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Direct RT-qPCR assay for the detection of SARS-CoV-2 in saliva samples V.1

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Equipment and reagents

Sample collection

- FluidX 48-format, 1.9-ml, external thread, next-gen jacket, tri-coded cryotubes (Brooks Life Sciences, 430128-1)
- Collection aid (single-use straws or funnels)
- Leakproof, transparent Ziploc bag
- Lidded container appropriate for transportation of UN 3373 biological substances category B
- Samples were donated voluntarily by staff and students of the University of Nottingham, UK in accordance with Faculty of Medical and Health Science Ethical Approval, University of Nottingham.Informed consent was used throughout the process.

Heat-inactivation

- Class I microbiological safety cabinet (MSC I)
- 20x20 metal storage rack
- Multipurpose benchtop oven (Genlab, OV/50/TDIG/SS/F)
- Thermometer with submersible probe (ThermoFisher, 200590998)
- 70% industrial methylated spirits (IMS) for surface decontamination

Sample registration

- FluidX Perception HD whole rack reader (Brooks Life Sciences, 20-4018)
- FluidX Intellicode decoding software (Brooks Life Sciences, 20-3503)
- FluidX 8x6 barcoded storage rack for 48-format, 1.9-ml cryotubes (Brooks Life Sciences, 430128-1)

qPCR setup and analysis

- FluidX IntelliXcap 48-Format Screw Cap Tube Rack Decapper/Capper (Brooks Life Sciences, 46-8011)
- C1000 Touch thermal cycler with CFX96 optical reaction module for real-time PCR systems (Bio-Rad, 1841100, 1845097)
- Bio-Rad CFX Mestro 1.0 data analysis software (version 4.0.2325.0418)
- Class II microbiological safety cabinet (MSC II)
- E1-ClipTip electronic adjustable tip spacing multichannel equalizer pipette (ThermoFisher Scientific, 4672050BT)
- ClipTip 200 filtered pipette tips (Thermo Scientific, 13286269)
- Eppendorf twin.tec 96-well PCR plate (SigmaAldrich, EP951020401)
- Thermo Scientific adhesive PCR plate seals (ThermoFisher Scientific, AB0558)
- UltraPlex 1-Step ToughMix, 4X (Quantabio, 95166)
- SARS-Related Coronavirus 2 (SARS-CoV-2) External Run Control (Zeptometrix, NATSARS(COV2)-ERC)
- SARS-Related Coronavirus 2 (SARS-CoV-2) Negative Control (Zeptometrix, NATSARS(COV2)-NEG)
- CDC nCOV_N2 FWD primer (5’- GACCCCAAAATCAGCGAAAT -3’)
- CDC nCOV_N2 REV primer (5’- TCTGGTTACTGCCAGTTGAATCTG -3’)
- CDC nCOV_N2 probe (5’- FAM-ACCCCGCAT /ZEN/ TACGTTTGGTGGACC-IBFQ -3’)
- Charité/Berlin E_sarbeco FWD primer (5’- ACAGGTACGTTAATAGTTAATAGCGT -3’)
- Charité/Berlin E_sarbeco REV primer (5’- ATATTGCAGCAGTACGCACACA -3’)
- Charité/Berlin E_sarbeco probe (5’- HEX-ACACTAGCC /ZEN/ ATCCTTACTGCGCTTCG-IBFQ -3’)
- CDC RNase P FWD primer (5’- AGATTTGGACCTGCGAGCG -3’)

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- CDC RNase P REV primer (5’- GAGCGGCTGTCTCCACAAGT -3’)
- CDC RNase P probe (5’- ATTO647-TTCTGACCT /ZEN/ GAAGGCTCTGCGCG-IBFQ -3’)
- Nuclease-free water

Procedures

Sample submission and registration. Time for completion: 1-2 min per sample

1.To guarantee optimal results, samples should be submitted before or at least 1-2 hours after any meals, drinks, or oral hygiene practices, in order to avoid diluting the saliva or introducing contaminants or inhibitors into the final reaction.

2.Only oral saliva should be provided, as forcefully expelled deep-throat sputum (DTS) may negatively affect the sample transfer steps, leading to sub-optimal results.

3.An adequate amount of saliva should be submitted to ensure that the sample is representative and can withstand the heat inactivation process.

4.Sample should be provided into barcoded, external thread tubes that allow for sample traceability and minimise the risk of spills and cross contamination between sample tubes during processing.

5.A straw (or similar collection aid) should be used to provide the sample within the tube.

6.Filled sample tubes should be bagged in leakproof, transparent bags to avoid spills and allow for easy inspection before processing.

7.Bagged samples tubes can be transported to the testing laboratory in adequate rigid, lidded containers according to UN 3373 specifications.

Heat-inactivation of saliva samples. Time for completion: 1.5 h (for 400 samples)

8.Turn on the oven and preheat it to 110 °C.

9. Transfer the bagged sample tubes inside the MSC I for inspection.

10.Place a sterilised 20x20 metal rack inside the cabinet.

11. Inspect sample tubes for damage or obvious tampering and leakage, prior to unbagging and placing them into the metal rack for the inactivation step. It is recommended to keep one empty slot between tubes in the rack and to reserve one corner slot for the control tube (a tube with a pierced lid and filled with water). Up to 199 samples can be placed in a single 20x20 rack this way. If fewer than 199 samples need to be inactivated skip straight to step 13.

CRITICAL STEP: Distancing tubes from each other ensures that all samples heat uniformly and consistently, thus reducing the overall time required to reach the target temperature.

12. Once the metal rack has been filled, repeat steps 9-11with a second rack.No space will be reserved for the control tube in this rack.

13. Once all the tubes have been racked, spray the rack(s) with 70% IMS and transfer them to the oven, taking care to place the rack with the empty corner spot on the top shelf.

14.Insert the thermometer probe into the control tube and place it in the reserved spot of the top rack.

15.Close the oven and monitor the temperature of the control tube using the thermometer until it reaches 95 °C.

PAUSE STEP: The internal sample temperature can take up to 23 min to reach 95 °C. Whilst a timer can be set for this time span, it is advisable to closely monitor the temperature, particularly when fewer than 200 samples are being inactivated.

16.Once the control tube reaches 95 °C, the samples must remain in the oven for a further 5 min to complete inactivation.

17.Remove the rack(s) from the oven using heat resistant gloves and transfer the sample tubes into the original barcoded 48-well storage racks.

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18. Place the storage racks at 4 °C to allow the samples to cool.

PAUSE STEP: Inactivated samples can be stored safely at 4 °C for up to one week until processed.

19. Repeat steps 9-18 for any remaining samples.

20. Once transferred in barcoded storage racks, all the inactivated samples can be scanned using the FluidX Perception HD rack reader with the provided Intellicode software to obtain CSV files that can be imported into the information management software of choice.

3.3. Setting up and running qPCR analyses on heat-inactivated samples. Time for completion: 2 h (for 90 samples)

21. Determine the number of reactions to set up, including the control reactions, making sure to add extra reactions to account for pipetting error.

22. Prepare an appropriate volume of primers/probe mix to add to the master reaction mix, according to Table 1.

23. Prepare the required volume of master mix for the reactions to be set up, as indicated in Table 2.

CRITICAL STEP: UltraPlex 1-Step ToughMix has been specifically selected for this application due to its resilience to PCR inhibition caused by template impurities, a major concern when analysing saliva samples. The use of this specific mix is strongly recommended.

Table 1. Primers and probes required in the reaction mix. The final concentration of each reagent is indicated. A primers/probes mix100. µM stocks.

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Reagent	Final concentration (nM)	Volume of stocks (100 µM) for primers/probes mix (µl)
nCOV_N2_F	250	N x 0.05
nCOV_N2_R	250	N x 0.05
nCOV_N2_P (FAM)	62.5	N x 0.0125
E_Sarbeco_F1	200	N x 0.04
E_Sarbeco_R2	200	N x 0.04
E_Sarbeco_P1 (HEX)	100	N x 0.02
RNase P_F	62.5	N x 0.0125
RNase P_R	62.5	N x 0.0125
RNase P_P (ATTO647)	62.5	N x 0.0125

Table 2. Reagents constituting the master reaction mix required for N individual tests.

Reagent	Volume for N reactions (µl)
Nuclease-free water	N x 6.75
Primers/probes mix	N x 0.25
UltraPlex 1-Step ToughMix (4X)	N x 5.00

Table 3. Reagents required for a 1.5-ml working stock of positive control containing 2 viral particles (vp) per microlitre.

Reagent	Volume (µl)
SARS-CoV-2 External Run Control (50 vp/µl)	60
SARS-CoV-2 Negative Control	200
Nuclease-free water	1,240

24. Dispense 12 µl of master mix into the wells of a 96-well PCR plate, including all the control wells.
25. De-cap one or more racks of tubes containing the samples to be tested.
26. Using the E1-ClipTip electronic adjustable multichannel pipette and 20-µl clip-tips, transfer 8 µl of saliva from the sample tubes to the appropriate wells of the qPCR plate.
27. **CRITICAL STEP:** Adding an accurate amount of saliva is paramount to the correct outcome of the assay. Whilst heat treatment helps to reduce the intrinsic viscosity of the medium, thus allowing the vast majority of the samples to be transferred with satisfactory accuracy, specimens that do not comply with the submission requirements (e.g., provided after a meal or containing sputum rather than saliva) may occasionally prove problematic to pipette with the multichannel pipette. These samples will need to be transferred with a single-channel pipette, or the entire pipetting step will need to be repeated using the multichannel pipette. **NOTE:** We suggest that investigators explore chemical or enzymatic treatments to improve the consistency of saliva and the transferability of the samples.
28. Re-cap the sample tubes.
29. Add 8 µl of nuclease-free water or SARS-CoV-2-positive control sample to the non-template and positive control wells, respectively. To ensure the accuracy of the results and monitor the performance of the assay, the positive control should be loaded at double the viral particle concentration of the expected limit of detection for the assay being used (e.g., 1-2 viral particles/µl
30. Seal the plate with transparent, PCR-grade adhesive film.
31. Centrifuge the plate for 10-20 seconds to remove any bubbles generated during the pipetting steps.

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32.Once all bubbles have been removed, transfer the plate into the thermocycler.
33.Set up and start a cycle as described below

Table 4. Thermocycler settings for each step of the PCR cycle.

	Step	Description	Temperature	Time (min:sec)
	1	Reverse transcription	50 °C	10:00
	2	Initial denaturation	95 °C	3:00
	3	Denaturation	95 °C	0:03
4	Annealing	55 °C	0:30	
5	Extension	72 °C	0:15	

3.4. Data analysis and expected results. Time for completion: 5-15 min (for 90 samples).
34.Once the PCR run is complete, export the data and analyse it with the software provided by the thermocycler manufacturer.
CRITICAL STEP: The correct detection of each target (i.e., N, E or RNase P) in the analysed sample will be indicated by the presence of sigmoidal amplification curves in the sample's well, as visible in one (or more) fluorophore detection channels of the qPCR machine (i.e., FAM, HEX, Cy5). Furthermore, each generated curve will provide a threshold cycle (Ct) value that semi-quantitatively indicates the starting quantity of target in the sample.
35.To confirm the purity of reagents and exclude the chance of widespread contamination, make sure that no amplification is detectable for any of the negative controls (2A).
36.To confirm that the reaction setup and the assay have been performed correctly, make sure that N, E and RNase P amplification is detectable for all the positive controls (2A).
37.To confirm that all samples have been loaded correctly, make sure that RNase P amplification is detectable in all the loaded sample wells. Properly submitted samples that have been loaded optimally should yield amplification curves that cluster within a range of 5-10 Ct values, and also reach a plateau at similar RFU levels (2B).
38.To determine the presence of SARS-CoV-2 within any sample, inspect all wells for N and/or E amplification curves. The detection of a sigmoidal curve for at least one target is indicative of the presence of virus within the sample (2C).
CRITICAL STEP: It is strongly recommended to re-test samples that only yield amplification for one target, as to exclude the possibility of false positive results caused by a contamination event.
39.To discern between a COVID-positive and -negative status for samples in which one or both SARS-CoV-2 targets have been detected, compare the Ct value(s) for each detected target against the cut-off value established for the assay.

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