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SARS Direct Saliva Protocol Takarabio V.1

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ABSTRACT

Diagnostic testing for SARS-CoV-2 by RT-qPCR has steadily increased since the COVID-19 pandemic began, resulting in the development of protocols for testing from a variety of biological matrices. Initial testing focused on nasopharyngeal and oropharyngeal swabs, which are invasive and require trained health care professionals to perform the sample collection, resulting in a bottleneck in testing. Saliva has emerged as a viable sample specimen for virus detection and can be self-collected with little instruction. Further, it has been demonstrated that virus detection is possible without the need for complex extraction protocols, including commercially available kits. However, a potential pitfall of available kits is the lack of comprehensive testing during development using contrived biological samples containing intact, heat inactivated SARS-CoV-2 virus in the appropriate matrix (e.g., saliva).

We have found the Takara Direct One-Step RT-qPCR Mix for SARS-CoV-2 kit to be a cost-effective alternative for testing the direct detection of virus in saliva when compared with magnetic bead-assisted extraction systems, with minimal modifications from the manufacturers protocol. We have found that successful detection of virus requires the addition of a proteinase K digestion step, an increase of the reverse transcriptase time, and 3-step cycle (compared with a 2-step cycle in the manufacturers documentation). These changes allow for the successful detection of SARS-CoV-2 virus with a minimal increase in Ct values for RNase P and viral N-gene transcripts when compared with magnetic bead-assisted extractions kits. Implementing these critical steps will help end-users avoid false negatives.

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Abstract

Diagnostic testing for SARS-CoV-2 by RT-qPCR has steadily increased since the COVID-19 pandemic began, resulting in the development of protocols for testing from a variety of biological matrices. Initial testing focused on nasopharyngeal and oropharyngeal swabs, which are invasive and require trained health care professionals to perform the sample collection, resulting in a bottleneck in testing. Saliva has emerged as a viable sample specimen for virus detection and can be self-collected with little instruction. Further, it has been demonstrated that virus detection is possible without the need for complex extraction protocols, including commercially available kits. However, a potential pitfall of available kits is the lack of comprehensive testing during development using contrived biological samples containing intact, heat inactivated SARS-CoV-2 virus in the appropriate matrix (e.g., saliva).

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Materials

- Zymo Proteinase K, 20 mg/mL in storage buffer, store at +4 °C for 1 month or make aliquots and freeze for longer storage (Cat D3001-2-20, 20 mg)
- Takara Direct One-Step RT-qPCR Mix for SARS-CoV-2 (Cat 638329, 200 Rxns)
- IDT SARS-CoV-2 CDC qPCR Probe Assay Kit (Cat 10006713, 500 Rxns)
- IDT Plasmid Control, 2019-nCoV_N_Positive Control (Cat 10006625)
- IDT Plasmid Control, Hs_RPP30 Positive Control (Cat 10006626)
- SARS-CoV-2 External Control Kit (ATCC MP-32)
- Pooled human saliva (Lee Biosolutions, Cat 991-05-P-250) or self-collected saliva with IRB approval

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Protocol

A. Extraction of RNA from saliva samples. [Note: ensure all supplies and bench space are treated with RNase Zap to inhibit RNase activity.]

1. Heat in-activate saliva samples at 65 °C for 30 minutes in a water bath.
2. In a biosafety cabinet, aliquot 4 µL of proteinase K to each well of a 96-well plate.
3. Add 100 µL of heat inactivated saliva samples to 96-well plate, ensure a negative and positive extraction control are included for each preparation.
4. Negative extraction control (NEC) = naïve saliva.
5. Positive extraction control (PEC) = naïve saliva spiked with SARS-CoV-2 65 °C heat inactivated virus prepared in a BSL3.
6. Cover plate with plate seal and mix sample, briefly spin (1000 rpm for 1 minute) to ensure sample is at the bottom of the well.
7. Place plate in a thermocycler and incubate at 37 °C for 5 minutes.
8. Remove plate from thermocycler, discard plate seal, then add 15 µL Sample Preparation Solution.
9. Cover plate with a new plate seal, mix gently. Place plate in thermocycler with program set to incubate at 95 °C for minutes, followed by incubation at +4 °C for infinity.

A	B
Reagent	Vol (µL)
One Step PrimeScript III RT-qPCR Mix (2x)	10.0
Primer/Probe Mix	0.4
DNase/RNase Free H2O	8.6

qPCR Master Mix

B. Prepare qPCR Master Mixes

1. Set up the following master mixes for testing of human RNase P (control), and SARS-CoV-2 N1 and N2 primer/probe sets (as above).
2. Suggested plate layout:

- Columns 1, 4, 7 and 10 contain RNase P master mix
- Columns 2, 5, 8 and 11 contain N1 master mix
- Columns 3, 6, 9 and 12 contain N2 master mix

C. Plating

1. Aliquot 19 µL of each master mix for each sample, including NEC, PEC, no template controls (NTC) and positive amplification controls (PAC).If more than one plate is required to run all samples, prepare one at a time, keeping samples at +4 °C until they are analyzed.
2. Using a multi-channel (8 channel) pipette, transfer 1 µL of each sample to wells containing RNase P, N1 and N2 master mixes.
3. For example, from extraction plate, transfer all samples in column 1 to columns 1, 2 and 3 of the qPCR plate. From the extraction plate, transfer all samples in column 2 to columns 4, 5 and 6 of the qPCR plate.
4. Ensure that each plate contains the following controls:

- Negative extraction control (transfer to wells E10, E11, and E12 of qPCR plate)
- Positive extraction control (transfer to wells F10, F11, and F12 of qPCR plate)
- No template controls (add DNase/RNase H_2O to wells G10, G11, and G12 of qPCR plate)
- Positive amplification controls (add RNase P plasmid to well H10, add SARS-CoV-2 N-gene plasmid to wells H11 and H12 of qPCR plate)

Example plate layout shown below:

D. Run the qPCR

1. Cover plate with plate seal, mix briefly, then centrifuge at 1000 rpm for 1 minute to ensure all liquid is at the bottom of the well. Keep plate on ice when transporting.

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2. Turn on qPCR instrument and allow it to go through startup protocols. Once initialized, open qPCR protocol or make a new one, with the following conditions:

A	B	C	D	E
	Cycles	Temperature	Time	
Stage 1	-	50 °C	20 mins	RT step
Stage 2	-	94 °C	2 mins	Denaturation
Stage 3	45	94 °C	15 secs	Denaturation
		55 °C	30 secs	Anneal
		68 °C	10 secs	Extension
		-	-	Plate read

E. Data analyses

1. Analyze data to ensure that all samples produced a Cq value of <40 for RNase P, including NEC and PEC. Ensure no signal for N1 or N2 is observed for NEC, but that signals are observed for PEC. Finally, ensure that no signal is observed for NTC, and signals are observed for PAC.
2. If NEC, PEC, NTC, or PAC do not fall in the above parameters, plate results are not valid, and samples need to be re-run.
3. Any test samples that do not give signal for RNase P are not valid and should be re-extracted/re-run.

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