Molecular assay development

Applicability of the lyo-ready one-step RT-qPCR system for direct RT-qPCR

Introduction

Sensitive and accurate detection of viruses has become a key factor in controlling the spread of infectious diseases, especially after the emergence of SARS-CoV-2. Therefore, it is no surprise that one-step RT-qPCR, with its high accuracy and sensitivity, is considered an excellent choice for the detection of viruses in different sample types, even those with low viral copy numbers. Today's molecular assay developers require a technology that enables getting the maximal number of results with minimal usage of reagents in a short period of time. In response, Thermo Fisher Scientific has developed the lyo-ready one-step RT-qPCR system, which together with Applied Biosystems™ SalivaReady™ Solution allows fast pathogen detection without the need for an additional nucleic acid extraction step. The system consists of Invitrogen™ Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase and Invitrogen™ Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR, combined with specially designed Invitrogen™ 5X Lyo-ready Platinum™ II PCR Buffer. Here we demonstrate that the system is suitable for direct nucleic acid detection from saliva, blood plasma, and various types of viral transport medium (VTM). Additionally, we provide reaction setup recommendations and cycling protocols for direct RT-qPCR.

Materials and methods

Reagents

- **Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase**—an engineered *Taq* DNA polymerase with an antibody-mediated hot-start mechanism. The enzyme is fast, highly sensitive, and designed to be resistant to many PCR inhibitors, such as RNA isolation reagents, hemin, isopropanol, and universal transport medium (UTM). The lyo-ready (glycerol-free) formulation makes the enzyme compatible with the lyophilization process, which is frequently used for stabilization of RT-qPCR mixes.
- **Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR**—an engineered version of Moloney murine leukemia virus (MMLV) reverse transcriptase; it has an intrinsic RNase H activity and greater thermostability than the wild-type MMLV, and it is resistant to many reverse transcription (RT) inhibitors, such as guanidine hydrochloride, heparin, and hemin. The lyo-ready (glycerol-free) formulation allows the enzyme to be compatible with lyophilization of the reaction mix.
- **Lyo-ready Platinum II PCR Buffer**—designed to achieve optimal one-step RT-qPCR performance of Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase and Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR. To enable compatibility with the lyophilization process, the buffer formulation contains no glycerol.
- **SalivaReady Solution**—specially formulated lysis buffer that enables preparation of raw saliva samples for qPCR without the need for an additional nucleic acid extraction step.

Evaluation of compatibility of the lyo-ready one-step RT-qPCR system with various types of VTM

A set of tests using the lyo-ready one-step RT-qPCR system was performed by amplifying two different genes (N and S) from 20 copies of the Twist Synthetic SARS-CoV-2 RNA Control (Twist Bioscience). RT-qPCR reactions contained 5–25% of VTM. As a control, a reaction mixture without VTM was used. Four different types of VTM were evaluated: Thermo Scientific™ MicroTest™ M4RT Medium (Thermo Fisher Scientific), UTM-RT™ Medium (Copan), VIR-Swab Virus Medium (Herenz), and Transport Medium (Vircell). As heating of samples present in VTM might be used before RT-qPCR, an additional set of experiments was performed using preheated VTM (5 min at 95°C) for RT-qPCR. Each RT-qPCR reaction was performed in triplicate on the Applied Biosystems™ QuantStudio™ 7 Flex Real-Time PCR System. Reaction setup conditions are summarized in Table 1. The recommended cycling protocol is provided in Table 2.

Direct RT-qPCR using the lyo-ready one-step RT-qPCR system and SalivaReady Solution

Before use in direct RT-qPCR, saliva or plasma samples were mixed with an equal volume of SalivaReady Solution and then heat inactivated following the thermal conditions provided in Table 3 (for more information about SalivaReady Solution, see Pub. No. MAN0019847, Rev. B.0). Reaction mixtures contained 5% or 10% of either saliva or plasma samples. After heat inactivation, samples were mixed with the RT-qPCR reaction mixture, aliquoted into a PCR plate, and then spiked with 20,000, 2,000, 200, and 20 copies of Twist Synthetic SARS-CoV-2 RNA Control. Two different genes of the SARS-CoV-2 RNA control (N and S) were amplified. Reaction mixtures without saliva or plasma samples were used as controls. Each sample was tested in triplicate. One-step RT-qPCR was performed on the QuantStudio 7 Flex Real-Time PCR System. Reaction setup conditions are summarized in Table 1. The recommended cycling protocol is provided in Table 2.

Table 1. Reaction setup for one-step RT-qPCR.

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	0.5 µL	0.25 µM each
10 µM reverse primer	0.5 µL	0.25 µM each
10 µM probe	0.4 µL	0.20 µM each
50 µM ROX™ reference dye	0.02 µL	50 nM
Thermo Scientific™ 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
RNA-containing sample	5 µL	Various

* Optional component.

Table 2. Cycling protocol for one-step RT-qPCR.

Step	Time	Temperature	No. of cycles
Reverse transcription	15 min*	50°C	1
Reverse transcription inactivation, initial denaturation, polymerase activation	2 min	95°C	1
Denaturation	5 sec	95°C	45 cycles*
Annealing and extension	30 sec*	60°C	

* Time and number of cycles can vary depending on the sample volume and sample type.

Table 3. Heat inactivation of saliva or plasma samples mixed with SalivaReady Solution.

Step	Time	Temperature
1	5 min	62°C
2	5 min	92°C
3	Hold*	4°C

* If necessary, the sealed plate may be stored at 4°C until using for real-time PCR.

Results

Compatibility of the lyo-ready one-step RT-qPCR system with various types of VTM

To shorten time-to-results, analytes present in VTM might be amplified in direct PCR. Since VTM might contain PCR inhibitors, we checked the compatibility of the lyo-ready one-step RT-qPCR system with various VTM, as described in the methods. Results demonstrated (Figure 1) that the presence of up to 3 µL of various unheated VTM in the reaction mixture (equal to 15% of reaction mixture volume) did not interfere with the detection of 20 copies of SARS-CoV-2 RNA for N and S gene targets. In all cases, the ΔC_q value between the test reaction (unheated VTM was added) and the control (no VTM was added) was less than 1 cycle. Higher amounts of VTM were shown to generate greater ΔC_q value differences for the S gene target, demonstrating the negative impact of VTM on RT-qPCR results. As can be seen in Figure 1A, 4 or 5 µL (20–25% of reaction mixture volume) of different VTM inhibit S gene amplification by a different extent, with 5 µL of unheated VIR-Swab Virus Medium generating a 6.7-cycle difference compared to control. On the other hand, the negative impact of VTM was demonstrated to be dependent on the target and primers since even 5 µL of all VTM were shown to have no negative impact on the second target's (N gene) amplification results (Figure 1B).

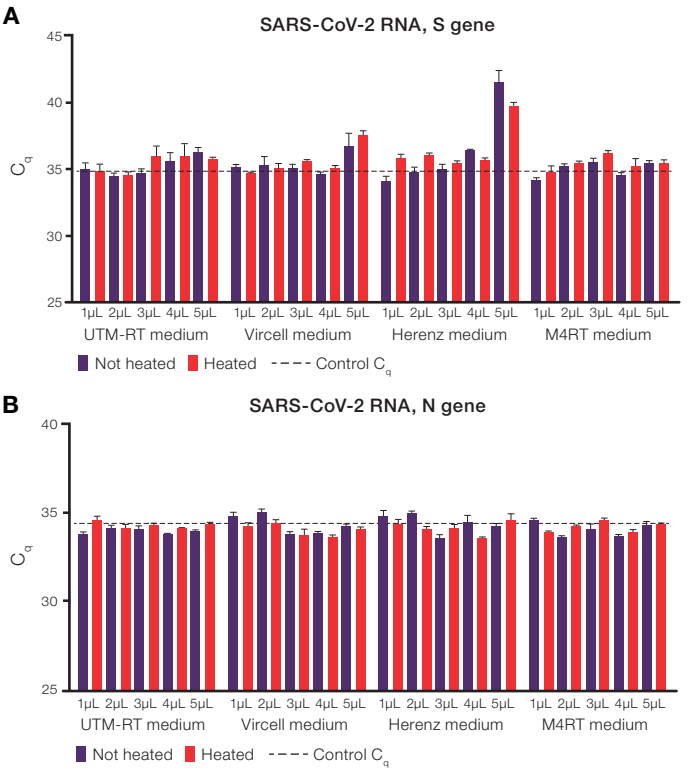

Figure 1. Comparison of quantification cycle (C_q) values of RT-qPCR, with various volumes of different types of VTM added in the reaction mixtures. Each reaction mixture contained 20 copies of synthetic SARS-CoV-2 RNA for detection of the S gene **(A)** and N gene **(B)**. The dashed line marks the C_q of the control reaction, in which VTM was replaced by nuclease-free water.

As viruses in VTM are usually thermally inactivated before RT-qPCR analysis, we have tested the impact of heating of VTM on RT-qPCR results. Data provided in Figure 1 show that the presence of 2–4 µL of different heated VTM could generate higher than 1 cycle difference in comparison to control (where no VTM was added). But overall, there was no consistent difference in RT-qPCR results when comparing heated or unheated VTM.

Together, these results demonstrate that the lyo-ready one-step RT-qPCR system tolerates 1–3 µL (5–15% of the reaction volume) of various types of VTM without negative impact on PCR results. Higher volumes of VTM can alter results depending on target gene, reaction composition, primers, and the VTM itself.

Performance of the lyo-ready one-step RT-qPCR system in the presence of saliva

SalivaReady Solution was used to prepare raw saliva samples for real-time PCR. Saliva samples were mixed with an equal volume of SalivaReady Solution and then added to the reaction mixture to a final concentration of 5% or 10% of saliva in the mixture. Results demonstrated that 20 copies of virus RNA can be detected in the presence of 5% or 10% of saliva in the reaction mixture (Figure 2). The C_q values of saliva-containing reactions were very similar to the C_q values of the control, where saliva was replaced with nuclease-free water. All reactions had acceptable reaction efficiency and R^2 values.

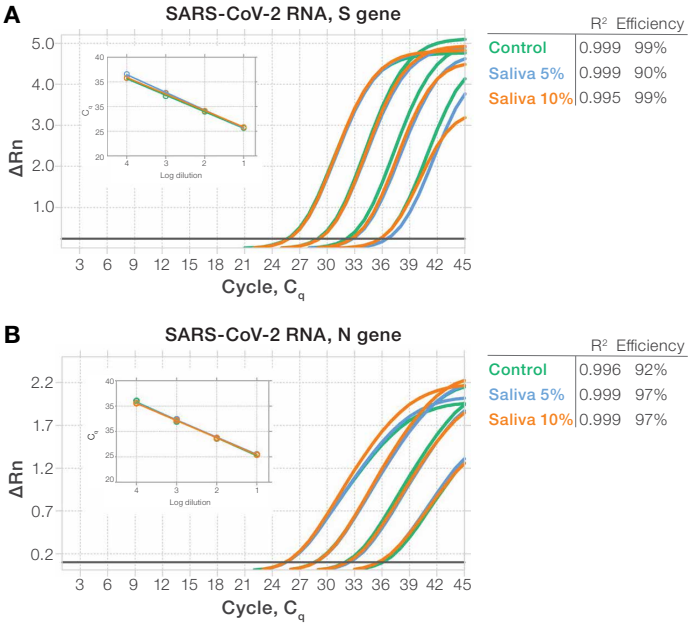

Figure 2. Amplification of S and N genes encoded by SARS-CoV-2 RNA, in the presence of 0% (control), 5%, or 10% saliva. Saliva samples were mixed with SalivaReady Solution and heat inactivated before adding them to the reaction mix. Reaction mixtures contained 20,000, 2,000, 200, and 20 copies of synthetic SARS-CoV-2 RNA. A control reaction contained nuclease-free water instead of a saliva sample. Amplification plots are shown for the S gene **(A)** and N gene **(B)**. The threshold is marked by the gray line. Standard curves with their parameters (R^2 and efficiency) are shown in the inset graphs and in the tables.

In another set of experiments, the performance of the lyo-ready one-step RT-qPCR system, in combination with the pretreatment step using SalivaReady Solution, was compared with the performance of products for direct RT-qPCR from other vendors (Figures 3 and 4). Results demonstrated that 20 copies of the N or S gene could be detected in the presence of 10% saliva. On the other hand, the sensitivity of the RT-qPCR system from vendor 1 was decreased for both N and S genes even in the presence of only 5% saliva. The RT-qPCR system from vendor 2 detected 20 copies of both genes in the presence of 5% saliva, but with 10% saliva in the reaction mixture, 3 out of 3 replicates generated positive signals only when at least 2,000 copies (S gene) or 200 copies (N gene) of target genes were amplified.

Analysis of C_q differences (ΔC_q) between control and saliva-containing reaction mixtures revealed that RT-qPCR systems from vendor 1 and vendor 2 are sensitive to saliva-borne inhibitors. ΔC_q for the system from vendor 1 was ≥4 for S gene and N gene detection in the presence of 10% saliva. The system from vendor 2 was more resistant to inhibitors in saliva—ΔC_q was between 2 and 4 for detection of the S gene (5% saliva) and N gene (10% saliva). Overall, the lyo-ready 1-step RT-qPCR system outperformed products from vendor 1 and vendor 2, showing no inhibition even in the presence of 10% saliva.

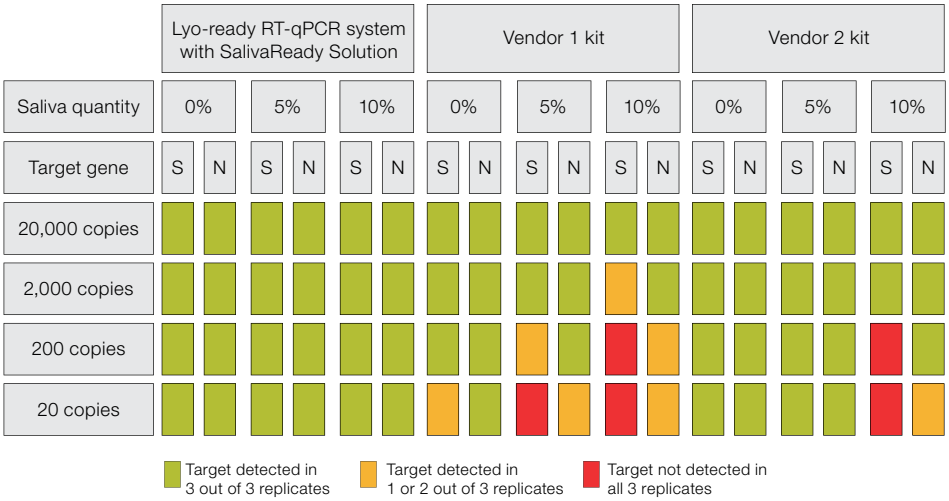

Figure 3. Comparison of sensitivity of RT-qPCR systems in the presence of saliva. 20 to 20,000 copies of the S or N gene of synthetic SARS-CoV-2 RNA were amplified in the presence of 0%, 5%, or 10% saliva. Saliva samples were mixed with SalivaReady Solution and were heat inactivated before adding them to the lyo-ready one-step RT-qPCR reaction mix. All reactions were performed in triplicate following suppliers' recommendations.

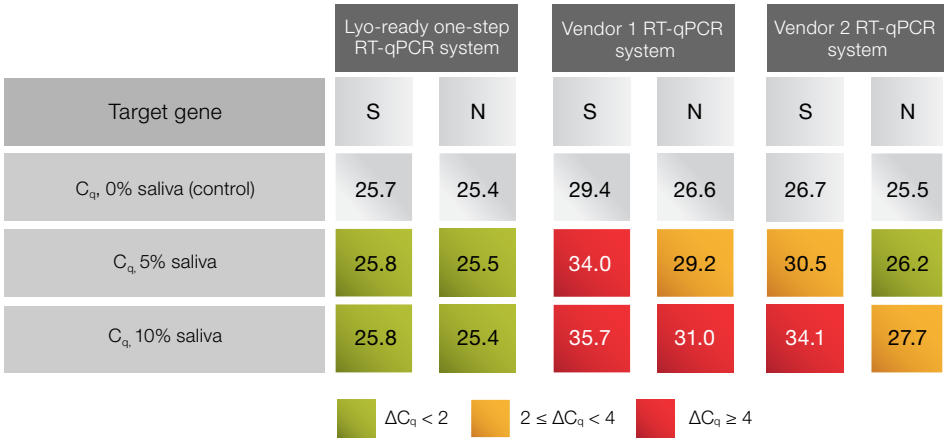

Figure 4. Comparison of C_q values of RT-qPCR when detecting 20,000 copies of synthetic SARS-CoV-2 RNA. 20,000 copies of the S or N gene of synthetic SARS-CoV-2 RNA were amplified in the presence of 0%, 5%, or 10% saliva. Saliva samples were mixed with SalivaReady Solution and were heat inactivated before adding them to the lyo-ready one-step RT-qPCR reaction mix. All reactions were performed in triplicate following suppliers' recommendations.

One-step RT-qPCR with the lyo-ready one-step RT-qPCR system and SalivaReady Solution in the presence of blood plasma

The lyo-ready one-step RT-qPCR system can be adapted to detect targets not only in inhibitor-enriched samples like saliva but also in plasma. The lyo-ready one-step RT-qPCR system, in combination with SalivaReady Solution, enabled the detection of 20 copies of synthetic SARS-CoV-2 RNA in the presence of 5% and 10% plasma (Figure 5).

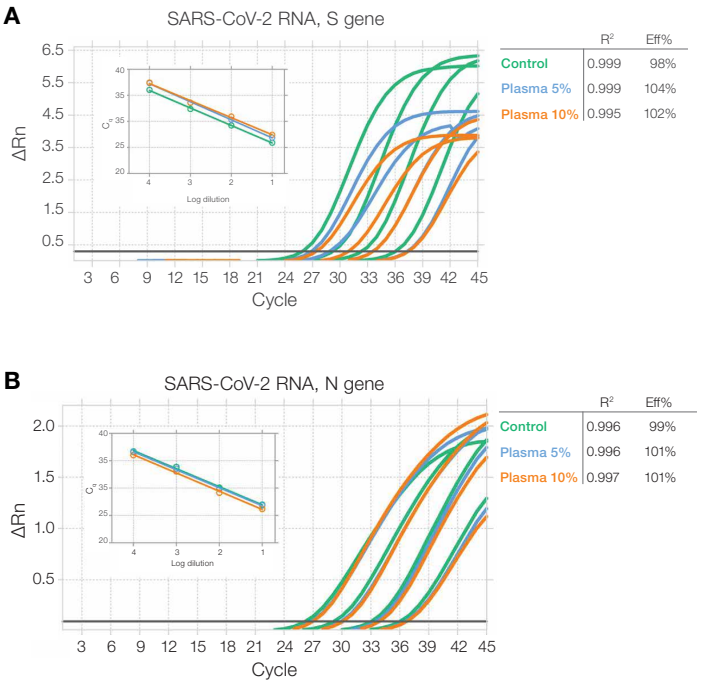

Figure 5. Amplification of SARS-CoV-2 S and N genes in the presence of 0% (control), 5%, or 10% plasma. Plasma samples were mixed with SalivaReady Solution and heat inactivated before adding them to the reaction mix. Reaction mixtures contained 20,000, 2,000, 200, and 20 copies of synthetic SARS-CoV-2 RNA. A control reaction contained nuclease-free water instead of a plasma sample. Amplification plots are shown for the S gene **(A)** and N gene **(B)**. The threshold is marked by the gray line. Standard curves with their parameters (R^2 and efficiency) are shown in the inset graphs and in the tables.

Conclusions

Due to exceptional tolerance to inhibitors and quick time-to-results, the optimized enzyme pair—Lyo-ready Platinum II *Taq* Hot-Start DNA Polymerase and Lyo-ready SuperScript Reverse Transcriptase, 1-Step RT-qPCR—enables fast and sensitive detection of nucleic acids directly from saliva, blood plasma, and VTM by one-step RT-qPCR. While the lyo-ready one-step RT-qPCR system was evaluated using synthetic SARS-CoV-2 RNA, it can be adapted for the detection of other viral or bacterial targets (e.g., Zika virus, data not shown). The methods described, which do not require nucleic acid extraction, can help streamline direct RT-qPCR–based workflows to provide fast and sensitive pathogen detection.

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