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Saliva Collection and RNA Extraction for SARS-CoV-2 Detection V.2

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Coronavirus Method Deve…

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Protocol status: Working
We use this protocol in our group, and it is working.

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Abstract

This protocol details recommendations for collecting and processing saliva for SARS-CoV-2 detection, as used in **Wyllie et al. 2020**, which details results of testing saliva collected from COVID-19+ inpatients and asymptomatic healthcare workers.

COVID-19 inpatients. Saliva samples were self-collected by the patient. Upon waking, patients were asked to avoid food, water and brushing of teeth until the sample was collected. Patients were asked to repeatedly spit into a sterile 90 mL specimen collection cup until roughly a quarter full of liquid (excluding bubbles), before securely closing it. For patients unable to provide saliva (such as those on mechanical ventilation), clinical teams were advised that suction could be used to collect saliva into the cup (or a sputum trap container for improved containment and safety). Collected volumes ranged from 0.5 - 20 mL. All saliva samples were stored at room temperature and transported to the research lab at the Yale School of Public Health within 5 hours of collection, with RNA for SARS-CoV-2 detection extracted within 12 hours of collection. When possible, saliva samples were stored at +4°C, otherwise they were kept at room temperature.

Asymptomatic healthcare workers. Asymptomatic healthcare workers were asked to collect ~10 mL of saliva into a sterile specimen collection cup. No specific instructions were given regarding food intake etc prior to collection. Samples were delivered to the research lab at the Yale School of Public Health within 6 hours of collection and stored for up to 6 hours at +4°C until aliquoting for RNA extraction. Samples collected on overnight shifts were stored at +4°C before delivery to the research lab.

Materials

MATERIALS

MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit **Thermo Fisher Catalog #**A42352

Sample collection

- Sterile collection tube: Depending on the volume of saliva required for experiments, various container types can be used, as long as they are sterile, screw shut, and have a wide enough opening to pipette from without introducing contamination. Examples of effective container types include 50, 25, or 5mL conical tubes. *Note: flat-bottomed specimen cups are difficult to vortex and are therefore not recommended.*
- Appropriate PPE (at minimum, gloves and a face mask)

Sample aliquotting

- P1000 pipettor and tips
- Sterile o-ring screw-cap tubes for storage
- Appropriate PPE and biosafety cabinet

RNA extraction

- KingFisher Flex System
- Molecular grade 100% EtOH and water, for preparation of fresh 80% EtOH
- P1000 and P200 pipettors and tips
- Optional: repeater pipette with 500µL, 5mL, and 12.5mL tips
- Secondary transport container
- Sterile o-ring screw-cap tubes for storage
- Appropriate PPE and biosafety cabinet

Safety warnings

Processing of any sample which could potentially be positive for SARS-CoV-2 should be conducted in BSL-2 settings. Before starting work with these samples, contact your local biosafety office for proper guidance on how to safely work with these samples in your laboratory.

Before start

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.
Saliva can be difficult to pipette, which can slow down processing or clog liquid handling robots. Adding proteinase K directly to the samples can make them less viscous and easier to pipette.

1 Saliva can either be collected independently by the individual providing the sample ('Self-collection') or with the assistance of a healthcare worker ('Assisted').

STEP CASE

Assisted Collection 35 steps

For collection of saliva by a healthcare worker or technician. The '**sample provider**' is the individual providing saliva and may include patients, individuals undergoing screening, or research study participants.

2 The **sample collector** must don PPE (at minimum, mask and gloves) prior to contact with the **sample provider.**

3 The **sample provider** must clean their hands using alcohol-based sanitizer or soap and water (no fragrances).

4 The **sample collector** should verify all collection material labeling (at minimum, patient name/identifier and date of collection) with the **sample provider.**

4.1 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.

5 The **sample collector** will give the collection container to the **sample provider**.

6 The **sample provider** will remove the lid of the collection container and gently expel saliva into the collection tube until at least 1 mL has been collected.

Note

The total volume measured should exclude any bubbles.

6.1 Once at least 1 mL has been collected, the **sample provider** will securely replace the lid of the collection container.

7 Following collection, the **sample provider** should again clean their hands using alcohol-based sanitizer or soap and water. The **sample collector** will sterilize the collection tube with 70% ethanol or a disinfecting wipe.

8 The **sample collector** will register the sample collection, and place the sample in a secondary container or a biohazard bag with a biohazard label.

9 The **sample collector** will transfer samples at room temperature to the laboratory for sample processing. The virus RNA in saliva remains stable at room temperature for 3-5 days.

10 Store samples in the laboratory 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.

Sample aliquotting

11 Transfer samples to the biosafety cabinet (BSC). If samples are frozen, thaw on ice.

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.

12 Vortex or swirl sample to mix.

13 Open the collection container, tipping it slightly towards you to pool the saliva on one side. Using a P1000 pipettor, pipette saliva up and down a few times to mix.

13.1 Aliquot saliva into labelled sterile o-ring screw cap tubes for storage. Working volumes of 500 µL to 1 mL could be considered, depending on the volume required for downstream applications.

Note

Work carefully to ensure that the pipettor does not come into contact with the internal surfaces of the sample collection container.
- If this does happen, wipe down the end of the pipettor with 70% ethanol before proceeding to the next sample.

Saliva can be thick and viscous, so:
- Take your time pipetting the saliva – make sure that it reaches the target volume and that there are no bubbles in the tip or at the end of the tip. Sometimes only part of the pipette tip will fill, regardless of efforts. If this occurs, make multiple transfers until the target aliquot volume is reached.
- If a sample is too difficult to pipette, the end of the pipette tip can be cut off with sterile scissors to give a wider opening for collecting the sample.
- If the sample will *only* be used for an RNA extraction that includes a proteinase K digest step: **10-20 µL proteinase K** can be added directly to the collection container, which should then be vortexed vigorously for 20-30 seconds, to reduce sample viscosity.

14 Aliquots for RNA extraction can be stored at 2-8°C. Aliquots for longer-term storage should be stored at -80°C.

RNA extraction

15 Transfer samples to the biosafety cabinet (BSC). If samples are frozen, thaw on ice.

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.

15.1 Prepare a plate map of samples being processed, designating an additional well as the negative extraction control.

16 Prepare a clean workspace by wiping down the RNA extraction bench and pipettes first with 10% bleach, and then with 70% ethanol.

17 Prepare fresh 80% ethanol in a 50 mL conical tube. (The extraction requires 1.5 mL per sample; prepare that volume +10%.)

18 Label and prepare the processing plates using a handystep repeating pipettor according to the following table:

	Plate	Plate type	Reagent	Volume per well	Handy step tip
	Sample Plate	Deep Well	See step 19	860 µL (total)	500 µL and 5 mL
	Wash 1	Deep well	Wash Buffer	1,000 µL	12.5 mL
	Wash 2	Deep well	80% Ethanol	1,000 µL	12.5 mL
	Wash 3	Deep well	80% Ethanol	500 µL	Same as above
	Elution plate	Standard	Elution Solution	75 µL	5 mL
	Tip plate	Deep well (seal and reuse)			

For the tip plate: Place a 96 deep-well tip comb into a deep-well plate. After the run, seal the Tip plate with an adhesive plate seal. Replace the Tip plate once per week.

18.1 Turn on the KingFisher Flex, open the **BindIt 4.0 for KingFisher program**, select the **MVP_Flex protocol** and press start. When prompted, load the tip, elution and wash plates into position.

19 Prepare the sample plate.

19.1 Immediately prior to use, vortex Magnetic Beads vigorously to ensure they are homogenous. Using the 500 µL handystep tip, add 20 µL of Magnetic Beads to each required well of the Sample Plate, vortexing the beads to resuspend well before refilling the tip each time.

Note

Beads settle out of solution rapidly, so vortexing before every refill and pipetting efficiently are crucial to ensure even bead distribution throughout the plate.

19.2 Using the 5 mL handystep tip, add 530 µL of Binding Solution to each required well of the Sample Plate.

Note

Since the Binding Solution is viscous, do this slowly and carefully. Watch the pipette tip to ensure the correct amount is being added into each well.

19.3 Use a secondary container to transfer the Sample Plate and an aliquot of nuclease-free water and proteinase K to the biosafety cabinet (BSC).

19.4 Vortex each sample vigorously for 10 seconds immediately before its addition to the sample plate.
Using the p1000, transfer 300 µL of each sample to its designated well. Add 300 µL nuclease-free water to the negative extraction control well.

Note

If a saliva sample is too thick to easily pipette, transfer ~315 µL to a 1.5 mL snap-cap tube. This may take a couple of attempts. Add 10 µl of proteinase K to this sample and vortex for 20 seconds. If the sample does not become thinner, add another 10 µL and vortex for another 20 seconds. This sample should now be easy to pipette into the sample plate.

19.5 Add 10 µL of proteinase K to each sample well using a multi-step pipettor.

19.6 Once all samples have been prepared, wipe down all surfaces and pipettes - including all sides and bottom of the Sample Plate - with 70% ethanol. Place the sample plate back in the secondary container, securely close the container, and spray down the outside with 70% ethanol.

19.7 Transfer the secondary container with the Sample Plate inside to the KingFisher Flex. Remove the Sample Plate from the secondary container, load it into the KingFisher Flex, and start the RNA extraction run.

20 Five minutes after the run is finished, put on proper PPE and transfer all plates into the secondary container.

Note

An extra five minutes is allowed after cycle completion to allow any aerosols that may have been generated during the procedure to settle.

21 Return the secondary container to the biosafety cabinet.

Note

If magnetic beads carried over into the elution plate for any samples, bring in a magnetic plate rack. The presence of magnetic beads in PCR sample reduces detection sensitivity.

21.1 If RNA will be stored long term, transfer the samples to pre-labelled o-ring screw cap tubes, using the magnetic plate rack to avoid bead carry-over to the stored RNA.

21.2 If RNA will only be used for RT-PCR detection of SARS-CoV-2, samples can be temporarily stored in a foil-sealed elution plate.

22 Store RNA templates and negative control on ice for while preparing for same-day RT-PCR testing. RNA templates which will not be tested within 24 hours or which have already undergone RT-PCR testing should be immediately stored at -80°C.