Direct One-Step RT-qPCR Mix for SARS-CoV-2 Protocol-At-A-Glance

I. Introduction

Direct One-Step RT-qPCR Mix for SARS-CoV-2 (Cat. Nos. 638329 and 638330) enables the user to perform accurate one-step RT-qPCR on unpurified saliva samples to determine the presence of virus. (See note below.) In this protocol, the sample and pretreatment reagent are mixed and heat treated, and then an aliquot of this mixture is combined with the RT-qPCR master mix. The protocol from pretreatment to detection takes less than one hour (~5 minutes for pretreatment and ~50 minutes for real-time RT-qPCR), enabling fast time to results.

NOTE: This protocol provides guidelines for SARS-CoV-2 detection in saliva samples, but no primers are included. The protocol was optimized using the primers and probes recommended by the CDC for N1, N2, and RNase P targets in a multiplex reaction. Direct One-Step RT-qPCR Mix for SARS-CoV-2 could be used to detect other viruses using primer/probe mixes specific to the desired targets, with optimization of the reaction conditions performed by the user.

II. Materials and Reagents

A. Components

The following components are included in the kits:

Direct One-Step RT-qPCR Mix for SARS-CoV-2 (Cat. No. 638329; 200 Rxns*)

- 4 ml — Sample Prep Solution (Cat. No. 638331) (Not sold separately)
- 1 each — One Step PrimeScript™ III RT-PCR Kit (Cat. No. RR600A)

One Step PrimeScript III RT-PCR Kit (Cat. No. RR600A; included in Cat. No. 638329)

- 4 x 625 µl — One Step PrimeScript III RT-qPCR Mix (2X)
- 2 x 1.25 ml — RNase Free H2O
- 100 µl — ROX Reference Dye (50X)
- 100 µl — ROX Reference Dye II (50X)

Direct One-Step RT-qPCR Mix for SARS-CoV-2 (Cat. No. 638330; 4,000 Rxns*)

- 19 x 4 ml — Sample Prep Solution (Cat. No. 638332) (Not sold separately)
- 1 each — One Step PrimeScript III RT-PCR Kit (Cat. No. RR60TW)

One Step PrimeScript III RT-PCR Kit (Cat. No. RR60TW; included in Cat. No. 638330)

- 50 ml — One Step PrimeScript III RT-qPCR Mix (2X)
- 50 ml — RNase Free H2O
- 2 ml — ROX Reference Dye (50X)
- 2 ml — ROX Reference Dye II (50X)

*The reaction number is based on a 25-µl reaction (total volume).

B. Additional Materials Required

- Primers and probes
- Micropipette and tips (with hydrophobic filters)
- Vortex mixer
- Benchtop centrifuge for tubes or plates
- Saliva sample collection tubes (e.g., disposable 50-ml conical tube)
- 1.5 ml Eppendorf tubes, 200-µl PCR tubes, or 200-µl PCR plates for sample pretreatment
- Tubes or plates for real-time PCR
- A real-time PCR machine

C. Storage and Handling

- Store Sample Prep Solution at room temperature.
- Store components of the One Step PrimeScript III RT-PCR Kit at –20°C.

III. Precautions

- Potentially infectious human samples must be handled according to the regulations at your facility.
- Handling of samples containing live virus should be conducted in a safety cabinet in a BSL2 laboratory, using appropriate personal protective equipment (PPE; i.e., mask, goggles, lab coat, gloves, and close-toed shoes). In addition, please perform all protocol steps through pretreatment (end of Section IV.A) in a BSL2 laboratory, since there is potential for a sample to be infectious until then. It is not necessary to prepare the RT-qPCR reaction mixture in a BSL2 laboratory.
- Perform all procedures through pretreatment (end of Section IV.A) in a way that minimizes the creation of splashes and/or aerosols. Vortexing, spinning, pipetting, or mixing of any sort should be performed in a safety cabinet.
- Cross-contamination between samples or from the user's sweat or saliva must be avoided to prevent false-results. Take necessary actions (e.g., change gloves or repeat the experiment) as needed.
- Discard samples, assuming infection risk, using appropriate PPE and according to the regulations for infectious waste disposal at your facility.
- Remember to remove gloves and any potentially contaminated clothing prior to exiting the lab. Wash hands thoroughly after working with samples and before leaving the facility.

IV. Protocol

A. Saliva Sample Pretreatment

1. Collect ~1 ml of saliva in a sample collection tube and vortex well.

NOTE: The test subject should not eat, drink, or smoke for at least 30 min prior to saliva collection.

2. In a 1.5-ml Eppendorf tube, 200-µl PCR tube, or well of a PCR plate, mix the saliva sample with Sample Prep Solution.

Pretreatment mixture (for 1 reaction):

100 µl	Saliva sample
15 µl	Sample Prep Solution
115 µl	Total volume

NOTE: The sample and the Sample Prep Solution can be added in any order.

3. Vortex the tube/plate well for 10 sec, and spin down briefly.

4. Incubate the tube/plate on a heat block or in a thermal cycler at 95°C for 2 min.

5. Incubate at 4°C until ready to combine with RT-qPCR master mix (up to 2 hr).

NOTES:

- The resulting RNA in the mixture should be stable at 4°C for up to 2 hr.
- Precipitate or sample debris may appear, but mixing or removing it is not necessary.

B. RT-qPCR Reaction Mix Preparation

1. Prepare the reaction mix for the RT-qPCR reaction as illustrated below. Following sample addition, vortex and spin down the tube/plate briefly. Then, immediately place the tube/plate into the thermal cycler.

RT-qPCR reaction mixture (for 1 reaction):

Components*	20-µl reaction	50-µl reaction
One Step PrimeScript III RT-qPCR Mix (2X)	10 µl	25 µl
Primer/probe mix (10 µM each; provided by user)**	0.4 µl	1 µl
RNase Free H2O	8.6 µl	21.5 µl
Pretreatment mixture (heat-treated sample from Section IV.A, Step 5)†	1 µl	2.5 µl
Total volume‡	20 µl	50 µl

*If using a machine that performs fluorescent signal correction between wells, add either ROX Reference Dye or ROX Reference Dye II to the reaction mix. Which ROX dye is used depends on the machine. Use ROX Reference Dye at a final concentration of 1X with the following ABI machines: StepOne, StepOnePlus, and 7300/7700/7900HT real-time PCR systems. Use ROX Reference Dye II at a final concentration of 0.5X with the following ABI machines: 7500, 7500 Fast, and QuantStudio series real-time PCR systems.

**A final concentration of 0.2 µM worked well for the multiplex reaction of N1, N2, and RNase P targets in SARS-CoV-2 detection. However, further optimization may be required depending on your chosen target(s).

†Always add the pretreatment mixture (sample) to the reaction mix last. If you have multiple samples, prepare the required amount of master mix first. Pretreatment mixture volume must not exceed 7.5% of the total reaction volume (e.g., ≤1.5 µl for the 20-µl reaction or ≤3.75 µl for the 50-µl reaction). Due to PCR inhibition, assay sensitivity will not improve by further increasing the pretreatment mixture input volume.

‡The total reaction volume depends on the qPCR instrument used. It is recommended to use the higher reaction volume (e.g., 50-µl reaction) to maximize the sample volume input.

C. RT-qPCR Reaction Protocol

1. Run the following standard cycling protocol.

NOTE: This protocol was developed using a fast ramp mode (6.5°C/sec); good results were obtained when multiplexing N1, N2, and RNase P targets for SARS-CoV-2 detection. However, further optimization may be required.

52°C	5 min
95°C	10 sec
45 cycles:	
95°C	5 sec
60°C	30 sec

2. After the reaction is complete, check the amplification curve. Confirm that the analytical parameter is appropriate and that the Ct value has been calculated.

NOTE: Please follow the instruction manual of each individual real-time qPCR machine. If the default setting analysis does not work, perform manual analysis per the instruction manual.

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This document has been reviewed and approved by the Quality Department.