Version 2

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Direct-lysis Saliva SARS-CoV-2 Detection V.2

Suzie Alarcon[1], Hannah Dose[1], Arturo Hernandez[1], Kyle Tanguay[1], Stephen Wilson[1]

[1]La Jolla Institute for Immunology (LJI)

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

Suzie Alarcon

ABSTRACT

A One-Step RT-qPCR assay based on the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel has been developed which can effectively detect SARS-CoV-2 particles from saliva samples which undergo both heat inactivation and direct lysis with detergent, eliminating the need to extract RNA

Assay has been automated on the Beckman BiomekFXpliquid handler

Heat inactivation of virus in saliva does not impact assay results and reduces overall risk

6ul Low Volume reaction allows for high-throughput screening at reduced cost

DOI

dx.doi.org/10.17504/protocols.io.bqwpmxdn

PROTOCOL CITATION

Suzie Alarcon, Hannah Dose, Arturo Hernandez, Kyle Tanguay, Stephen Wilson 2020. Direct-lysis Saliva SARS-CoV-2 Detection . **protocols.io**
https://dx.doi.org/10.17504/protocols.io.bqwpmxdn
Version created by Suzie Alarcon

WHAT'S NEW

Added alternate enzyme Added primer/probe set (outside nucleocapsid) to mitigate false positives from plasmid positive control (nucleocapsid gene) for confirmation of positives/inconclusives.

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45743

GUIDELINES

Ensure proper Biosafety standards are followed

1. Prepare for work in BSL2+ space
2. PPE required:
3. Cloth lab coat
4. Disposable lab coat
5. Disposable sleevelets
6. N95 mask
7. Goggles or glasses
8. Face shield
9. Two pairs of gloves
10. Put thumb of first pair of gloves through the end of the sleeve of the disposable coat
11. Put second pair of gloves over the sleevelet, tucking the sleevelet into the outer pair of gloves
12. Decontaminate all non-porous equipment with quaternary ammonium or similar according to manufacturer's instructions. Follow with 70% Ethanol.
13. Aspirate all saliva samples into bleach with a final concentration (taking aspirated volume into account) of 10%

MATERIALS TEXT

MATERIALS

☒ Triton X-100 **Bio-rad Laboratories Catalog #1610407**

☒ 1L TE Buffer [1X], pH 8.0, Low EDTA (Tris-EDTA; 10mM Tris base, 0.1mM EDTA) **G-Biosciences Catalog #786-152**

☒ UltraPlex 1-Step ToughMix (4X) **Quantabio Catalog #95166-01K**

☒ 2019-nCoV Charite/Berlin RdRP_SARSr_P2 Probe and Primers **IDT Catalog #10006886; 10006860; 10006881**

☒ TaqPath™ 1-Step RT-qPCR Master Mix, CG **Thermo Fisher Catalog #A15299**

☒ 2019-nCoV CDC RUO Primers and Probes **IDT Catalog #10006713**

☒ 2019-nCoV CDC RUO Plasmid Controls **IDT Catalog #10006625**

BEFORE STARTING

1. Before opening biohazard specimen bags, inspect each tube carefully through the bag to check for any signs of stress, cracks, or leakage
2. If any of this is evident, reject samples. Scan barcode into results tab and assign rejection code
3. Dispose of unopened bag into Biohazard waste

Ensure barcodes on tubes from same bag match

Saliva Collection

1 Prepare saliva collection packets

Include:

- Large RNase, DNase-free tube into which participant can deposit saliva
- 2ml Safe-Lock Eppendorf tube containing 1.5 ml TE pH 8
- 2ml screw-cap gasketed cryo-safe tube
- Transfer pipette (plastic bulb type)
- Barcodes for tubes, or human readable labels
- Alcohol wipes
- Biohazard specimen transport bag

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2 Instruct participant to collect saliva and create Working Dilution

Instructions to participant:

1. Ideally, complete the saliva collection before your first meal and within one hour of drop-off. Saliva collection may be aided by being well-hydrated.
2. If you have eaten, brushed your teeth, smoked, or chewed gum, please wait at least one hour before collecting your saliva. Refrain from using mouth wash for 12 hours before collecting your saliva.
3. Wash hands thoroughly.
4. Open clear bag and retrieve the packet containing the large collection tube.
5. Open the large collection tube and passively drool into the large collection tube until 2 mls of saliva have been collected. This is best done by allowing saliva to pool in the mouth, then tip your head forward and allow saliva to enter the tube.
6. Passive saliva collection, as opposed to stimulated and expectorated saliva is preferred and samples collected passively may contribute to better detection of CoV particles. Any samples that appear viscous or containing mucus will be rejected and will not be tested.
7. Remove some of the saliva with the transfer pipette and place into the small collection tube (MUST BE ALMOST FULL) AND into the working tube (MUST BE FULL) with the saliva you just collected
8. Close tubes and **wipe both with the alcohol pads - thank you for helping to protect the researchers running this screening!**
9. Make sure to close both tubes tightly! Any tubes that come partially opened, leaking, or otherwise not tightly sealed will be rejected and not tested.
10. Affix barcode to tubes and place back into biohazard bag. Dispose of large tube and transfer pipette
11. Close bag
12. Collect bag from participant and place on ice.

3 Prepare Saliva Samples

1. Heat-inactivate samples at 98C x 5 minutes or 65C x 30 minutes in a dry heat block with appropriate adapters.
2. Allow tubes to cool
3. Centrifuge 25% working dilution for 20 minutes at 300g
4. Lyse 25% working dilution with 30% Triton-X:

- Add 5 parts 25% saliva dilution to 1 part 30% Triton-X (example, 50ul saliva to 10ul 30% Triton-X)
- Mix by slowly pipetting. Triton is a foaming detergent, take care not to foam.
- Incubate for 5 minutes at room temperature.
- Add 5 parts 25% saliva dilution to 1 part 30% Triton-X (example, 50ul saliva to 10ul 30% Triton-X)
- Mix by slowly pipetting. Triton is a foaming detergent, take care not to foam.
- Incubate for 5 minutes at room temperature

4 Prepare Plasmid dilution.

Plasmid stock is 200,000 copies/ul. Prepare 200 copies/ul dilution and treat as sample. (Example 50 ul of 200 copies/ul is lysed with 10 ul 30% Triton X, as with samples). Aliquot in separate clean space to mitigate chance of false positives.

Prepare Master Mix

5 Primer and Probe Preparation

If received lab-ready at 100uM, skip to dilution and aliquoting**:**

1. Upon receipt, store dried primers and probes at 2-8°C.
2. Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
3. Using aseptic technique, suspend dried reagents in nuclease-free water to 100uM and allow to rehydrate for 15 min at room temperature in the dark.
4. Mix gently and aliquot primers/probe in 300 µL volumes into pre-labeled tubes. Store a single, working aliquot of primers/probes at 2-8C in the dark. Store remaining aliquots at ≤ - 20C in a non-frost-free freezer. Do not refreeze thawed aliquots (stable for up to 4 months at 2-80C).
5. Prepare working dilutions as follows:
6. Primers: 20uM
7. Probes: 5uM
8. Store aliquots of working dilutions at -20C in the dark in black microcentrifuge tubes.

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6 Prepare Master Mix (MM), working on ice

A	B	C	D	E
Component	**ul x1**	**[Stock] uM**	**[Final] nM**	**Reporter Dye**
N1 Fw	0.15	20	500	
N1 Rv	0.15	20	500	
N1 Probe	0.15	5	125	FAM
N2 Fw	0.15	20	500	
N2 Rv	0.15	20	500	
N2 Probe	0.15	5	125	SUN
RP Fw	0.15	20	500	
RP Rv	0.15	20	500	
RP Probe	0.15	5	125	ATTO 647
TaqPath or UltraPlex Tough Mix	1.5	4X	1X	
H2O	0.15			
Total	**6**			

7 Distribute 3 or 6.5ul (see below) MM to clean 384 well optical PCR plate (internally validate sensitivity of white vs clear plate)
- Mix 3ul lysed saliva sample with 3ul MM in plate
- OR
- If adding technical replicates, mix 6.5ul lysed saiva sample with 6.5ul MMand dispense 6ul from well containing 13ul of lysed saliva+MM to empty well

8 Visually inspect plate to ensure all expected wells contain reagent.
Seal plate with optically clear film and centrifuge for 1 minute at 1200 RPM

qPCR and Data Export

9 Run qPCR according to software and manufacturer instructions. Ensure all probe fluorophores are selected.
Step 4 can be 3-5 seconds

A	B	C	D
Step	Deg, C	Time	Purpose
1	25	2 min	UNG Incubation
2	50	15 min	T Incubation
3	95	2 min	Enzyme Activation
4	95	5 sec	Amplification
5	56	30 sec	Amplification/ Read
7	Go to step 4 45 times		

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10 Export data. If cycler has background dye like ROX, account for that in machine's settings.

Evaluate and Report

11
1. Evaluate Data: Where "+" is Ct <40 and "-" is Ct >40 or not a number (NaN), Expected batch control = "+" for both N1 and N2 for plasmid and "-" for NTC
2. In the case of Positive or Inconclusive, repeat sample with fresh 25% dilution from stock tube, and with a 12.5% dilution using MM as listed above. Add an additional reaction with RdRP_SARSr_P2 Probe and primers at following concentrations to rule out plasmid contamination/false positive. Multiplex with RNAseP primer and probe set as internal control.

A	B	C
Component	[Final] nM	Reporter Dye
RdRP_SARSr_F2 Forward Primer,	600	-
RdRP_SARSr_R1 Reverse Primer	800	-
RdRP_SARSr_P2 Probe	100	FAM (can order with SUN)

- If any probe produces artifacts in the plasmid control, reduce primer and probe concentrations by 15%. Validate on positive samples.
- Evaluate NTCs manually to inspect for sigmoidal curve - any sigmoidal curve in the NTC wells indicate possible contamination and plate should be repeated.

N1	N2	RP	Plasmid Positive Control	NTC	Test Type	Result	Next Step
+	+	+	+	-	Initial	Positive	Re-Test
+	+	+	+	-	Confirmatory	Positive	Release Data
+	-	+	+	-	Initial	Positive	Re-Test
+	+	+	+	-	Confirmatory	Positive	Release Data
-	+	+	+	-	Initial	Positive	Re-Test
+	+	+	+	-	Confirmatory	Positive	Release Data
-	-	+	+	-	Confirmatory	Inconclusive	Re-collect sample
-	-	+	+	-	Initial	Negative	Release Data
-	-	-	+	-	Initial	Sample Fail	Re-collect sample
any	any	any	If any batch control returns unexpected result, assay fail		Any	Assay Fail	Re-run plate

Decontamination

12 **Complete thorough decontamination of all working areas as designated by local, federal, and or institutional guidelines.**

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1. Submerge any racks or holders in quaternary ammonium at appropriate concentration for time recommended by manufacturer. Do not use quaternary ammonium near NGS equipment
2. Follow with 70 Ethanol decontamination
3. Any non-submergable equipment should be sprayed with quaternary ammonium and allowed a 20 minute contact time, followed by 70% EtOH
4. All residual saliva samples, if not being stored (-80C storage recommended), should be aspirated into a bleach solution with a final concentration of 10%.
5. Dispose of all consumables and PPE according to local, federal, and or institutional guidelines.

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