1.0 INTENDED USE

The AzureSeq Direct Saliva SARS-CoV-2 Assay is an RT-qPCR test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in saliva collected without collection tubes containing preservatives. The AzureSeq Direct Saliva SARS-CoV-2 Assay is available for use under the Emergency Use Authorization (EUA) by Yale School of Public Health's SalivaDirect™ protocol for detection of SARS-CoV-2 in symptomatic or asymptomatic patients under healthcare supervision or unsupervised collection. 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. The simplicity and flexibility of SalivaDirect™ means that it is not as affected by supply chain bottlenecks as some other assays.

Results are for the detection and identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in saliva samples during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA. Clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results do not rule out bacterial infection or co-infection of other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all results to the appropriate public health authorities.

Negative results do not preclude SARS-CoV-2 infection and should not be used as the sole basis for patient management. Negative results must be combined with clinical observations, patient history, and epidemiological information. Negative results for SARS-CoV-2 RNA from saliva should be confirmed by testing of an alternative specimen type if clinically indicated.

AzureSeq Direct Saliva SARS-CoV-2 Assay is intended for use by qualified laboratory personnel specifically instructed and trained in the techniques of RT-qPCR and in vitro diagnostic procedures. The assay is only for use under the Food and Drug Administration's Emergency Use Authorization.

2.0 TEST PRINCIPLE

The AzureSeq Direct Saliva SARS-CoV-2 Assay utilizes RT-qPCR to detect SARS-CoV-2 RNA in saliva samples by reverse transcribing viral RNA into cDNA. The master mix contains probes that anneal to a specific target sequence located between the forward and reverse primers. During the elongation phase of the PCR cycle, Taq polymerase degrades the probe via 5' nuclease activity, causing the reporter dye to detach from the quencher dye, generating a fluorescent signal. After each subsequent qPCR cycle, additional reporter dye molecules are removed from their respective dyes, increasing the fluorescent intensity, which is monitored during the qPCR cycling.

3.0 MATERIALS

COMPONENT	DESCRIPTION	VOLUME	STORAGE
BT-ITMP-MM-1000	2X Inhibitaq HotStart Multiplex Master Mix	2 x 1 mL	-20°C
BT-DRT-1000	RT Mix	1 x 250µL	-20°C
BT-N1RP-PPM	20X N1 RP Primer/Probe Mix	2 x 100µL	-20°C
BT-SDNFW	Saliva Direct Nuclease Free Water	2 x 350µL	-20°C
BT-CVPC-SAL	Saliva Direct Covid Positive Control*	1 x 150µL	-20°C
BT-CVNC-150	Saliva Direct Covid Negative Control	1 x 150µL	-20°C
BT-CVNC-SAL	Saliva Direct Negative Extraction Control	1 x 150µL	-20°C

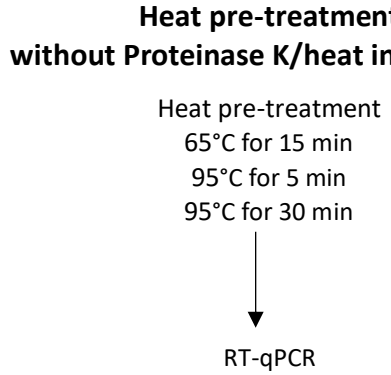
*Controls are Research Use Only

4.0 PROCEDURE

4.1. Collection

1. Saliva should be self-collected under the observation of a trained 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.
4. The patient should not have food or drink for 30 minutes prior to sample collection.
5. 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.
6. The patient can rinse their mouth with water about 10 minutes before collection, then spit into a collection container until 0.5 to 1 mL is collected.
7. 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.
8. Turn in samples to the lab. Samples are stable at room temperature for more than a week but can be refrigerated or frozen if longer term storage is needed.

4.2. Heat Pre-Treatment



Note: This work should be completed under **BSL-2 conditions** (samples potentially containing SARS-CoV-2 should only be handled in a biosafety cabinet).

1. Vortex each saliva sample until homogenous.
2. Remove 50µL of saliva from each sample and pipette into new reaction tubes (200µL PCR strip tubes are appropriate).
3. Place 50µL nuclease free water into new reaction tube (this will be a control to ensure no cross contamination occurs).
4. Vortex tubes for 1 minute at 3000-5000 RPM.
5. Briefly spin down tubes.
6. Incubate tubes in a thermocycler, water bath, oven or similar at 65°C for 15 minutes, 95°C for 5 minutes **OR** 95°C for 30 minutes.
7. ADVICE: Now is a good time to proceed to Master Mix and qPCR setup.
8. Vortex samples then proceed immediately to RT-qPCR testing.

4.3. Master Mix Setup

Perform this part of the protocol in a PCR laboratory hood to minimize cross-contamination.

1. Thaw 2x InhibiTaq Master Mix (orange cap), CoVi Primer/Probe mix 3 (brown tube/cap) and Nuclease-free water (yellow cap) on ice for about 30min or until completely thawed. For best results, do not thaw Direct RT mix (blue cap)—enzyme in RT is activated at 50°C.
    a. When ready to add RT to MM, simply remove from freezer and add to mix (RT is viscous and should be fine to pipette without thawing).
    b. ADVICE: Now is a good time to complete the RT-qPCR setup and plate layout while reagents thaw.

2. After thawing, vortex reagent tubes at max speed for few seconds, then spin down briefly.
3. Proceed to setup master mix as follows in clean or designated setup area:

MASTER MIX COMPONENTS	1 rxn (10µl Total Volume)	1 rxn (20µl Total Volume)
2X Inhibitaq Master Mix	5µl	10µl
RT Mix	0.625µl	1.25µl
Primer Probe Mix	0.5µl	1µl
Nuclease Free Water	1.375µl	2.75µl
Total Volume	**7.5µl**	**15µl**

4. Calculate number of reactions needed based on table above.
5. Pipette each component into a new tube (1.5mL or 2.0mL Eppendorf tubes) and label as Master Mix.
6. After adding all components of Master Mix, vortex until homogenous and spin down briefly.
7. Place tube on ice until ready for use.
8. In 96 or 384 well plate, add 7.5µL of Master Mix per well and 2.5µL sample (i.e., saliva sample, Positive Control, Negative control, etc) per well for total volume of 10µL per well.
9. If 20µL reaction, then add 15µL of Master Mix per well and 5µL sample (i.e., saliva sample, positive control, negative control, etc) per well for total volume of 20µL per well.
10. Mix each well 2-3x with pipette, be careful not to introduce air bubbles.
11. Seal and spin down plate for 30 seconds.
12. Proceed to qPCR instrument and place sealed plate into the qPCR instrument.

4.4. Polymerase Chain Reaction

Setup RT-qPCR parameters and plate map

1. Launch qPCR software and setup run parameters, select fluorophores and plate map.

2. Add the following qPCR conditions:

	A	B	C
1	**Step**	**Temperature**	**Time**
2	1	52°C	10 min
3	2	95°C	2 min
4	3	95°C	10 sec
5	4	55°C	30 sec
6	5	Read plate (FAM & Cy5 channels)	
7	Repeat steps 3-5 for 44 cycles.		

3. Select the following fluorophores for qPCR run:
    a. FAM channel: N1 (SARS-CoV-2)
    b. Texas Red (or equivalent channel ROX): RNase P
4. Typical plate setup as follows (96 well plate as example):

	1	2	3	4	5	6	7	8	9	10	11	12
A	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples
B	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples
C	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples
D	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples
E	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	RB
F	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Control +
G	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Control -
H	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	Patient samples	H2O

RB= Reagent blank (extraction control) or could be Nuclease Free Water (PCR control)

5. Select start/run to begin qPCR run.

4.5. Results Interpretation

1. Set the threshold detection line above the background/basal signal.
2. Test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted.
    a. The positive control should have N1 Ct <40 and RP Ct value <35.
    b. The negative control should have RP value <35.
    c. The reagent blank and water should have N1 as NA and RP as NA.
3. Patient results interpretation is shown in table below:

	A	B	C
1	**Result**	**Ct value N1**	**Ct value RP**
2	Positive	<40.0	Any value
3	Negative	≥40.0	<35.0
4	*Invalid	≥40.0	≥35.0

Note: Invalid test results will be repeated by retesting the primary specimen from the beginning of the protocol. Results from retested samples will follow the same interpretation as listed in the table above.

N1 Ct values <40 and RP Ct values <35 above background are interpreted as positive. If N1 is detected, RNase P may be negative. Use the following table to interpret results:

SARS-CoV-2 N1 (FAM)	RNaseP (Texas Red or ROX)	Result Interpretation	Report	Actions
+	+/-	2019-nCov detected	Positive 2019-nCov	Report results to healthcare provider and appropriate public health authorities.
-	+	2019-nCov not detected	Not Detected	Report results to healthcare provider and appropriate public health authorities.
-	-	Invalid Result	Invalid	Repeat test. If still invalid, collect another specimen. If another specimen is not available, report to healthcare provider.

5.0 MANUFACTURING INFORMATION:

Empirical Bioscience

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Grand Rapids, MI 49508

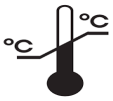
6.0 SUPPORT

Phone: +1-626-460-3050

Email: info@seqonce.com

7.0 EXPLANATION OF SYMBOLS:

Symbol	Definition
	*In vitro* diagnostic medical device
	Lot Number

Symbol	Definition
	Manufacturer
	Date of Manufacture
	Storage & Transport Conditions
	Consult instructions for use
	Use by Date