One-step RT-qPCR

SARS-CoV-2 RNA detection with the lyo-ready one-step RT-qPCR system

Abstract

Reverse-transcription quantitative PCR (RT-qPCR) is one of the most powerful tools researchers have in the fight against SARS-CoV-2. The lyo-ready system for one-step multiplex RT-qPCR includes Invitrogen™ Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase, Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR, and 5X Lyo-ready Platinum™ II PCR Buffer. With the lyo-ready one-step RT-qPCR system, several SARS-CoV-2 targets can be detected simultaneously in a single well. The lyo-ready system is tolerant of PCR inhibitors that are commonly found in biological samples and PCR inhibitors like RNA isolation reagents. Lyo-ready enzymes and buffer are virtually glycerol-free, which provides users with the flexibility to modify concentration, volume, and even packaging.

Introduction

Lyo-ready RT-qPCR enzymes and buffer enable simultaneous reverse transcription (RT) and quantitative PCR in the same tube, which saves time and reduces the risk of contamination. Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase and Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR are new and highly processive next-generation enzymes. They have been developed specifically for one-step RT-qPCR applications and perform optimally with 5X Lyo-ready Platinum II PCR Buffer. Assay development can be accomplished in less time with the optimized lyo-ready system than it can with other ready-to-use RT-qPCR products on the market. The lyo-ready system enables multiplexing, so several targets can be analyzed at once. Lyo-ready enzymes and buffer also have glycerol contents of less than 0.01%, so they can be lyophilized to simplify logistics and storage requirements.

Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase

Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase is a specially engineered *Taq* polymerase with antibody-mediated hot-start activity. Hot-start technology blocks DNA polymerase activity at room temperature, which prevents nonspecific amplification and provides a high degree of specificity and sensitivity for low-abundance targets. Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase is highly resistant to inhibitors that are often introduced during sample preparation, so it is ideal for testing sub-optimal samples when used together with an RNase inhibitor. Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase remains stable after multiple freeze-thaw cycles, and it can be lyophilized and still retain all of the performance features of standard *Taq* DNA polymerase. This polymerase synthesizes DNA faster than other *Taq* DNA polymerases and can extend 1 kb of nucleic acid in 15 seconds. Like standard *Taq* DNA polymerase, Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase lacks 3′ to 5′ exonuclease activity. Because it has both 5′ to 3′ polymerase and 5′ to 3′ exonuclease activity, it is compatible with Applied Biosystems™ TaqMan™ technology.

Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR

Lyo-ready SuperScript Reverse Transcriptase is an engineered version of Moloney murine leukemia virus reverse transcriptase with greater thermostability and intrinsic RNase H activity. The glycerol-free formulation can be lyophilized without impacting any of the desired performance features of the enzyme. Lyo-ready SuperScript Reverse Transcriptase synthesizes first-strand cDNA optimally at 50°C, but it can be used at temperatures up to 55°C to denature RNA with GC-rich regions or strong secondary structures. Inhibitors like hemin, heparin, and guanidine hydrochloride compromise the performance of many reverse transcriptases and reduce RT efficiency. Lyo-ready SuperScript Reverse Transcriptase is more tolerant of inhibitors and more sensitive than other commercially available reverse transcriptases, so it is ideal when sample quantity is limited or RNA quality is sub-optimal.

5X Lyo-ready Platinum II PCR Buffer

5X Lyo-ready Platinum II PCR Buffer has been designed to optimize the performance of lyo-ready enzymes for one-step RT-qPCR. This specially formulated buffer contains glycerol-free reagents that enable optimal sensitivity, resistance to reaction inhibitors, and compatibility with multiplex applications.

Advantages of the lyo-ready one-step RT-qPCR system

- Glycerol-free enzymes and buffer that are compatible with lyophilization
- Multiplexing capability to test multiple targets in one reaction
- Tolerance to reaction inhibitors to help ensure high sensitivity and accuracy
- An optimized reaction buffer and a protocol that reduces time and resources needed for assay development

One-step multiplex RT-qPCR for SARS-CoV-2 RNA detection

One-step multiplex RT-qPCR is an important tool for the detection of SARS-CoV-2 RNA, because analyzing multiple targets simultaneously saves time and reduces the likelihood of generating false negative results. We have developed a one-step, multiplex RT-qPCR protocol for the detection of two SARS-CoV-2 S gene targets and one nucleocapsid (N) gene target. SARS-CoV-2 RNA was isolated from nasopharyngeal swabs collected from individuals who tested positive for the virus using the Applied Biosystems™ MagMax™ Viral/Pathogen Nucleic Acid Isolation Kit. One-step RT-qPCR was then performed on the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System. The reaction mixtures were prepared with the reagents and volumes listed in Table 1. The recommended thermal cycling protocol is summarized in Table 2.

Each SARS-CoV-2 RNA target was quantified using a standard curve consisting of a 10-fold dilution series prepared with a synthetic SARS-CoV-2 RNA control (Twist Bioscience). The concentration of viral RNA in the samples was relatively low, so each standard curve ranged from 20,000 to 20 synthetic RNA copies per reaction. Each sample was tested in triplicate for all three targets at each dilution point. The results obtained using the lyo-ready one-step RT-qPCR system were then compared to results obtained using two RT-qPCR kits from different vendors under the conditions recommended by the respective manufacturers.

Table 1. Reaction setup for one-step RT-qPCR to detect SARS-CoV-2 RNA.*

Reagent	Volume	Final concentration
5X Lyo-ready Platinum II PCR Buffer	4 µL	1X
10 mM dNTP mix	1.2 µL	0.6 mM each
50 mM $MgCl_2$	4.0 µL	10 mM
10 µM forward primers	0.4 µL	0.3 µM
10 µM reverse primers	0.4 µL	0.3 µM
10 µM probes	0.3 µL	0.2 µM
50 µM ROX reference dye	0.02 µL	50 nM
Lyo-ready RiboLock RNase Inhibitor (40 U/µL)**	0.5 µL	1 U/µL
Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase (20 U/µL)	0.12 µL	0.12 U/µL
Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR (200 U/µL)	0.1 µL	1 U/µL
Template RNA	5 µL	variable
Nuclease-free water	to 20 µL	–

* For additional recommendations, see the guidelines for reaction setup in the lyo-ready one-step RT-qPCR system user guide.

** Thermo Scientific™ RiboLock RNase Inhibitor inhibits the activity of RNase A, B, and C by noncompetitive binding in a 1:1 ratio. It does not inhibit eukaryotic RNase T1, T2, U1, U2, or CL3 activity or prokaryotic RNase I or H activity.

Table 2. Recommended cycling conditions for the lyo-ready one-step RT-qPCR system.

Step	Time	Temperature	Cycles
Reverse transcription	15 min	50°C	1
qPCR			
RT termination, initial denaturation, polymerase activation	2 min	95°C	1
Denaturation	5 s	95°C	45
Annealing and extension	20 s	60°C	

Performance in the presence of inhibitors

Another set of tests were conducted to evaluate the performance of the lyo-ready one-step RT-qPCR system in the presence of PCR inhibitors that are commonly found in sample materials and PCR inhibitors that can be introduced during nucleic acid extraction. A 10-fold dilution series was prepared with the synthetic SARS-CoV-2 control RNA and used to generate four-point standard curves with the highest concentration at 20,000 synthetic RNA copies per reaction. To test performance in the presence of an inhibitor, the master mix was spiked with 10 µM hemin, 2% isopropanol, or 2.5 µL universal transport medium (UTM). Each point on the curve was evaluated with the control master mix and the spiked master mixes. We compared the results obtained with the lyo-ready one-step RT-qPCR system to results obtained with the other two RT-qPCR products in the presence of the same inhibitors. One-step RT-qPCR was performed with each system under the conditions recommended by the respective manufacturer, and the C_q values at the highest point on each curve were recorded for all three SARS-CoV-2 targets. We also calculated ΔC_q, which was the difference between the C_q of a reaction prepared with a control master mix and the C_q of a reaction prepared with the same master mix spiked with an inhibitor.

Results

One-step RT-qPCR sensitivity and efficiency

The amplification efficiency, coefficients of correlation, and sensitivity of the lyo-ready one-step RT-qPCR system were superior to those of the other systems. The lyo-ready system enabled reliable detection of all three targets with as few as 20 SARS-CoV-2 RNA copies per reaction. At least one of the three targets went undetected with RT-qPCR products from the other vendors (Figure 1). The unbiased amplification efficiency of the lyo-ready system was nearly 99% (±3%) for all three gene targets with R^2 values of 0.99 (Figure 2). The lyo-ready system also generated the highest ΔRn values of all the systems tested.

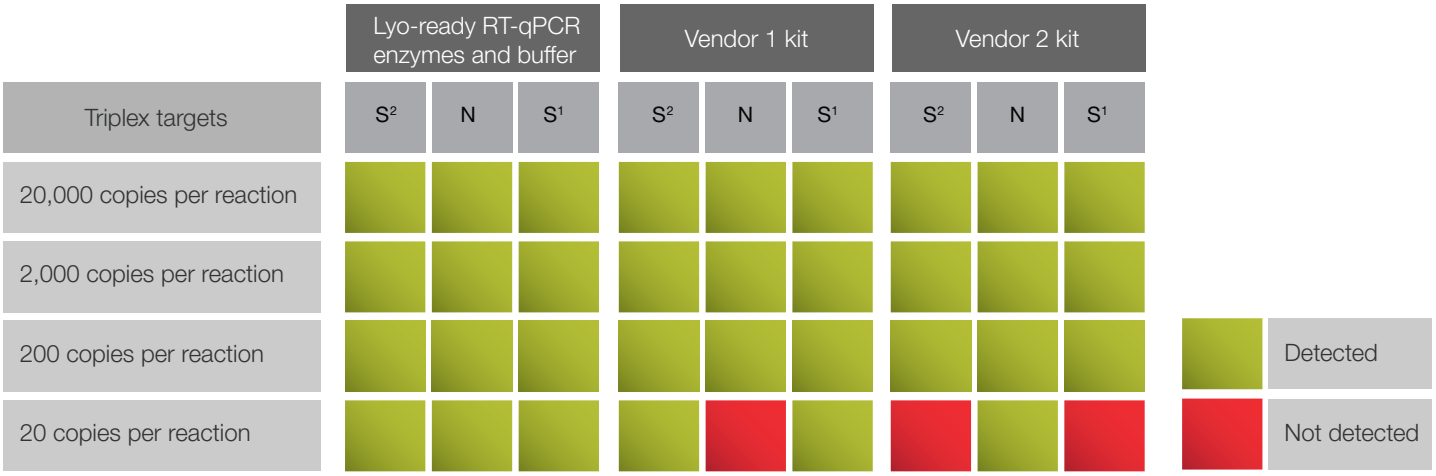

Figure 1. Comparison of triplex RT-qPCR sensitivity for detection of the SARS-CoV-2 N gene target and S gene targets S¹ and S².

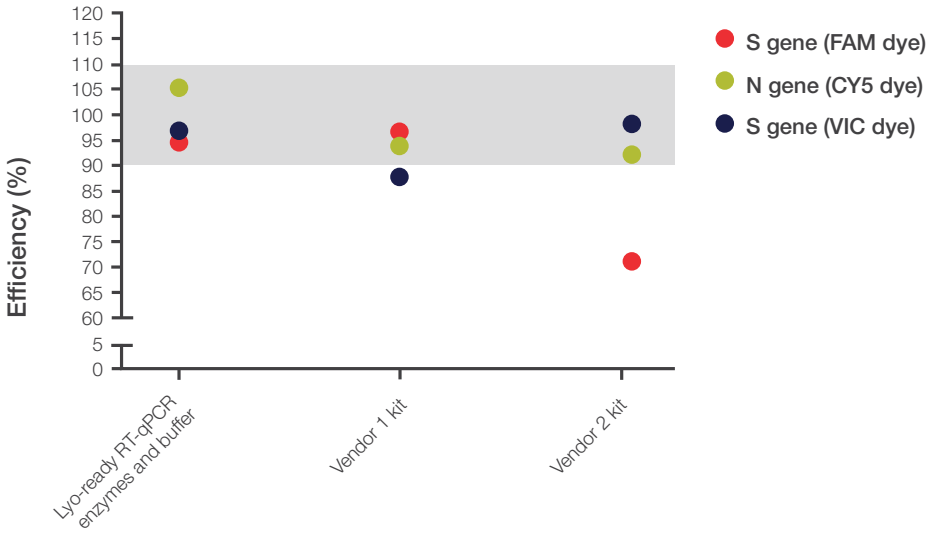

Figure 2. One-step triplex RT-qPCR efficiency for detection of the SARS-CoV-2 N gene target and S gene targets S¹ and S².

Inhibitor tolerance

Results obtained with the lyo-ready one-step RT-qPCR system in the presence of inhibitors are compared to the results obtained with the other systems in Figure 3. The amplification efficiency of the lyo-ready system was largely unaffected by the inhibitors, even when viral RNA copy number was low. The lyo-ready ΔC_q values were ≤1 for all three targets in the presence of isopropanol or UTM. In the presence of hemin, only two of the lyo-ready ΔC_q values were ≤1, and one fell between 1 and 2. No amplification was observed in reactions prepared with products from the other two vendors in the presence of hemin. The S^2 gene target was not detected in a control prepared with the master mix from one of the vendors, even though it did not contain an inhibitor. The performance of the lyo-ready system was fairly robust in the presence of each inhibitor, which confirmed that it could be used to analyze RNA in samples affected by handling and nucleic acid extraction.

Conclusion

The optimized lyo-ready one-step RT-qPCR system enables extremely sensitive, specific, and quantitative detection of SARS-CoV-2 RNA. The lyo-ready system is designed to provide users with the flexibility to optimize their own master mixes and assays, which can help them save time and conserve resources. The lyo-ready one-step RT-qPCR system out-performs commercially available one-step RT-qPCR kits, and it can enable molecular diagnostic tests to be developed more easily in a shorter period of time. The lyo-ready system also enables sensitive and reliable analysis of samples that contain a low number of copies of target RNA, even in the presence of inhibitors.

		Lyo-ready RT-qPCR enzymes and buffer			Vendor 1 kit			Vendor 2 kit		
Triplex targets		S^2	N	S^1	S^2	N	S^1	S^2	N	S^1
Control C_q with 20,000 synthetic SARS-CoV-2- RNA per reaction		25.1	25.4	24.8	25.3	25.2	25.0	0.0	25.8	26.6
Control + Inhibitors	10 µM hemin	25.9	26.3	26.0	0.0	0.0	0.0	0.0	0.0	0.0
	2% isopropanol	25.4	25.3	25.3	25.1	24.9	25.0	0.0	26.3	27.6
	2.5 µM UTM	25.2	25.2	25.4	28.6	27.7	30.2	0.0	27.4	29.2

ΔC_q value corresponds to the difference between control and C_q value of the inhibitor treated samples:

- $\Delta Cq \leq 1$
- $1 < \Delta C_q \leq 2$
- $\Delta C_q > 2$

Figure 3. Comparison of C_q values generated with a synthetic SARS-CoV-2 RNA control in the absence of PCR inhibitors and C_q values generated after spiking the master mixes with hemin, isopropanol, or UTM. Each reaction mixture contained 20,000 copies of the synthetic RNA control.

Learn more at **thermofisher.com/lyo-ready-1-step-RT-qPCR**