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High-throughput Sars-CoV-2 detection from self-collected saline gargle samples

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2 *Works for me* dx.doi.org/10.17504/protocols.io.bsr3nd8n

Coronavirus Method Development Community

Karl Frontzek

SUBMIT TO PLOS ONE

ABSTRACT

The corona virus disease 19 (COVID19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a worldwide health crisis. A rigid test, trace and isolate strategy is obstructed by a global shortage in lab supplies such as RNA extraction kits, RT-PCR reagents or other consumables. We opted to develop a cheap and easily scalable molecular Sars-CoV-2 test.

Nasopharanygeal and/or throat swabs and subsequent quantitative RT-PCR (RT-qPCR) are the current gold standard for detection of SARS-CoV-2. Swabs need to be taken, however, by skilled personell and exposure to infected individuals poses a serious health risk to health care professionals.

Here, we describe a protocol for high-throughput Sars-CoV-2 detection from self-collected saline gargle samples, a rapid (collection to result < 3 hours), scalable (up to several 1'000 samples per day) and sensitive (10 copies Sars-CoV-2 per reaction) molecular test that based on self-collected saliva. Unstimulated saliva after wake-up or fasting for 1 hour is collected by gargling with common saline (0.9% NaCl) solution. Saliva is stored in plastic buckets without additives and is stable even after 12 hours at 4°C plus at least one freeze-thaw cycle.

When compared to the best available method for Sars-CoV-2 detection, our protocol reaches a positive percent agreement of 95 % and a negative percent agreement of 98.9 % at a cost of ca. 5 $ per test. Rapidly scalable, molecular detection of Sars-CoV-2 from saline/saliva mixtures using alternative reagents could help reduce disease burden when implemented in a test, trace and isolate regimen.

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COVID, Chelex, Saliva Sample preparation, RT-PCR, Saliva sample collection, SARS-CoV-2

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GUIDELINES

Sample collection for validation of samples was performed in accordance with Swiss legislation in the framework of the ACCURATE-BL-COVID-19 study (https://clinicaltrials.gov/ct2/show/NCT04625257).

Personal protective equipment (PPE) for sample handling is essential. Use double pair of gloves, face masks, protective glasses and lab coats.

Every sample has to be considered as infectious!

Sample collection at home (5 ml 0.9% NaCl solution, plastic collection cup):

- After wake-up, do not drink, eat, smoke or brush your teeth.
- Gargle with saline solution for 30 seconds, then collect in cup, e.g. 50 ml falcon tube. Keep at 4°C if possible.
- If collection after wake-up is not possible, fasting for 1 hour (no drinking, eating, smoking) and subsequent collection is possible. **CAUTION: do not gargle in public spaces due to generation of infectious aerosols.**
- For validation, our sampes were kept on ice for up to 12 hours and then frozen at -80°C until thawed on ice before processing.

Our test was compared with the best available method and reached a positive percent agreement of 95 % and a negative percent agreement of 98.9 %.

The use of 15ml Falcon tubes is not recommended due to the narrow opening. We observed difficulties of patients to donate saliva into the 15 ml tubes. If 50 ml falcon tubes are not feasible, any other wide-bore collection cup can be used.

MATERIALS TEXT

Order list for the RNA extraction

A	B	C
Product	**Company**	**Cat#**
Chelex® 100 Resin	BioRad	142-1253
TaqPath™ 1-Step Multiplex Master Mix (No ROX)	Thermo Fisher	A28523
8 Molar Guanidine HCl	Thermo Scientific	24115
1 Molar Tris pH 8.0	Invitrogen	AM9855G
Nuclease-Free water	Invitroegn	AM9930
Ethanol absolute	VWR	20821.321
384 well PCR Microplate, Clear	Axygen	321-29-051
SpeedBead	cytiva	65152105050250
ViiA 7 Real-Time PCR System	Thermo Fisher	4453545

Amounts have to be calculated depending on the planned sample load.

All chemicals were obtained from Sigma.

We used common laboratory equipment such as:

- Pipettes (P10, P20, P200, and P1000 - optional P10 multichannel)
- Pipette tips (10 µL, 20 µL, 200 µL, and 1000 µL), RNAse-, RNA- and DNA-free
- Plate centrifuge or spinner
- Eppendorf tubes, RNAse-, RNA- and DNA-free
- 8-well PCR strips, RNAse-, RNA- and DNA-free
- Equipment and solutions to ensure an RNAse free working environment
- Adhesive film applicator
- Autoclave to inactivate BSL2 waste

SAFETY WARNINGS

All steps involving patient samples have to be performed in accordance with local biosafety and ethic guidelines. Saliva samples were considered hazardous - Switzerland: biosafetly level 2 - prior to 65°C inactivation for 15 minutes.

Wearing personal protective equipment *e.g. FFP2 masks, protective eyewear, two pairs of gloves, lab coat* is necessary and strongly adivised.

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

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BEFORE STARTING

Make sure it is possible in your lab to adhere to good laboratory practice under biosafety label 2 conditions.

Padma Nambisan (2017). Laboratory Biosafety and Good Laboratory Practices. An Introduction to Ethical, Safety and Intellectual Property Rights Issues in Biotechnology.. http://10.1016/B978-0-12-809231-6.00011-9

Lankes HA, Makhlouf H (2021). Biospecimen Collection During the COVID-19 Pandemic.. American journal of clinical pathology. https://doi.org/10.1093/ajcp/aqaa171

World Health Organization (2020). Laboratory biosafety guidance related to coronavirus disease (COVID-19): interim guidance. World Health Organization, 2020..

Prepare reagents and instruments for nucleic acid extraction

1 Set plate thermoshaker at **65 °C** .

1.1 Prepare bead binding buffer 20m

For a **1x** working solution mix following chemicals:

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A	B	C	D
Item	**Conc. (stock)**	**Volume**	**Conc. (final)**
Sodium Chloride	5 Molarity (M)	25 mL	2.5 Molarity (M)
Nuclease-free Water		4.672 mL	
Trisodium citrate	1 Molarity (M)	50 µL	1 Milimolar (mM)
Tween 20	20% w/v	250 µL	0.05% volume
PEG 8000	50% w/v	20 mL	20% volume
HCl	1 Molarity (M)	28 µL	0.56 Milimolar (mM)

Components for a 1x working solution for **50 mL**

Titrate the Buffer to pH **6.4** with HCl.

Approximately **35 µl** of 37% HCl for a **100 mL** Volume.

Link: https://openwetware.org/wiki/SPRI_bead_binding_buffer

1.2 Prepare a 30% w/v Chelex stock solution: 5m

For 16 Samples add **4.5 g** Chelex to a 15 ml falcon tube.

Fill to **15 mL** with nuclease-free, deionized water.

Shake the tube well until no clumps are visible.

Chelex stock solution is stable at 4°C for up to 4 weeks.

Michael J. Flynn, Olga Snitser, James Flynn, Samantha Green, Idan Yelin, Moran Szwarcwort-Cohen, Roy Kishony, Michael B. Elowitz (2020). A simple direct RT-LAMP SARS-CoV-2 saliva diagnostic. medRxiv.
https://doi.org/10.1101/2020.11.19.20234948

2 Prepare GdnHCl / Chelex mix. 2m

Note: GdnHCl helps to stablize the RNA at 65°C during Chelex incubation.

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Prepare per sample plus 15% overage: **250 µl** GdnHCl **8 Molarity (M)** + **900 µl** Chelex 30%.

CHELEX beads settle quickly and need to be resuspended directly prior to pipetting

3 Prepare nucleic acid extraction plate: **2 mL** - **2.5 mL** 96 deep well plate. 2m

Pre-fill wells with **1150 µl** GdnHCl / CHELEX mix.

Put on ice **On ice** .

4 Prepare SpeedBeads in SPRI RNA bead binding buffer. 3m

SpeedBeads need to be vortexed thouroughly before pipetting to ensure a good suspension

For **100 µl** of SpeedBeads, add **4.9 mL** of SPRI RNA bead binding buffer.

Bead suspension can be stored at **4 °C** for 4 weeks.

Prepare per sample + 15% overage **480 µl** SpeedBead-Mix

Put **On ice**

Nucleic acid extraction ** **IN BSL2 LAMINAR FLOW HOOD** ** 1m

5 Add saliva to Chelex / GdnHCl mix. 1m

Using a multichannel pipette, add **450 µl** of saliva-saline solution to **1150 µl** CHELEX / GdnHCl.

Mix thouroughly.

6 Seal 96 deep well plate tightly with aluminium plate seal or sealing mat. 1m

Ensure that no cross-contamination can occur during shaking

7 Incubate samples for **00:15:00** at **65 °C** under constant shaking at **700 rpm** - **900 rpm** and put **On ice** . 15m

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Magnetic bead clean up 28m

8 Briefly spin down 96 deep well plate at **200 x g** for **00:01:00** to pellet CHELEX resin. 1m

9 Prepare a fresh **1.1 mL** - **1.5 mL** 96 deep well plate with **480 µl** of SpeedBead-Mix per well. 1m

10 Transfer **600 µl** of supernatant from CHELEX / GdnHCl incubation to SpeedBead-Mix, resuspend thouroughly. 1m

11 Seal plate, incubate for **00:10:00** **at** **Room temperature** . 10m

During incubation, prepare **1 mL** fresh 80% EtOH per sample in nuclease-free water.

12 Put deep well plate on magnetic rack, wait for **00:05:00** or until all beads have pelleted. 5m

13 2m

Remove supernatant, add **500 µl** of 80% EtOH to beads, briefly shake plate and repeat this step once.

Shake plate gently to avoid cross-contamination.

14 Remove residual EtOH, dry plate for **00:02:00** or until beads no longer look "shiny". 2m

Do not let beads overdry and crack

15 Remove from magnet, add **100 µl** **10 Milimolar (mM)** Tris **pH8** per well. 5m

Incubate for **00:05:00** at **Room temperature** .

Resuspend well

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16 Pellet on magnet for **00:05:00** . 5m

Collect up to 75 µl of supernatant in clean tubes or plates and immediately proceed with RT-qPCR. Alternatively, samples can be stored at -80°C for further use.

Technical note: We observed that it may help leave some elution buffer in the plate to avoid resuspension of beads.

RT-PCR 1h 26m

17 For the PCR step, following primers are used:

A	B	C	D	E
Name	**Sequence:5'->3'**	**Stock solution [µM]**	**Stock solution for primer master mix [µl]**	**Final reaction conc. [nM]**
CDC N1 fwd	GAC CCC AAA ATC AGC GAA AT	100	4	400
CDC N1 rev	TCT GGT TAC TGC CAG TTG AAT CTG	100	4	400
CDC N1 FAM	ACC CCG CAT TAC GTT TGG TGG ACC	100	2	200
Charité E-Sarbeco fwd	ACA GGT ACG TTA ATA GTT AAT AGC GT	100	4	400
Charité E-Sarbeco rev	ATA TTG CAG CAG TAC GCA CAC A	100	4	400
Charité E-Sarbeco HEX	ACA CTA GCC ATC CTT ACT GCG CTT CG	100	2	200
CDC RNase P fwd	AGA TTT GGA CCT GCG AGC	100	4	400
CDC RNase P rev	GAG CGG CTG TCT CCA CAA GT	100	4	400
CDC RNase P Cy5	TTC TGA CCT GAA GGC TCT GCG CG	100	2	200

Add 70 µl nuclease-free H_2O.

Stocks can be kept at 100 uM.

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Vogels CBF, Brito AF, Wyllie AL, Fauver JR, Ott IM, Kalinich CC, Petrone ME, Casanovas-Massana A, Catherine Muenker M, Moore AJ, Klein J, Lu P, Lu-Culligan A, Jiang X, Kim DJ, Kudo E, Mao T, Moriyama M, Oh JE, Park A, Silva J, Song E, Takahashi T, Taura M, Tokuyama M, Venkataraman A, Weizman OE, Wong P, Yang Y, Cheemarla NR, White EB, Lapidus S, Earnest R, Geng B, Vijayakumar P, Odio C, Fournier J, Bermejo S, Farhadian S, Dela Cruz CS, Iwasaki A, Ko AI, Landry ML, Foxman EF, Grubaugh ND (2020). Analytical sensitivity and efficiency comparisons of SARS-CoV-2 RT-qPCR primer-probe sets.. Nature microbiology. https://doi.org/10.1038/s41564-020-0761-6

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18 Following table is for one PCR reaction: 5m

A	B
TaqPath™ 1-Step Multiplex Master Mix (No ROX)	2.5 µl
Primer Mix (step 17)	1 µl
Nuclease-free water	1.5 µl
Sample	5 µl
Total	**10 µl**

Prepare **On ice** .

The primers are light sensitive. Avoid long exposure to light. Cover up with aluminum foil.

Use RNAse + nucleic acid free tubes.

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19 Load onto a RT-PCR plate - we usually run all samples and controls in duplicates. 1m

Note: we used 500 copies of TWIST synthetic RNA as positive control and nuclease-free water as negative control.

Spin down quickly before loading the plate into the RT-PCR machine.

20 PCR Program used will take **01:20:00** : 1h 34m 40s

UNC incubation and Hold Stage:

25 °C **00:02:00**

53 °C **00:10:00**

95 °C **00:02:00**

PCR Stage:

95 °C **00:00:10**

55 °C **00:00:30**

Repeat for 45 cycles.

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21 Interpretation of results

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A	B	C	D
E Ct (both replicates)	**N1 Ct (both replicates)**	**RNase P Ct (both replicates)**	**Interpretation**
≤ 40	≤ 40	< 35	positive
any	any	≥ 35	invalid
> 40	> 40	< 35	negative

Interpretation guideline:
- *positive test result*: all four Sars-CoV-2 genes, e.g. both E and both N1 replicates, must show Ct values < 40.
- *negative test result:* between 0 and 3 Sars-CoV-2 genes with Ct values < 40 **AND** RNase P Ct < 35.

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