Roche Lightcycler® Qiagen Rotor-Gene™ Eppendorf Mastercycler® Cepheid® SmartCycler®	Not recommended	Not necessary
Applied Biosystems® 7500, 7500 Fast, QuantStudio™, ViiA7™, Agilent Mx™	Low ROX (50nM final concentration)	Turn off ROX passive reference dye
Applied Biosystems® 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne™, StepOnePlus™	High ROX (500nM final concentration)	Turn off ROX passive reference dye

Note: Turn off ROX passive reference dye button when setup assays on Applied Biosystems/ThermoFisher instruments.

Table 2. Compatible instruments to our kits with ROX reference dye (Low ROX and High ROX added)

RT-PCR Instrument	ROX required by instrument
Applied Biosystems® 7500, 7500 Fast, QuantStudio™, ViiA7™, Agilent Mx™	Low ROX (50nM final concentration)
Applied Biosystems® 5700, 7000, 7300, 7700, 7900, 7900HT, 7900HT Fast, StepOne™, StepOnePlus™	High ROX (500nM final concentration)

Note: This kit contains High or Low concentration of ROX passive dye. Turn on ROX passive reference dye button when setup assays.

Notes: Applied Biosystems recommends using a passive reference dye (typically ROX) to overcome well-to-well variations that can be caused by machine limitations such as “edge effect”, bubbles, small

differences in volume, and autofluorescence from dust or particulates in the reaction. However, ROX normalization doesn't eliminate the variations caused by pipetting errors of templates/primers, heterogeneous Master Mix, and evaporation/condensation issues.

FAQ: Can I use shorter cycling times?

Standard thermo cycling conditions (e.g., 60 seconds for annealing and extension step) are recommended as a starting choice.

For many targets, fast thermo cycling conditions (e.g., 30 seconds for annealing and extension step) can be used. However, short time usually needs much higher concentrations of primers.

FAQ: Can I use fast thermo-cycling conditions with the One-Step RT-PCR Kits?

Yes. The One-Step RT-PCR Kits is compatible with both fast and standard thermo-cycling conditions.

If you use fast or standard thermo-cycling conditions, you should optimize the reaction parameters, such as primer concentrations, under each condition.

FAQ: What RNA samples can be used in my One-Step RT-PCR kit?

For the best results, we recommend using a qualified silica-based method to extract RNA from biological samples.

FAQ: How much RNA template should I use in my One-Step RT-PCR reaction?

The One-Step RT-PCR Kit works well with purified RNA templates including total RNA, mRNA, and in vitro transcription RNA samples.

Type of RNA	Amount in 20 µl reaction
total RNA	0.001-1µg
mRNA	0.0001-0.1µg
In-vitro transcripts	1-10^9 copies

FAQ: Does the One-Step RT-PCR kits contain dUTP?

No. The One-Step RT-PCR kits do not contain dUTP.

However, the thermophilic reverse transcriptase can efficiently incorporate dUTP and deaza-dGTP into newly synthesized cDNA.

FAQ: How should I determine RNA integrity?

RNA integrity of a total RNA sample can be estimated by the comparison between 28S and 18S rRNA amounts.

The total RNA is denatured and then resolved by size by gel electrophoresis. The ratio of intensities of 28S rRNA to 18S rRNA is then assessed, with a 2:1 ratio indicative of intact RNA (Figure 1A).

A more quantitative method of Agilent Technologies uses microfluidics to assess RNA integrity. The method produces a digital readout, called the RNA Integrity Number, or RIN, where values ranging between 8 and 10 indicate high-quality RNA [1,2] (Figure 1B).

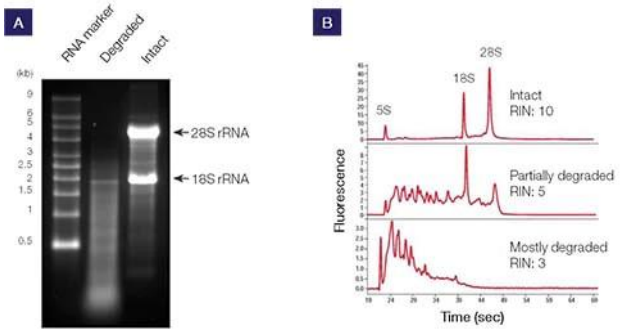

Figure 1. Analysis of RNA integrity by (A) gel electrophoresis and (B) microfluidics.

Reference

Schroeder A, Mueller O, Stocker S et al. (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3.

Mueller O, Lightfoot S, Schroeder A (2016) RNA Integrity Number (RIN) – Standardization of RNA Quality Control. Agilent Technologies Publication 5989-1165EN.

FAQ: What are RT inhibitors in extracted RNA samples?

Common RT inhibitors include SDS, EDTA and guanidium salts from RNA extraction, and their presence at increased concentrations can inhibit the thermophilic reverse transcriptase and DNA polymerase.

To remove the common RT inhibitors, an additional 70% ethanol wash step in the RNA extraction can be used.

FAQ: Can I run multiplex RT-PCR with your probe-based One-Step RT-PCR kit?

Yes, a probe-based One-Step RT-PCR kit (Cat 143) is designed for singleplex assay, and in many cases it can also be applied to duplex assays.

Another probe-based One-Step RT-PCR kit (Cat 146, 149) is designed for multiplex (up to quadruplex) assays in which more reverse transcriptase and DNA polymerase are added, and it can also be applied to singleplex assay.

FAQ: Should I include a No-RTase control?

The no-RTase control should not amplify from a residual amount of genomic DNA because your designed primers across splicing sites (exon-exon junctions) or span two neighboring exons to prevent such amplification from genomic DNA.

Even so, you should treat RNA samples with DNase to remove genomic DNA, but DNase (e.g., DNase I) must be removed completely prior to RT-PCR, since any residual enzyme would degrade single-stranded DNA, such as primers and newly synthesized cDNA.

Furthermore, in order to demonstrate no any amplification from genomic DNA, place an aliquot of the master mix into a thermocycler or dry bath and hold at 94°C for 2 minutes to eliminate the reverse transcriptase activity but keep the DNA polymerase activity. Then the heat-inactivated master mix can be used to amplify the no-RTase control.

General topics

FAQ: How do I choose between one-step RT-PCR and two-step RT-PCR?

There are two methods available: 1) one-step RT-PCR performed in a single tube , and 2) two-step RT-PCR performed in two separate tubes.

Table 2. Comparison of one-step and two-step RT-PCR

RT-PCR	RT Primers	Advantages	Disadvantages	Ideal uses
One-Step	Gene-specific primers	Simple setup Single closed-tube, reducing contamination	Need starting RNA sample(s) per reaction	Test many RNA samples High-throughputs
Two-Step	1) Oligo(dT) primers 2) Random hexamer primers 3) Gene-specific primers	Choice of RT primers Choice of enzyme and its amount	More setup steps Greater variation, potential contamination due to open-tube step	Test multiple targets from few RNA samples Save cDNA product for future re-use

FAQ: How do I use RT-PCR to determine the concentration of my RNA material?

Quantitative RT-PCR (RT-PCR) uses real-time fluorescence to measure the quantity of DNA present during a RT-PCR.

A wide variety of approaches have been developed for generating a fluorescent signal, and the most common approach is to use either hydrolysis probes (e.g., TaqMan), or a double-stranded DNA binding dye, (e.g., SYBR Green).

At a cycling point where the RT-PCR fluorescence signal is detectable over the background fluorescence, termed as a quantification cycle, or C_t value, can be determined. C_t values can be used to measure relative target quantities between two or more samples. Alternatively, they can be used to calculate absolute target quantities in reference to an standard curve derived from a series of dilutions of a DNA sample with a predetermined copy number.

FAQ: How many dilutions should I use to make a standard curve?

Typical RT-PCR experiments include standard samples spanning 5 orders of magnitude (five 10-fold serial dilutions) to generate a standard curve. Choose the lowest standard amount that will be approximately equal to one copy of target, if maximum sensitivity is desired. The number of points on the standard curve can be increased or decreased as desired, and as few as three points can be used when the target, assay, and sample are well characterized.

FAQ: Why do I have multiple peaks in my melt curve?

A typical melt curve will give rise to a single peak in the plot of the negative derivative of fluorescence vs. temperature. This indicates that the amplified double-stranded DNA product is a specific product. The presence of multiple products can give rise to multiple peaks in the melt curve, typically indicating the presence of primer dimer, false-primering or over-amplified (mega-priming) products.

FAQ: How can I distinguish non-template amplification (NTC) from real products?

These no-template control (NTC) reactions may generate primer dimer, which will usually be of a different size and/or sequence content than the desired DNA product and may therefore generate a different melt curve.

FAQ: Why do I see amplification curves in my NTC samples?

Non-template control (NTC) reactions may produce primer dimer, a source of the amplification curve.

Another common source of non-specific amplification is contamination from RT-PCR products in earlier RT-PCR experiments. This is a significant concern when the same assay is used repeatedly, and in particular when the reaction tubes are opened after the RT-PCR is completed.