Molecular assay development

Multiplexed detection of respiratory viruses with the lyo-ready one-step RT-qPCR system

Introduction

Reverse-transcription quantitative PCR (RT-qPCR) plays a pivotal role in detecting pathogens such as SARS-CoV-2. Fast, precise, and multiplexed pathogen testing has become an increasingly vital need in recent years. In response, Thermo Fisher Scientific has developed the lyo-ready one-step RT-qPCR system that integrates Invitrogen™ Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase and Invitrogen™ Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR, with specially designed Invitrogen™ 5X Lyo-ready Platinum™ II PCR Buffer. This system allows simultaneous detection of multiple targets, including different SARS-CoV-2 genes, within a single well. By combining reverse transcription and quantitative PCR in a single tube, the lyo-ready one-step RT-qPCR system helps save time and reduce contamination risks. Its exceptional processivity and multiplexing capability help streamline molecular assay development compared to other products. The system's tolerance to PCR inhibitors, use of glycerol-free components, and compatibility with lyophilization and cartridge-based technologies make it a valuable tool for accurate and efficient nucleic acid detection. It enables timely and confident development of molecular assays, helping to advance future research and clinical applications.

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

- Glycerol-free enzymes and buffer that are compatible with lyophilization
- Compatibility with microfluidics solutions for point-of-care (POC) testing
- Multiplexing capability to test up to 5 targets in one reaction
- Tolerance to reaction inhibitors to help ensure high sensitivity and accuracy
- An optimized reaction buffer and a protocol that reduces the time and resources needed for assay development

Materials and methods

Reagents

- **Lyo-ready Platinum II Taq Hot-Start DNA Polymerase**—an engineered *Taq* DNA polymerase that shows increased resistance to reaction inhibitors originating from sample materials or nucleic acid purification steps. Like the standard *Taq* DNA polymerase, it has both 5′ to 3′ polymerase and 5′ to 3′ exonuclease activities but lacks 3′ to 5′ exonuclease activity and can be used with hydrolysis probes. The lyo-ready enzyme formulation offers the option to lyophilize reaction components while retaining all favorable properties. Due to an antibody-mediated hot-start mechanism, polymerase activity is blocked at ambient temperatures and restored after the initial denaturation step at 95°C. This automatic "hot start" provides increased sensitivity, specificity, and yield while allowing reaction assembly at room temperature.
- **Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR**—an engineered version of Moloney murine leukemia virus (MMLV) reverse transcriptase, designed especially for the one-step RT-qPCR application. The enzyme has increased thermal stability and RNase H activity. The lyo-ready enzyme formulation provides the option to lyophilize the enzyme or use it in cartridge-based technologies. The reverse transcriptase can be used to synthesize first-strand cDNA at temperatures up to 55°C, providing high sensitivity, specificity, and yields of cDNA.
- **5X Lyo-ready Platinum II PCR Buffer**—an optimized buffer designed to achieve superior performance of the reverse transcriptase and polymerase when they are used as a pair in one-step RT-qPCR. The buffer's composition helps ensure high reaction sensitivity, tolerance to reaction inhibitors, and compatibility with multiplexing and lyophilization.
- **Invitrogen™ Uracil-DNA Glycosylase**—enzyme produced in a recombinant *E. coli* strain that contains a modified *UNG* gene from Atlantic cod. At room temperature, the enzyme removes uracil from DNA by catalyzing hydrolysis of the N-glycosylic bond between uracil and the sugar. Uracil-DNA glycosylase (UNG) allows previous PCR amplicons and nonspecific products to degrade, leaving intact the native nucleic acid templates intended for amplification. *Taq* DNA polymerase and other PCR components are unaffected by UNG. The enzyme remains stable after multiple freeze-thaw cycles but is completely and irreversibly inactivated by heat (at 55°C). Efficient heat inactivation helps ensure that the integrity of PCR products is maintained for the long term regardless of storage conditions.

Reaction setup and cycling conditions

One-step RT-qPCR was performed on the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System. Reaction conditions for viral RNA detection are summarized in Table 1. The recommended cycling protocol is shown in Table 2.

Table 1. Reaction setup for one-step RT-qPCR to detect viral RNA.*

Component*	Volume	Final concentration
Water, nuclease-free	To 20 µL	–
5X Lyo-ready Platinum II PCR Buffer	4.0 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 primer	0.6 µL	0.3 µM each
10 µM reverse primer	0.6 µL	0.3 µM each
10 µM probe	0.4 µL	0.2 µM each
50 µM ROX™ reference dye†	0.02 µL	50 nM
Thermo Scientific™ Lyo-ready RiboLock™ RNase Inhibitor, 40 U/µL (optional)	0.5 µL	1 U/µL
UNG (1 U/µL)** (optional)	0.02 µL	0.001 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	Various

* For additional recommendations on reaction setup, see the lyo-ready one-step RT-qPCR system user guide (Pub. No. MAN0026633).

** Adjustments should be made when samples are being treated with UNG. Note that when using UNG, 50% of dTTP is replaced with dUTP. A higher percentage of replacement could result in a lower fluorescence intensity.

† For additional recommendations on using ROX dye, see the lyo-ready one-step RT-qPCR system user guide (Pub. No. MAN0026633).

‡ The volume of template RNA can be increased with a corresponding decrease of water in the master mix.

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

Step	Time	Temperature	No. of cycles
UNG activity* (optional)	5 min	25°C	1
Reverse transcription	5–15 min	50°C	
Quantitative PCR			
Reverse transcription inactivation, initial denaturation, polymerase activation	2 min	95°C	
Denaturation	5 sec	95°C	45
Annealing and extension	20 sec**	60°C	

* Step is added when the reaction mix contains UNG.

** Annealing and extension time depends on the number of targets and the qPCR instrument used.

Triplex one-step RT-qPCR assays

To evaluate assay sensitivity and efficiency of the lyo-ready one-step RT-qPCR system, multiplex reactions were set up with synthetic SARS-CoV-2 RNA Control* at 20,000, 2,000, 200, and 20 copies per reaction. Three targets were amplified, one in the N gene (N) and two in the S gene (S1 and S2). Results were compared to those of RT-qPCR systems from two other vendors. One-step RT-qPCR was performed using the conditions of the respective manufacturers.

Performance in the presence of inhibitors

A set of tests was conducted to evaluate the performance of the lyo-ready one-step RT-qPCR system in the presence of PCR inhibitors. 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 copies per reaction. To test tolerance to inhibitors, 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 compared with the control master mix without inhibitor and the spiked master mix with inhibitor. Results obtained with the lyo-ready one-step RT-qPCR system were compared to results obtained with the two vendors' RT-qPCR systems in the presence of the same inhibitors. One-step RT-qPCR was performed with each system under the conditions recommended by the respective manufacturers. Quantification cycle (C_q) values for 20,000 copies per reaction were recorded for the three SARS-CoV-2 targets (N, S1, and S2). These values were used to determine 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.

Quadruplex one-step RT-qPCR assays

The lyo-ready one-step RT-qPCR system was tested in a quadruplex assay with synthetic SARS-CoV-2 RNA, parainfluenza virus type 1 RNA, and synthetic influenza A virus RNA. Human cervical adenocarcinoma (HeLa S3) total RNA was used as a control. All targets were tested with 5 concentrations of a 10-fold dilution series. Each sample was tested in triplicate for all four targets at each dilution.

Performance in the presence of UNG

A set of tests was performed to evaluate the compatibility of the lyo-ready one-step RT-qPCR system with UNG. The tests were performed using 10-fold dilution series of synthetic SARS-CoV-2 RNA, parainfluenza virus type 1 RNA, synthetic influenza A virus RNA, and HeLa S3 RNA. Five-point standard curves were generated and compared with the five-point standard curves of untreated samples. To assess the performance of all four targets, C_q values of the 1:100 dilution point were also compared with those of the untreated samples.

Pentaplex one-step RT-qPCR assays

The lyo-ready one-step RT-qPCR system was tested in a pentaplex assay with synthetic SARS-CoV-2 RNA, parainfluenza virus type 1 RNA, adenovirus type 4 DNA, and chikungunya virus RNA. HeLa S3 total RNA was used as a control. Nucleic acids from parainfluenza virus, adenovirus, and chikungunya virus were purified from Vircell™ inactivated virus suspensions using the Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Cat. No. A48310). All targets were tested with 4 concentrations of a 10-fold dilution series. Each sample was tested in triplicate for all five targets at each dilution.

* Twist Synthetic SARS-CoV-2 RNA Control (Twist Bioscience) was used in all multiplex assays.

Results and discussion

Sensitivity and efficiency of triplex one-step RT-qPCR assays

The sensitivity and amplification efficiency of the lyo-ready one-step RT-qPCR system were compared to those of two other vendors' systems, using synthetic SARS-CoV-2 control RNA. Three targets were detected (N, S1, and S2) in a triplex reaction. The lyo-ready one-step RT-qPCR system enabled reliable detection of all three targets with as few as 20 SARS-CoV-2 RNA copies per reaction. With the RT-qPCR systems from the other vendors, at least one of the three targets was not detected at 20 copies per reaction (Figure 1). The unbiased amplification efficiency of the lyo-ready one-step RT-qPCR system was nearly 99% (±3%) for all three gene targets (Figure 2), with R^2 values of 0.99. The lyo-ready system also generated the highest ΔRn values of all tested systems.

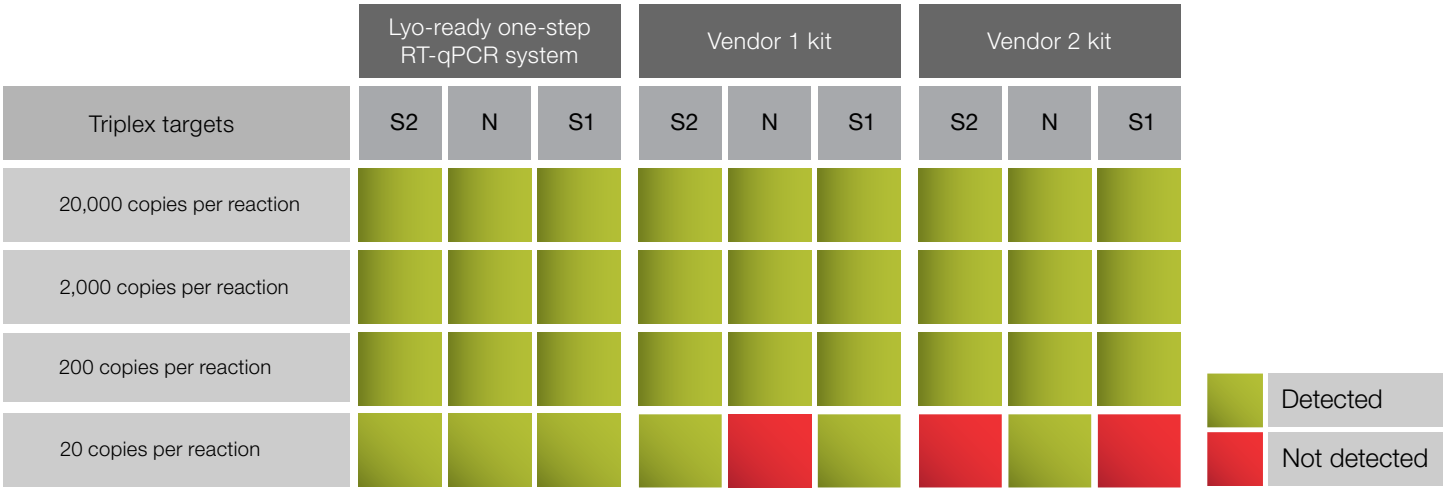


Figure 1. Sensitivity of one-step RT-qPCR for detecting three SARS-CoV-2 gene targets.

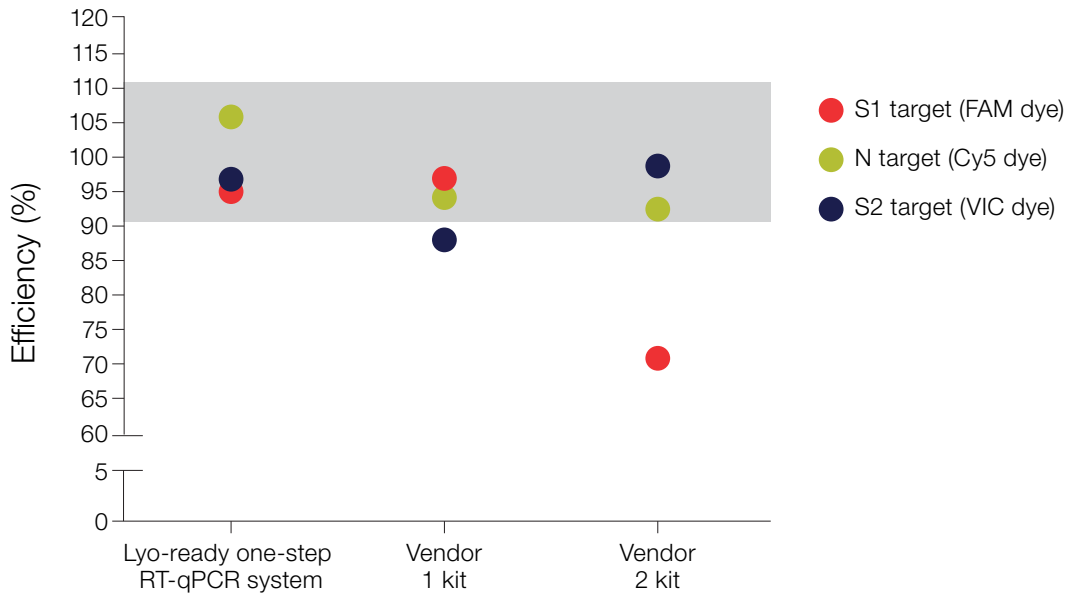


Figure 2. Efficiency of one-step RT-qPCR for detecting three SARS-CoV-2 gene targets.

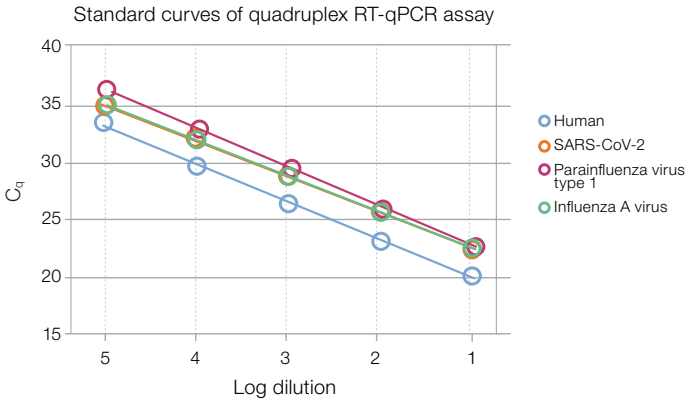
Inhibitor tolerance

The amplification efficiency of the lyo-ready one-step RT-qPCR system was largely unaffected by inhibitors (Figure 3). The ΔC_q values of the lyo-ready system were ≤1 for all three SARS-CoV-2 targets in the presence of isopropanol or UTM. In the presence of hemin, two of the ΔC_q values were ≤1, and one fell between 1 and 2. No amplification was observed in reactions prepared with the other two vendors' products in the presence of hemin. The S2 gene target was not detected in the control prepared with the master mix from vendor 2, even when it did not contain an inhibitor. The performance of the lyo-ready system was relatively 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.

Figure 3. C_q values for a synthetic SARS-CoV-2 RNA amplified with and without inhibitors. Each reaction mixture contained 20,000 copies of the synthetic RNA control.

Efficiency of quadruplex one-step RT-qPCR assays

One-step RT-qPCR is a vital tool for the detection of SARS-CoV-2 RNA and other viral targets. The lyo-ready one-step RT-qPCR system was used to develop a one-step multiplex RT-qPCR assay for detecting SARS-CoV-2 target together with other viruses. Results show that the system enables reliable detection of four different targets (Figure 4). Amplification efficiencies of at least 93% were achieved for all four gene targets, with R^2 values above 0.990. These results verify that the lyo-ready system can be used in applications where there is a need for fast and accurate detection and differentiation of respiratory viruses.



Target	Fluorophore (channel)	Template	Efficiency	R^2	Slope
Human	FAM™	HeLa S3 RNA	97.3%	0.998	–3.4
SARS-CoV-2	Cy®5	Synthetic SARS-CoV-2 RNA	97.8%	0.998	–3.4
Parainfluenza virus type 1	JOE™	Parainfluenza virus type 1 RNA	93.4%	0.996	–3.5
Influenza A virus	TAMRA™	Synthetic influenza A virus RNA	102.8%	0.997	–3.3

Figure 4. Linear dynamic range and parameters for each reaction in the quadruplex RT-qPCR experiment.

Performance in the presence of UNG

Quadruplex assays were performed with the lyo-ready one-step RT-qPCR system to evaluate its performance with and without UNG. Amplification plots show that samples treated with UNG share similar ΔRn values with those treated without the enzyme (Figure 5). Also, after setting the threshold and analyzing five-point standard curves (data not shown), there was no significant difference in C_q values for all four targets. Parameters such as slope, amplification efficiency, and R^2 values are compared in Table 3. These results confirmed that UNG can be successfully used as a contamination control with the lyo-ready system.

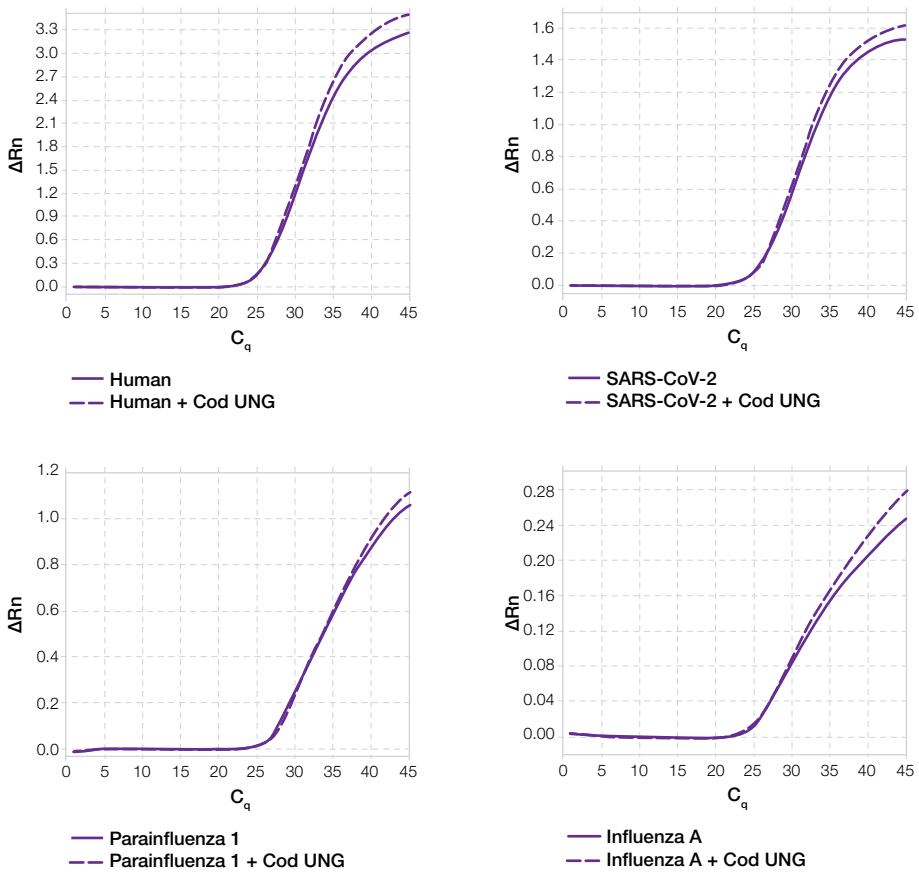


Figure 5. Amplification plots of quadruplex one-step RT-qPCR assays at the 1:100 dilution with and without UNG.

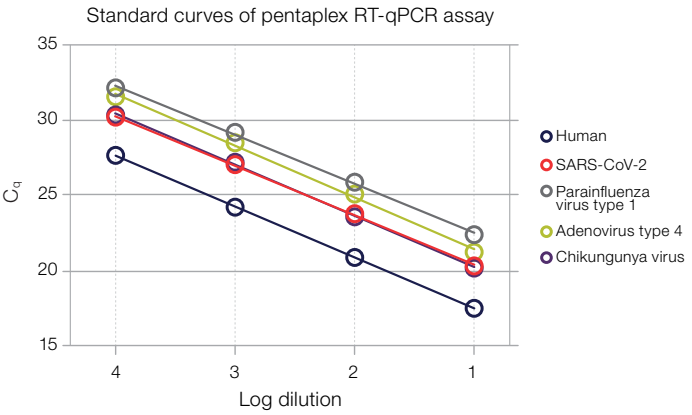
Table 3. Linear dynamic range parameters for each reaction in the quadruplex RT-qPCR experiment.

	Reactions without UNG			Reactions with UNG		
Target	**Efficiency**	**R^2**	**Slope**	**Efficiency**	**R^2**	**Slope**
Human	98.2%	0.999	–3.4	101.2%	0.999	–3.3
SARS-CoV-2	102.4%	0.999	–3.3	95.5%	1.000	–3.4
Parainfluenza virus type 1	92.4%	0.999	–3.5	99.6%	0.995	–3.3
Influenza A virus	106.3%	0.998	–3.2	99.4%	0.998	–3.3

Efficiency of pentaplex one-step RT-qPCR assays

The lyo-ready one-step RT-qPCR system was evaluated in a multiplex reaction with five different targets (Figure 6). Results show that reliable detection can be achieved across four 10-fold dilutions. Furthermore, one of the targets (adenovirus type 4) is a respiratory virus with a double-stranded DNA genome, indicating that the lyo-ready system can be used for simultaneous detection of RNA and DNA viruses.

Conclusions



Target	Fluorophore (channel)	Template	Efficiency	R^2	Slope
Human	TAMRA	HeLa S3 RNA	97.7%	1.000	–3.4
SARS-CoV-2	ROX	Synthetic SARS-CoV-2 RNA	100.1%	0.999	–3.3
Parainfluenza virus type 1	JOE	Parainfluenza virus type 1 RNA	102.7%	0.998	–3.3
Adenovirus type 4	FAM	Adenovirus type 4 DNA	95.3%	0.997	–3.4
Chikungunya virus	Cy5	Chikungunya virus RNA	96.3%	0.999	–3.4

Figure 6. Linear dynamic range and parameters for each reaction in the pentaplex RT-qPCR experiment.

The optimized lyo-ready one-step RT-qPCR system enables sensitive, specific, and quantitative detection of SARS-CoV-2 RNA as well as other viral nucleic acids. The lyo-ready system is designed to provide users with the flexibility to optimize their own master mixes and assays, which can help save time and conserve resources. The lyo-ready one-step RT-qPCR system outperforms other commercially available one-step RT-qPCR kits, and it can enable easier development of molecular assays in a shorter time. The lyo-ready system also enables sensitive and reliable analysis of samples that contain low numbers of copies of target RNA, even in the presence of inhibitors. Additional results highlight that the system can be used together with UNG, which minimizes the risk of false positives.

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