Sep 01, 2020 Version 5

SalivaDirect™: RNA extraction-free SARS-CoV-2 diagnostics V.5

JMIR Public Health and Surveillance

DOI
dx.doi.org/10.17504/protocols.io.bkjgkujw

Chantal Vogels[1], Doug E. Brackney[2,1], Chaney C Kalinich[1], Isabel M Ott[1], Nathan D Grubaugh[1], Anne Wyllie[1]

[1]Department of Epidemiology of Microbial Diseases, Yale School of Public Health;
[2]Department of Environmental Sciences The Connecticut Agricultural Experiment Station

Coronavirus Method De... ITTD

Nathan D Grubaugh
Department of Epidemiology of Microbial Diseases, Yale Schoo...

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.bkjgkujw

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

Protocol Citation: Chantal Vogels, Doug E. Brackney, Chaney C Kalinich, Isabel M Ott, Nathan D Grubaugh, Anne Wyllie 2020. SalivaDirect™: RNA extraction-free SARS-CoV-2 diagnostics. **protocols.io https://dx.doi.org/10.17504/protocols.io.bkjgkujw**

Manuscript citation:
Vogels CB, Watkins AE, Harden CA, Brackney DE, Shafer J, Wang J, Caraballo C, Kalinich CC, Ott IM, Fauver JR, Kudo E, Lu P, Venkataraman A, Tokuyama M, Moore AJ, Muenker MC, Casanovas-Massana A, Fournier J, Bermejo S, Campbell M, Datta R, Nelson A, , Cruz CSD, Ko AI, Iwasaki A, Krumholz HM, Matheus J, Hui P, Liu C, Farhadian SF, Sikka R, Wyllie AL, Grubaugh ND, SalivaDirect: A Simplified and Flexible Platform to Enhance SARS-CoV-2 Testing Capacity. Med (New York, N.y.) doi: 10.1016/j.medj.2020.12.010

Protocol status: Working
We use this protocol and it's working

Created: August 31, 2020

Last Modified: February 10, 2023

Protocol Integer ID: 41288

Keywords: SARS-CoV-2, COVID-19, saliva, extraction-free PCR, COVID-19 diagnostics, specialized equipment for rna extraction, qpcr for sar, processed saliva, rna extraction, saliva collection tube, qpcr method for sar, qpcr test, saliva, qpcr testing, probes for rt, other assay, dualplex assay, additional rp probe, free sar, rna, extraction, supply chain bottlenecks as some other assay, us cdc, qpcr, qpcr method

Disclaimer

Abstract

SalivaDirect™ is an RNA-extraction free, dual-plexed RT-qPCR method for SARS-CoV-2 detection. It can be broadly implemented as it (1) does not require saliva collection tubes containing preservatives, (2) does not require specialized equipment for RNA extraction, and (3) is validated for use with products from multiple vendors. Thus, the simplicity and flexibility of SalivaDirect™ means that it is not as affected by supply chain bottlenecks as some other assays. Our method is RNA-extraction free which enables testing of low volume and minimally processed saliva in dual-plexed RT-qPCR for SARS-CoV-2 detection. Saliva will be treated with proteinase K followed by a heat inactivation step, and is then directly used as input in the dual-plexed RT-qPCR test. Our aim was not to design new primers and probes for RT-qPCR testing, but rather to use validated primer and probe sets (N1 and RP) developed by the US CDC. The human Ribonuclease P (RP) probe was modified with a different fluorophore so that the primer/probe set could be combined in a dualplex assay, reducing the number of tests to 1 assay with 2 sets.

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.

Version 3 has been updated to remove steps for sample self-collection.

Version 4 has updated Ct thresholds for the ABI 7500 Fast Dx.

Version 5 has an updated description for use, additional RP probe with ATTO647 fluorophore, and a detailed table with catalog numbers.

Materials

SalivaDirect™ ordering shortlist

	Vendor	Item	Catalog number	Quantity	# Reactions
	Order one of the following Proteinases K				
	ThermoFisher Scientific	MagMAX Viral/Pathogen Proteinase K	A42363	10 mL	4.000 reactions
	New England Biolabs	Proteinase K, Molecular Biology Grade	P8107S	2 mL	320 reactions
	AmericanBio	Proteinase K	AB00925	100 mg	800 reactions
	Order one of the following RT-qPCR kits				
	New England Biolabs	Luna Universal Probe One-Step RT-qPCR Kit	E3006S	2 mL	200 reactions
			E3006L	5 mL	500 reactions
			E3006X	10 mL	1.000 reactions
			E3006E	25 mL	2.500 reactions
	Bio-Rad	Reliance One-Step Multiplex RT-qPCR Supermix	12010176	1 mL	200 reactions
			12010220	5 mL	1.000 reactions
			12010221	10 mL	2.000

					reactions
	ThermoFisher Scientific	TaqPath 1-Step RT-qPCR Master Mix, GC	A15299	5 mL	1.000 reactions
			A15300	10 mL	2.000 reactions
	Order the following primers and probes				
	Integrated DNA Technologies	nCOV_N1 Forward Primer Aliquot	10006821	50 nmol	6.250 reactions
			10006830	100 nmol	12.500 reactions
		nCOV_N1 Reverse Primer Aliquot	10006822	50 nmol	6.250 reactions
			10006831	100 nmol	12.500 reactions
		nCOV_N1 Probe Aliquot	10006823	25 nmol	6.250 reactions
			10006832	50 nmol	12.500 reactions
		RNase P Forward Primer Aliquot	10006827	50 nmol	16.600 reactions
			10006836	100 nmol	33.300 reactions
		RNase P Reverse Primer Aliquot	10006828	50 nmol	16.600 reactions

			10006837	100 nmol	33.300 reactions
		RNase P Probe	Custom probe (Cy5)	25 nmol	6.250 reactions
			Custom probe (Cy5)	50 nmol	12.500 reactions
			10007061 (ATTO647)	25 nmol	6.250 reactions
			10007062 (ATTO647)	50 nmol	12.500 reactions
	Order one of the following nuclease-free waters				
	Integrated DNA Technologies	Nuclease-free water	11-04-02-01	20 mL	
			11-05-01-14	300 mL	
			11-05-01-04	1 L	
	New England Biolabs	Nuclease-free water	B1500S	25 mL	
			B1500L	100 mL	
	Order the following positive control				
	Twist Bioscience	Synthetic SARS-CoV-2 RNA Control 2	102024	100 µL	

Sample collection

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

Note

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, they are difficult to vortex during the processing step, and thus we do not recommend 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)
  ThermoFisher
  NEB
  AmericanBio
- 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
- 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	GACCCCAAAATCAGCGAAAT
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG
		2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-IBFQ
	Human RNase P	RP-F	AGATTTGGACCTGCGAGCG
		RP-R	GAGCGGCTGTCTCCACAAGT

		RP-P*	Cy5-TTC TGA CCT GAA GGC TCT GCG CG-IBRQ
			or
			ATTO647-TTC TGA CCT GAA GGC TCT GCG CG-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.

* Use either the Cy5 or ATTO647 fluorophore for the RP probe.

Troubleshooting

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 start

The FDA issued Emergency Use Authorization (EUA) for SalivaDirect™ as a Laboratory Developed Test (LDT) on 15 August 2020. This version of the SalivaDirect™ protocol is for **RESEARCH ONLY**. Prior authorization and the official Instructions For Use are needed to use SalivaDirect™ as an FDA-authorized LDT. See our **website** for authorization instructions.

Latest information on SalivaDirect™ is available here.

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 should be collected with the assistance of a healthcare worker or technician.

2 Before collection, clean hands using alcohol-based sanitizer or soap and water (no fragrances) and don appropriate PPE (at minimum, gloves and a mask).

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

3.1 While preparing collection materials, direct the sample provider to begin pooling saliva in their mouth. Saliva production can be stimulated by thinking about food (favorite foods, upcoming meals, etc.) or about the saliva collection itself.

Note

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 direct the sample provider to gently expel saliva into the container until at least 0.5 mL has been collected.

Note

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.

4.2 Sterilize the container surface with 70% ethanol or a disinfecting wipe, and place the sample in a secondary container or an appropriately labeled biohazard bag.

4.3 Dispose of gloves, and register the sample collection (including date and time).

5 Transfer the sample to the laboratory for sample processing. If the time between sample collection and the initial processing steps (aliquoting) is likely to exceed 6 hours, samples can be stored at 2-8°C for up to 7 days, or -80°C for long-term storage, then later thawed on ice for testing.

Extraction-free sample processing

6 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 experiments for different Proteinase K options are available **here**.
Reagent order info: **ThermoFisher**, **NEB**, **AmericanBio**

Safety information

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.

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

7.1 Close the 8-strip tube lids tightly.

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

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

Note

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

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

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

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

RT-qPCR

10 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.

Note

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

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

	Component	Volume (1 reaction)	Volume (100 reactions)
	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.

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

Note

Briefly vortex and centrifuge reagents before use.

	Component	**NEB Luna**	**Bio-Rad Reliance**	**Thermo Taq Path**
	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.

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

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

Safety information

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.

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

Note

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

RT-qPCR

12.2 Add 5 µL of positive control (Twist synthetic SARS-CoV-2 RNA controls at 100 copies/uL) and no-template control (NTC - water) to designated PCR wells for the controls (1 NTC, and 2 positive controls per plate). Mix by pipetting, taking care to avoid introducing bubbles.

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

13 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 (FAM & Cy5 channels)	
	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**.

14 Report results per the following criteria:

	Bio-Rad CFX96 & ABI 7500 Fast			
	Output	**Significance**	**RP CT**	**N1 CT**
	0	Negative	<35	≥40
	1	Invalid	≥35	≥40
	2	Positive	any value	<40
	ABI 7500 Fast Dx			
	Output	**Significance**	**RP CT**	**N1 CT**
	0	Negative	<35	≥40
	1	Invalid	≥35	≥40
	2	Positive	any value	<37