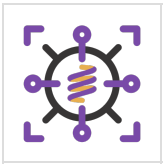

Version 2

Jul 01, 2020

SalivaDirect: RNA extraction-free SARS-CoV-2 diagnostics V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bh6jj9cn

Coronavirus Method Development Community

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ABSTRACT

The first section of this protocol details recommendations for collecting saliva for SARS-CoV-2 detection, either by self-collection or healthcare worker-assisted collection.
The second section details an extraction-free method for preparation of saliva samples for SARS-CoV-2 RNA detection. This involves the addition of proteinase K to reduce saliva viscosity, followed by a heating step to inactivate the proteinase K before RT-qPCR testing.
The third section describes a dualplex RT-qPCR assay for high-throughput SARS-CoV-2 diagnostics. The CDC_N1 (N1) primer/probe set targets the SARS-CoV-2 nucleocapsid gene, and is shown to be sensitive and specific. The human RNase P (RP) primer/probe set is included as an internal sample control - detection of RP at PCR cycle threshold (CT) <35 indicates that saliva of sufficient quantity and quality were tested. The combination of both N1 and RP in the dual-plex assay ensures that any negative SARS-CoV-2 PCR result occurred due to genuine absence of virus, and not because of invalid saliva collection or preparation.

Version 2 includes:

- Optimized thermocycler conditions
- Locally validated alternative options for Proteinase K, RT-qPCR master mix, and thermocyclers
- Use of 8-strip tubes for sample processing step, due to contamination issues in 96-well plates.

EXTERNAL LINK

https://covidtrackerct.com/about-salivadirect/

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PROTOCOL CITATION

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KEYWORDS

SARS-CoV-2, COVID-19, saliva, extraction-free PCR, COVID-19 diagnostics

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GUIDELINES

This protocol is still under development.

MATERIALS TEXT

Sample collection

- Wide-mouth collection tube labelled with unique subject identity number and collection data.
  For example: 5 ml screw cap tube, 25 ml conical tube, 50 mL Falcon tube.

> 15 mL conical tubes are not recommended due to their long, narrow opening. This design increases the risk of contaminating pipettes when aliquoting the collected sample.
> Urine cups can also be used for sample collection; however it makes sample vortexing during the processing step difficult and thus we do not recommend using them.

- Personal protective equipment (PPE) for **sample collector**. (at minimum, gloves and face mask)

Extraction-free sample processing

- Thermocycler or real-time PCR (qPCR) instrument
- Vortex mixer
- Plate centrifuge or spinner
- Pipette (P20 and P200)
- Pipette tips (20 µL and 200 µL)
- 8-strip PCR tubes (200 µL capacity)
- Proteinase K (volumes for different concentrations given in text)
- Saliva samples (see “sample collection”)

SARS-CoV-2 RNA Detection by Dual-Plex RT-qPCR

- Real-time PCR (qPCR) instrument. Validation data are currently available for:
  Bio-Rad CFX96
  ABI 7500 Fast
  ABI 7500 Fast Dx
- RT-qPCR kit. Validation data are currently available for:
  NEB Luna Universal Probe One-Step RT-qPCR kit
  Reliance One-Step Multiplex RT-qPCR Supermix
  TaqPath™ 1-Step RT-qPCR Master Mix
- 96 well optical PCR plate and adhesive film
  For Bio-Rad CFX96: plates and seals
  For ABI 7500 Fast (Dx): plates and seals
- Plate centrifuge or spinner
- 1.5 mL tube centrifuge
- Vortex
- Pipettes (P10, P20, P200, and P1000 - optional P10 multichannel)
- Pipette tips (10 µL, 20 µL, 200 µL, and 1000 µL)
- PCR cooler
- Adhesive film applicator
- 1.5 mL tubes (clear and LightSafe)
- Nuclease-free water

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- 70% ethanol, for cleaning
- Twist synthetic SARS-CoV-2 RNA controls at 100 copies/uL
- Extraction-free saliva samples (see “extraction-free sample processing”)
- Primers and probes, as follows.

Target	Primer/probe	Sequence
CDC-N1	2019-nCoV_N1-F	GACCCCAAAATCAG CGAAAT
	2019-nCoV_N1-R	TCTGGTTACTGCCA GTTGAATCTG
	2019-nCoV_N1-P	FAM-ACCCCGCATTACGT TTGGTGGACC-IBFQ
Human RNase P	RP-F	AGATTTGGACCTGC GAGCG
	RP-R	GAGCGGCTGTCTCC ACAAGT
	RP-P	Cy5-TTCTGACCTGAAGG CTCTGCGCG-IBRQ

Stocks can be kept at 100 uM, and will be diluted to working concentrations of 20 uM at the beginning of the RT-qPCR protocol section.

SAFETY WARNINGS

Processing of any sample type which could potentially be positive for SARS-CoV-2 should be conducted in BSL2+ settings. Before starting work with these samples, please contact your local EHS (environment, health and safety) or biosafety office for proper guidance on how to work with these samples in your laboratory.

BEFORE STARTING

This protocol is still under development. Results of ongoing validation studies are available here.

Version 2 includes:

- Optimized thermocycler conditions
- Locally validated alternative options for Proteinase K, RT-qPCR master mix, and thermocyclers
- Use of 8-strip tubes for sample processing step, due to contamination issues in 96-well plates.

While collecting saliva is significantly easier than swabs, saliva samples can be difficult to work with. It is important to follow the sample collection guidelines to ensure that saliva, not sputum, is being collected.

Sample collection

1 Saliva can either be collected independently by the individual providing the sample ('Self-collection') or with the assistance of a healthcare worker ('Assisted'). Alternatively, a saliva collection aid can be used.
Step 1 includes a Step case.
Self-collection
Assisted collection

step case

Self-collection

For independent collection of sample by the individual providing the sample.

2 Before collection, clean hands using alcohol-based sanitizer or soap and water (no fragrances).

3 Ensure all collection materials are labelled with the correct identifying information.

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3.1 While preparing collection materials, begin pooling saliva in your mouth. Saliva production can be stimulated by thinking about food (favorite foods, upcoming meals, etc.) or about the saliva collection itself. Thinking about this while preparing to collect the sample can help ensure enough can be pooled in the mouth quickly and easily.

This protocol is intended for the collection of the **normal** saliva that naturally pools into the mouth. **No coughing or sniffing prior to sample collection is required.** Ideally, water should be avoided 10 minutes prior to collection. Other drinks, food, and nasal sprays should be avoided for half an hour before sample collection.

4 Remove the lid of the collection container and gently expel saliva into the collection tube until at least 0.5 mL has been collected.

The total volume measured is to exclude any bubbles.

4.1 Once at least 0.5 mL has been collected, securely replace the lid of the collection container.

5 Following collection, clean hands using alcohol-based sanitizer or soap and water, and sterilize the collection tube with 70% ethanol or a disinfecting wipe.

6 Register the sample collection (including date and time), and place the sample in a secondary container or biohazard bag with a biohazard label.

7 Transfer the sample at room temperature to the laboratory for sample processing. The virus RNA in saliva remains stable at room temperature for 3-5 days.

8 Store samples at 2-8°C until sample transport or processing (up to 72 hours). If longer-term storage is required, samples can be kept at -20°C for 2-4 weeks, or at -80°C for longer term storage.

Extraction-free sample processing

9 Add Proteinase K (see table for volume per sample) to designated 8-strip PCR tubes (200 µL capacity).

Vendor	Concentration	Volume
ThermoFisher Scientific MagMAX™ Viral/Pathogen Proteinase K	50 mg/mL	2.5 µL
New England Biolabs Proteinase K, Molecular Biology Grade	20 mg/mL	6.25 µL
AmericanBio Proteinase K	Lyophilized (add 50 mg per 1 mL of nuclease-free water)	2.5 µL

Results of validation studies for different Proteinase K options are available here.
Reagent order info: ThermoFisher, NEB, AmericanBio

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This work should be completed under BSL-2 conditions, and samples potentially containing SARS-CoV-2 should only be handled in a biosafety cabinet. Please seek guidance from your local biosafety office on specific recommendations for working with samples which could contain SARS-CoV-2.

10 Vortex each saliva sample until homogeneous, and immediately transfer 50 µL saliva to each 8-strip PCR tube containing proteinase K.

10.1 Close the 8-strip tube lids tightly.

10.2 Place the 8-strip tubes in a rack and vortex for 1 minute at 3000-5000 RPM.

10.3 Briefly spin down the rack/tubes using a plate spinner or 8-strip tube microcentrifuge.

If no plate centrifuge or spinner is available, the plate can be gently tapped to get the samples at the bottom of each well.

11 Inactivate the proteinase K by heating samples for 5 minutes at 95°C on a PCR instrument or equivalent thermocycler.

11.1 Briefly spin down the tubes using a plate spinner or 8-strip tube microcentrifuge.

12 Store samples at -80°C or proceed immediately to RT-qPCR testing.

RT-qPCR

13 Prepare 20 uM working stocks of the primers and probes (sequences provided in Materials) by adding 20 µL of 100 uM stock to 80 µL nuclease-free water.

Briefly vortex and centrifuge reagents before use.
Probes are photosensitive and should be stored in the dark.

13.1 Use the 20 uM working stocks to prepare dualplex primer-probe-water mix containing the following:

Component	Volume (1 reaction)	Volume (100 reactions)

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2019-nCoV_N1-F (400 nM/reaction)	0.4 µL	40 µL
2019-nCoV_N1-R (400 nM/reaction)	0.4 µL	40 µL
2019-nCoV_N1-P (200 nM/reaction)	0.2 µL	20 µL
RP-F (150 nM/reaction)	0.15 µL	15 µL
RP-R (150 nM/reaction)	0.15 µL	15 µL
RP-P (200 nM/reaction)	0.2 µL	20 µL
Nuclease-free water	2.5 µL	250 µL

A larger volume of primer-probe-water mix can be prepared in advance, aliquoted in LightSafe microcentrifuge tubes, and stored at -20°C.

14 On ice, prepare a master mix containing the following (account for 10% extra lost during pipetting).

Briefly vortex and centrifuge reagents before use.

Component	NEB Luna	Bio-Rad Reliance	Thermo TaqPath
Master mix	10 µL	5 µL	5 µL
RT	1 µL	-	-
Primer-probe-water mix (see above)	4 µL	4 µL	4 µL
Nuclease-free water	-	6 µL	6 µL

5 µL Proteinase K-treated samples, standards, or controls will be added to each well individually in step 15, for a total 20 µL reaction.
Kit order info:
- NEB Luna Universal Probe One-Step RT-qPCR kit
- Reliance One-Step Multiplex RT-qPCR Supermix
- TaqPath™ 1-Step RT-qPCR Master Mix

Validation data, including demonstrated compatibility between kits and thermocyclers, are available here.

14.1 Place the 96-well PCR plate on the PCR plate cooler, and add 15 µL of mastermix to each designated well.

15 Bring the processed samples and the PCR mastermix plate to a biosafety cabinet.

Most institutions will require samples potentially containing full-length SARS-CoV-2 RNA to be handled in a biosafety cabinet. Please seek guidance from your local biosafety office on specific recommendations for working with samples which could contain full-length SARS-CoV-2 RNA.

15.1 Add 5 µL of extraction-free saliva sample to each well of the mastermix plate. Mix by pipetting, taking care to avoid introducing bubbles.

Extraction-free saliva samples can be added using a multichannel pipette for high-throughput testing.

15.2 Add 5 µL of positive control (Twist synthetic SARS-CoV-2 RNA controls at 100 copies/uL) and no-template control (NTC - water) to each designated PCR well. Mix by pipetting, taking care to avoid

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introducing bubbles.

15.3 Seal with a transparent plastic qPCR seal. Centrifuge briefly to remove bubbles, if present.

16 Load the plate into the qPCR machine, and run the following thermocycler conditions:

Step	Temperature	Time
1	52°C	10 min
2	95°C	2 min
3	95°C	10 sec
4	55°C	30 sec
5	Read plate	
Repeat steps 3-5 for 44 cycles.		

Real-time PCR (qPCR) instruments currently locally validated: Bio-Rad CFX96, ABI 7500 Fast, and ABI 7500 Fast Dx. Validation data, including demonstrated compatibility between kits and thermocyclers, are available here.

17 Report results per the following criteria:

Output	Significance	RP CT	N1 CT
0	Negative	<35	>40
1	Invalid	>35	>40
2	Positive	any value	<40

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