EMERGENCY USE AUTHORIZATION (EUA) SUMMARY
SARS-CoV-2 RT-PCR Assay

(Yale School of Public Health, Department of Epidemiology of Microbial Diseases)

For *In vitro* Diagnostic Use
Rx Only
For use under Emergency Use Authorization (EUA) only

(The SalivaDirect assay will be performed at laboratories designated by the Yale School of Public Health, Department of Epidemiology of Microbial Diseases, that includes the Clinical Molecular Diagnostics Laboratory, Department of Pathology, Yale School of Medicine, located at 310 Cedar St., New Haven, CT 06510, that are also certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a and meet the requirements to perform high complexity tests, as described in the Laboratory Instructions for Use that was reviewed by the FDA under this EUA.)

INTENDED USE

SalivaDirect is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2 in saliva collected without preservatives in a sterile container in the presence of a trained observer (adult trained on how to collect saliva samples) from individuals suspected of COVID-19 by their healthcare provider. This test is also for use with saliva specimens that are self-collected by individuals 18 years of age or older unsupervised at home, and dropped off at a collection site, using the SalivaDirect Unsupervised Collection Kit when determined to be appropriate by a healthcare provider or unsupervised at home using the SalivaDirect At-Home Collection Kit and mailed to a testing laboratory, when used consistent with its authorization. This test is also intended for use in individuals without symptoms or other epidemiological reasons to suspect COVID-19, using supervised saliva collection, or unsupervised saliva self-collection with the SalivaDirect Unsupervised Collection Kit or the SalivaDirect At-Home Collection Kit.

This test is also for the qualitative detection of nucleic acid from the SARS-CoV-2 in pooled samples containing up to five individual saliva specimens (using specified workflows) that are collected without preservatives in a sterile container in the presence of a trained observer (adult trained on how to collect saliva samples) from individuals suspected of COVID-19 by their healthcare provider or self-collected using the SalivaDirect Unsupervised Collection Kit or SalivaDirect At-home Collection Kit. Negative results from pooled testing should not be treated as definitive. If a patient's clinical signs and symptoms are inconsistent with a negative result or results are necessary for patient management, then the patient should be considered for individual testing. Specimens included in pools with a positive or invalid result must be tested individually prior to

reporting a result. Specimens with low viral loads may not be detected in sample pools due to the decreased sensitivity of pooled testing.

Testing is limited to laboratories designated by the Yale School of Public Health, Department of Epidemiology of Microbial Diseases, that includes the Clinical Molecular Diagnostics Laboratory, Department of Pathology, Yale School of Medicine, located at 310 Cedar St., New Haven, CT 06510, that are also certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a and meet the requirements to perform high complexity tests.

Results are for the detection and identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in saliva specimens 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 with 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 decisions. 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.

SalivaDirect is intended for use by qualified clinical 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.

DEVICE DESCRIPTION AND TEST PRINCIPLE

SARS-CoV-2 Assay

SalivaDirect is an RNA-extraction free, dualplex 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 nucleic acid extraction, and (3) is validated for use with products from multiple vendors. Thus, the simplicity and flexibility of SalivaDirect means that it is not as affected by supply chain bottlenecks as some other assays. The method is nucleic acid extraction-free, which enables testing of low volume and minimally processed saliva in dualplex RT-qPCR for SARS-CoV-2 detection. Saliva is first treated with proteinase K followed by a heat inactivation step and is then directly used as input in the dualplex RT-qPCR test using validated primer and probe sets (2019-nCoV_N1 and RP) developed by the US CDC. The human *Ribonuclease P* (RP) probe was modified with a different fluorophore so that the primer/probe set could be combined in a dualplex assay, reducing the number of tests to 1 assay with 2 sets.

The SalivaDirect assay is authorized for use with the SalivaDirect At-Home Collection Kit, which was authorized for use in a separate EUA (EUA210243), as well as the SalivaDirect Unsupervised Collection Kit, authorized in the current EUA.

SalivaDirect Unsupervised Collection Kit

The SalivaDirect Unsupervised Collection Kit enables the self-collection of a saliva specimen in a sterile container that will be sent to a laboratory designated by the Yale School of Public Health as authorized to run the SalivaDirect Assay when determined to be appropriate by a healthcare provider. The kit collects viral RNA saliva specimens and can be used for the short-term room temperature storage of a sample. The SalivaDirect Unsupervised Collection Kit is a non-invasive alternative for collection of viral RNA by/from individuals who are suspected of COVID-19 by their healthcare provider.

The self-collection kit consists of one of four different options for obtaining saliva specimens:

- Short straw (5-6 cm in length or of similar dimensions to the Salimetrics Saliva Collection Aid, catalog #5016.02 or the Mirimus SalivaClear Collection Kit, catalog #800100)
- Funnel
- Bulb Transfer Pipette (1 mL)
- Pipette Tip (1000 µl)

The SalivaDirect Unsupervised Collection kit will include the following components:

In a zip-lock bag (or similar):

- Self-collection instructions
- One identifying information form for patients to record their name, date of birth and date and time of sample collection (to be created and provided by the test laboratory)
- One of four different devices for obtaining saliva specimens
- One sterile plastic tube (1 to 5 mL in volume)
- One biohazard bag for specimen transport
- One alcohol wipe.

SALIVADIRECT UNSUPERVISED COLLECTION KIT ORDERING, PROCESSING AND MEDICAL OVERSIGHT

The unsupervised collection of saliva samples for use with the SalivaDirect assay can only occur for patients who have been previously qualified by their healthcare provider as needing SARS-CoV-2 testing. The healthcare provider will submit a prescription for testing to the designated laboratories authorized to run the SalivaDirect assay. The designated laboratories will then be

responsible for preparing the collection kits as described in the Instructions for Use and providing the Unsupervised Collection Kit to those individuals for whom testing has been ordered. The Unsupervised Collection Kit will contain one of the four authorized devices for obtaining the saliva specimens, one saliva collection tube, a form to gather identifying information (name, date of birth, date/time of sample collection), the unobserved self-collection instructions, a biohazard bag for specimen transport, and an alcohol wipe for contamination issues. The designated laboratory will also be responsible for informing the individual where to return the sample (i.e., the sample could be dropped off at the lab or a specified collection box for that lab; however, **the sample will not be mailed nor shipped**). Test results will then be communicated back to the ordering physician.

RT-qPCR INSTRUMENTS USED WITH TEST

SalivaDirect should be used with the following RT-qPCR instruments:

Vendor	Instrument	Software
Bio-Rad	CFX96 Touch Real-Time PCR Detection System	Bio-Rad CFX Maestro 1.1 V4.1.2435.1219
Bio-Rad	CFX384 Touch Real-Time PCR Detection System	Bio-Rad CFX Maestro 1.1 V4.1.2435.1219
ThermoFisher Scientific	Applied Biosystems StepOne Real-Time PCR System	StepOne Software v2.3
ThermoFisher Scientific	Applied Biosystems StepOne Plus Real-Time PCR System	StepOne and StepOnePlus Software v2.3
ThermoFisher Scientific	Applied Biosystems 7500 Fast Real-Time PCR System	7500 Software v2.3
ThermoFisher Scientific	Applied Biosystems 7500 Fast Dx Real-Time PCR System	7500 Fast System SDS software v1.4.1
ThermoFisher Scientific	Applied Biosystems PRISM 7000 Real-Time PCR System	PRISM 7000 Sequence Detection System version 1.0
ThermoFisher Scientific	ABI QuantStudio 5 Real-Time PCR system (96 or 384 well format)	QuantStudio Design and Analysis Software v2.4.3
ThermoFisher Scientific	ABI QuantStudio 6 Real-Time PCR system (96 or 384 well format)	QuantStudio Design and Analysis Software v2.4.3
ThermoFisher Scientific	ABI QuantStudio 7 Pro Real-Time PCR system (96 or 384 well format)	QuantStudio Design and Analysis Software v2.4.3
ThermoFisher Scientific	ABI QuantStudio 7 Flex Real-Time PCR system (96 or 384 well format)	QuantStudio Design and Analysis Software v2.4.3
ThermoFisher Scientific	ABI QuantStudio 12K Flex Real-Time PCR system (384 well format)	QuantStudio Design and Analysis Software v2.4.3
ThermoFisher Scientific	ABI QuantStudio Dx Real-Time PCR system (96 well format)	QuantStudio Design and Analysis Software v2.4.3
Ubiquitome	Liberty16	Liberty16 App Version 1.8 (iOS)
Roche	Cobas Z480	User Defined Workflow for cobas z 480
Roche	LightCycler 480	LightCycler 480 Software, Version 1.5

Vendor	Instrument	Software
CHAI	Open qPCR	Open qPCR software (HTML5/JavaScript web app)
Analytik Jena	qTower	qPCRsoft version 2.2
Agilent	AriaMX Real-Time PCR System	N/A (fully integrated)

INSTRUMENTS AND MATERIALS USED WITH SALIVADIRECT TEST IN THE HAMILTON AUTOMATED PROTOCOL (INSTRUCTIONS FOR USE, APPENDIX C)

The Hamilton automated protocol for SalivaDirect as detailed in Appendix C of the Instructions for Use should be used with the following instruments and materials:

Vendor	Item	Catalog #
Hamilton	Vantage 2.0 liquid handling robot equipped with 96-channel head and 8-channel spanner head. The Hamilton Venus 4 software package used for instrument programming and operation via the "Venus on Vantage" software utility.	Custom configuration
Applied Biosystems	384-Well Polypropylene PCR plate	4343814
Hamilton	50 µL filtered pipette tips	235948
ThermoFisher	1 mL sterile-internal threaded tube	3741
Hamilton	96 well PCR FramePlate	814302
Hamilton	LabElite DeCapper SL	193602
Rainin	BenchSmart 96-200 Semi-automated pipette system	BST 96-200

INSTRUMENTS AND MATERIALS USED WITH SALIVADIRECT TEST IN THE TECAN FLUENT 780 AUTOMATED PROTOCOL (INSTRUCTIONS FOR USE, APPENDIX D)

The Tecan Fluent 780 automated protocol for SalivaDirect as detailed in Appendix D of the Instructions for Use should be used with the following instruments and materials:

Vendor	Item	Catalog #
Tecan U.S. Group, Inc.	Fluent® 780 liquid handling robot equipped utilizing the FluentControl software package for instrument programming and operation	Custom Configuration
Tecan U.S. Group, Inc.	Fluent ID Barcode Scanners	30042504 30042505
Tecan U.S. Group, Inc.	Flexible Channel Arm (FCA) 8-Channel, Standard fixed tips	30042145
Tecan U.S. Group, Inc.	Robotic Gripper Arm (RGA) Long Z, Regular Finger	30042405
Tecan U.S. Group, Inc.	Runner, Eppendorf 2.0mL 1x32 Safe-Lock Tubes	30042509
QInstruments GmbH	Heated Adapter Plate BioShake 3000-T ELM	30127732
Agilent Technologies, Inc.	PlateLoc Thermal Microplate Sealer	30135829
Eppendorf North America, Inc.	Safe-Lock Eppendorf Tubes (2.0mL)	022363352
Bio-Rad Laboratories, Inc.	Bio-Rad Hard-Shell PCR Plates 96-well, thin wall	HSP9601

INSTRUMENTS AND MATERIALS USED WITH SALIVADIRECT TEST IN THE TECAN FLUENT 480 ASSISTED RT-qPCR PREPARATION PROTOCOL (INSTRUCTIONS FOR USE, APPENDIX E)

The Tecan Fluent 480 assisted RT-qPCR preparation for SalivaDirect as detailed in Appendix E of the Instructions for Use should be used with the following instruments and materials:

Vendor	Item	Catalog #
TECAN U.S.	Fluent® 480 liquid handling robot equipped utilizing the FluentControl software package for instrument programming and operation	Custom Configuration
TECAN U.S.	Flexible Channel Arm (FCA) 8-Channel, Standard fixed tips	30042145
TECAN U.S.	Multiple Channel Arm (MCA)	30042350
TECAN U.S.	MCA Head Adapter: Tipblock 96 tips, 125 µl	30032066
Applied Biosystems	MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1mL)	4483485
Applied Biosystems	MicroAmp® Optical 384-Well Reaction Plate with Barcode	4309849

REAGENTS AND MATERIALS

Designated laboratories should refer to the SalivaDirect website for a list of qualified reagent lots.

Vendor	Item	Catalog number	Quantity	# Reactions
Order one of the following Proteinases K				
ThermoFisher Scientific	MagMAX Viral/Pathogen Proteinase K	A42363	10 mL	4,000 reactions
New England Biolabs	Proteinase K, Molecular Biology Grade	P8107S	2 mL	320 reactions
AmericanBio	Proteinase K	AB00925	100 mg	800 reactions
Order one of the following RT-qPCR kits				
New England Biolabs	Luna Universal Probe One-Step RT-qPCR (2x) Kit	E3006S	2 mL	200 reactions
		E3006L	5 mL	500 reactions
		E3006X	10 mL	1,000 reactions
		E3006E	25 mL	2,500 reactions
New England Biolabs	Luna Probe One-Step RT-qPCR 4x Mix with UDG (for use with 384-well format PCR instruments)	M3019S	1.06 mL	200 reactions
		M3019L	2.5 mL	500 reactions
		M3019X	5 mL	1,000 reactions
		M3019E	10.5 mL	2,500 reactions
Bio-Rad	Reliance One-Step Multiplex RT-qPCR Supermix	12010176	1 mL	200 reactions
		12010220	5 mL	1,000 reactions
		12010221	10 mL	2,000 reactions
ThermoFisher Scientific	TaqPath 1-Step RT-qPCR Master Mix, GC	A15299	5 mL	1,000 reactions
		A15300	10 mL	2,000 reactions
Quantabio	UltraPlex 1-Step ToughMix	95166-100	500 µl	100 reactions
		95166-500	2.5 mL	500 reactions

Vendor	Item	Catalog number	Quantity	# Reactions
		95166-01K	5 mL	1,000 reactions
GS Biomark, LLC	2X Inhibitaq Multiplex HotStart Master Mix	BM-ITMP-MM-1000	1 mL	1,000 reactions (100 per tube)
	Direct RT Mix (order with the Master Mix)	BM-DRT-1000	250 µl	1,000 reactions (200 per tube)
Order one of the following primer and probe sets				
Eurofins Genomics	SalivaDirect primer and probe set (complete set of the 6 primers and probes), Cy5 probe	12YS-010YST	50-100 nmol	12,500 reactions
	SalivaDirect primer and probe set (complete set of the 6 primers and probes), HEX probe	12YS-000YST	50-100 nmol	12,500 reactions
Integrated DNA Technologies	nCOV_N1 Forward Primer Aliquot	10006821	50 nmol	6,250 reactions
		10006830	100 nmol	12,500 reactions
	nCOV_N1 Reverse Primer Aliquot	10006822	50 nmol	6,250 reactions
		10006831	100 nmol	12,500 reactions
	nCOV_N1 Probe Aliquot	10006823	25 nmol	6,250 reactions
		10006832	50 nmol	12,500 reactions
	RNase P Forward Primer Aliquot	10006827	50 nmol	16,600 reactions
		10006836	100 nmol	33,300 reactions
	RNase P Reverse Primer Aliquot	10006828	50 nmol	16,600 reactions
		10006837	100 nmol	33,300 reactions
	RNase P Probe	Custom order (Cy5)	25 nmol	6,250 reactions
		Custom order (Cy5)	50 nmol	12,500 reactions
		10007061 (ATTO647)	25 nmol	6,250 reactions
		10007062 (ATTO647)	50 nmol	12,500 reactions
LGC Biosearch Technologies	nCOV_N1 Forward Primer	nCoV-N1-F-100	100 nmol	12,500 reactions
		nCoV-N1-F-1000	1000 nmol	125,000 reactions
	nCOV_N1 Reverse Primer	nCoV-N1-R-100	100 nmol	12,500 reactions
		nCoV-N1-R-1000	1000 nmol	125,000 reactions
	nCOV_N1 Probe	nCoV-N1-P-25	25 nmol	6,250 reactions
		nCoV-N1-P-250	250 nmol	62,500 reactions
	RNase P Forward Primer	RNP-F-20	20 nmol	6,660 reactions
		RNP-F-100	100 nmol	33,300 reactions

Vendor	Item	Catalog number	Quantity	# Reactions
		RNP-F-1000	1000 nmol	333,300 reactions
	RNase P Reverse Primer	RNP-R-20	20 nmol	6,660 reactions
		RNP-R-100	100 nmol	33,300 reactions
		RNP-R-1000	1000 nmol	333,300 reactions
	RNase P Probe	RNP-PQ670-25	25 mol	6,250 reactions
		RNP-PQ670-250	250 nmol	62,500 reactions
GS Biomark, LLC	N1 RP Primer/Probe Mix (Primers and probes come pre-mixed)	BM-N1F-100-550uL	20X	550 reactions
Lighthouse Lab Services	SalivaNow SARS-CoV-2 Assay (Primers and probes come pre-mixed)	9731816-S	-	2,000 reactions
Order one of the following nuclease-free waters				
Integrated DNA Technologies	Nuclease-free water	11-04-02-01	20 mL	
		11-05-01-14	300 mL	
		11-05-01-04	1 L	
New England Biolabs	Nuclease-free water	B1500S	25 mL	
		B1500L	100 mL	
Order one of the following positive controls				
Twist Bioscience	Synthetic SARS-CoV-2 RNA Control 2	102024	100 µL	
Integrated DNA Technologies	2019-nCoV_N_Positive Control	10006625	250 µL	
Lighthouse Lab Services	Positive CoV-2 Control	9731816PC	80 µL	
Optional negative extraction control (NEC)				
Lighthouse Lab Services	Negative Control	9731816EC	10 mL	

CONTROLS RUN WITH THE COVID-19 RT-PCR

The following controls are run with the SalivaDirect assay:

Control	Description	Purpose	Frequency
Negative Extraction Control (NEC)	Nuclease-free water	To monitor for contamination during saliva processing	Every batch of up to 93 saliva samples

	Lighthouse Labs Negative Control (synthetic RNAse P control)	To monitor for effective proteinase K treatment and contamination during saliva processing	
Negative Template Control (NTC)	Nuclease-free water	To monitor for contamination of PCR reagents	Every PCR plate with up to 93 saliva samples
Positive Control	Twist Synthetic SARS-CoV-2 RNA control 2 (**Dilute to 100 copies/µL**)	To monitor functioning of RT-qPCR reagents	Every PCR plate with up to 93 saliva samples
	IDT 2019-nCoV_N_Positive Control (**Dilute to 100 copies/µL**)		
	Lighthouse Lab Services Positive CoV-2 Control (synthetic SARS-CoV-2 RNA control, 100 copies/µl)		
Internal Process Control	Primer/Probe set detecting RNaseP	To ensure that saliva of a sufficient quantity and quality was tested	Every sample

INTERPRETATION OF RESULTS

1. SARS-CoV-2 RT-PCR Test Controls – Positive, Negative, and Internal

Positive control: The positive control should yield a "detected" result for the N1 target and "not detected" for the RNaseP control.

Negative Extraction Control (NEC): If using nuclease-free water, the NEC should yield a "not detected" result for both the N1 and RNaseP targets. If using the Lighthouse Lab Services Negative Control, the NEC should yield a "not detected" result for the N1 target and a Ct value <30 Ct for the RNaseP target.

Negative Template Control: The NTC should yield a "not detected" result for both the N1 and RNaseP targets.

Internal Control: Detection of RNaseP below a specified cut-off (see tables below) indicates that saliva of sufficient quantity and quality were tested. Detection of RNaseP is required to report a negative SARS-CoV-2 result.

2. Examination and Interpretation of Patient Specimen Results

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted. Results of individual sample testing from a primary, individual saliva specimen or as a reflex to pooled sample testing will be interpreted according to the tables below:

16-Well and 96-Well Formats

Bio-Rad CFX96 Touch ABI 7500 Fast ABI 7500 Fast Dx ABI PRISM 7000 ABI QuantStudio Dx ABI QuantStudio 5 ABI QuantStudio 7 Flex ABI StepOne Plus Analytik Jena qTower CHAI Open qPCR Ubiquitome Liberty16		
Result	**Ct value N1**	**Ct value RP**
Positive	<40.0	**Any value**
Negative	≥40.0	<35.0
*Invalid	≥40.0	≥35.0

ABI StepOne ABI QuantStudio 6 ABI QuantStudio 7 Pro		
Result	**Ct value N1**	**Ct value RP**
Positive	<37.0	**Any value**
Negative	≥37.0	<35.0
*Invalid	≥37.0	≥35.0

Roche LightCycler 480		
Result	**Ct value N1**	**Ct value RP**
Positive	<35.0	**Any value**
Negative	≥35.0	<35.0
*Invalid	≥35.0	≥35.0

Agilent AriaMX Roche Cobas Z480		
Result	**Ct*** value N1**	**Ct value RP**
Positive	<34.0	**Any value**
Negative	≥36.0	<30.0
**Inconclusive	≥34.0 - <36.0	<30.0
*Invalid	≥34.0	≥30.0

*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.

**When the Ct value for RP is <30 and the Ct is in the range of ≥34.0 - <36.0 for N1, the sample will be retested from the beginning of the protocol to potentially convert an inconclusive result to a confirmed negative or positive, if desired by the requesting healthcare provider. Results from retested samples will follow the same interpretation as listed in the table above.

***Cq values are qualified cycle thresholds reported by the Agilent AriaMX system and can be interpreted synonymously to Ct values.

384-Well Format

Results of individual sample testing from a primary, individual saliva specimen or as a reflex to pooled sample testing will be interpreted according to the table below.

CFX384 Touch ABI QuantStudio 5 ABI QuantStudio 6 ABI QuantStudio 7 Pro ABI QuantStudio 7 Flex ABI QuantStudio 12K Flex		
Result	**Ct value N1**	**Ct value RP**
Positive	<40.0	Any value
Negative	≥40.0	<35.0
*Invalid	≥40.0	≥35.0

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

Pooled Sample Results and Reflex Testing

The interpretation of results for pooled SalivaDirect testing is the same for all thermocyclers used. Results of pooled testing should be interpreted according to the following (and summarized in the table below):

- **Negative Result**: If samples were pooled and the SARS-CoV-2 N1 target is not detected at all (not detected, ND; NaN; Undetected; ≥45.0; etc.), then all samples in that pool should be reported as Negative. Negative results from pooled sample testing should not be treated as definitive. If the patient's clinical signs and symptoms are inconsistent with a negative result and if results are necessary for patient management, then the patient should be considered for individual testing. The utilization of sample pooling should be indicated for any specimens with reported negative results.
- **Not Negative Result (i.e., positive or invalid)**: If samples were pooled and determined not negative through generating a Ct value of any value for N1 (<45.0) or returns an invalid result (poor or no RP detection), then all samples in that pool should be tested individually by the laboratory's standard SalivaDirect protocol prior to result reporting. Only the results

of the individually tested samples (as interpreted depending on the thermocycler used; tables above) should be reported.

Pooled SalivaDirect results, interpretation and action (for testing conducted on all thermocyclers).

Ct value		Interpretation	Action
N1	**RP**		
≥45.0	<30.0	Negative	Report all samples as Negative
≥45.0	≥30.0	Not negative: invalid	Reflex test all samples individually
Any value	Any value <45	Not negative: positive	Reflex test all samples individually

If a pool is reported as not negative but all samples from the pool return negative results when tested individually, the occurrence should be referred to the laboratory director and an investigation should be initiated, including assessment of the potential for: a) contamination/false-positive pool result; b) assay inhibition upon individual testing; c) differences in assay reagents between pooled and individual testing. If no root cause is identified, the individual samples will be retested once (assuming adequate volume remains) and the results will be reported. If insufficient volume remains for retesting, the subjects will be informed of a test error and encouraged voluntarily to re-test. Recollected samples will be processed according to the standard SalivaDirect protocol used in the designated laboratory.

SALIVADIRECT UNSUPERVISED COLLECTION KIT SAMPLE ACCESSIONING

In order for the designated laboratory to perform testing, the received samples shall meet the following criteria:

- **Proper return of sample**: sample is present, identifying information form is present and filled out, the sample tube is not broken, sample is not leaking.
- **Verification of patient information**: the patient information on the collection tube matches the information on the identifying information form.
- **Sample acceptability**: sufficient sample volume, sample received within 72 hours from sample collection date and time (as per identifying information form).

PERFORMANCE EVALUATION

1. Analytical Sensitivity

Limit of Detection (LoD)

A positive saliva specimen from a confirmed COVID-19 healthcare worker with a known virus concentration (3.7×10^4 copies/µL) was spiked into saliva collected from healthcare workers who tested negative for SARS-CoV-2 using the CDC assay. The following 2-fold dilution series was tested in triplicate to determine the preliminary limit of detections: 400, 200, 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. Spiked saliva specimens were tested according to the SalivaDirect protocol. In total, three different proteinase K reagents, three different RT-qPCR kits, and three different RT-qPCR thermocyclers were validated with the assay. Input volumes, matrices and RT-qPCR programs were the same for each combination of proteinase K, RT-qPCR kit, and RT-qPCR instrument. The preliminary limit of detection was then confirmed with 20 additional replicates. The table below shows the final limit of detection for the different reagents/instruments used with SalivaDirect.

Proteinase K					
Proteinase K	*RT-qPCR kit*	*RT-qPCR instrument*	*LOD*	*Positive replicates*	*Mean Ct value (SD)*
Thermo	NEB Luna (2x)	Bio-Rad CFX96 Touch	6 copies/µL	100% (20/20)	36.7 (1.0)
NEB	NEB Luna (2x)	Bio-Rad CFX96 Touch	3 copies/µL	100% (20/20)	36.6 (1.0)
AmericanBio	NEB Luna (2x)	Bio-Rad CFX96 Touch	3copies/µL	100% (20/20)	33.51 (0.4)
RT-qPCR kit					
Proteinase K	*RT-qPCR kit*	*RT-qPCR instrument*	*LOD*	*Positive replicates*	*Mean Ct value (SD)*
Thermo	Bio-Rad Reliance	Bio-Rad CFX96 Touch	6 copies/µL	100% (20/20)	36.4 (0.6)
Thermo	Thermo TaqPath	Bio-Rad CFX96 Touch	12 copies/µL	100% (20/20)	35.9 (1.2)
RT-qPCR instrument					
Proteinase K	*RT-qPCR kit*	*RT-qPCR instrument*	*LOD*	*Positive replicates*	*Mean Ct value (SD)*
Thermo	Thermo TaqPath	ABI 7500 Fast	12 copies/µL	95% (19/20)	36.8 (1.2)
Thermo	Thermo TaqPath	ABI 7500 Fast Dx	6 copies/µL	95% (19/20)	32.4 (0.9)

Additional LoD studies were conducted to validate the Agilent AriaMX 96-well format thermocycler, the Liberty16 16-well format thermocycler, and the CFX384 Touch 384-well format thermocycler. Samples were prepared by spiking saliva from a confirmed positive patient into negative clinical matrix. The following dilutions were tested in triplicate in the range finding study: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. The LoD was then confirmed by testing 20 replicates and determined to be 6 copies/µL for the Agilent AriaMx and the CFX384 Touch thermocyclers, and 12 copies/µL for the Liberty16.

Proteinase K	*Primer/Probe*	*RT-qPCR kit*	*RT-qPCR instrument*	*LOD*	*Positive replicates*	*Mean Ct value (SD)*
Thermo	IDT	NEB Luna (2x)	Agilent AriaMX	6 copies/µL	100% (20/20)	30.3 (0.4)
Thermo	Eurofins	NEB Luna (2x)	Liberty16	12 copies/µL	100% (20/20)	35.18 (0.7)
Thermo	IDT	NEB Luna (2x)	CFX384 Touch	6 copies/µL	100% (20/20)	36.25 (0.4)

In addition, 22 weak positive clinical samples were tested in both the CFX96 Touch and CFX384 Touch PCR instruments with the NEB Luna 2x RT-PCR kit, with 100% concordance. Additionally, 9 clinical samples were tested on both the CFX96 Touch and QuantStudio 5 (384) PCR instruments with NEB Luna 2x RT-PCR kit, with 100% concordance. These results demonstrate similar detection in clinical samples when using either the 96 or 384 well formats Results are summarized below:

Thermocycler	*Positive Replicate*	*Mean Ct Value*
CFX96 Touch	100% (22/22)	35.78
CFX384 Touch	100% (22/22)	36.68

Thermocycler	*Positive Replicates*	*Mean Ct Value*
CFX96 Touch	100% (9/9)	28.62
QuantStudio 5 (384)	100% (9/9)	27.76

Additional RT-PCR Mixes

In addition to the 2x NEB Luna RT-PCR mixture validated above, a 4x concentration was also validated via an LoD study on the CFX384 Touch using the Thermo Proteinase K. The LoD of 6 copies/mL previously confirmed for the NEB Luna 2x was confirmed on the CFX384 Touch, as shown below:

	6 copies/ul		3 copies/ul	
	Positive Replicates	Mean Ct	Positive Replicates	Mean Ct
NEB Luna (4x)	100% (20/20)	35.77	85% (17/20)	36.57

The Quantabio UltraPlex 1-Step ToughMix PCR mixture was also validated via an LoD study on the CFX96 Touch using the Thermo Proteinase K and was found to have a confirmed LoD of 3 copies/mL, as shown below:

	6 copies/ul		3 copies/ul	
	Positive Replicates	Mean Ct	Positive Replicates	Mean Ct
UltraPlex 1-Step ToughMix	100% (20/20)	36.42	95% (19/20)	37.45

A bridging study was performed to validate the GS Biomark 2X Inhibitaq Multiplex HotStart Master Mix (with Direct RT Mix). A 2-fold dilution series was tested in triplicate with the new Master Mix in parallel with a previously validated Master Mix to establish equivalent performance. Samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. All samples were tested through the standard SalivaDirect workflow using the Thermo Proteinase K and tested in RT-qPCR using either the previously validated 2x NEB Luna RT-PCR mix or the new GS Biomark 2X Inhibitaq Multiplex HotStart Master Mix (with Direct RT Mix). The table below lists the positivity rates for each concentration when tested using validated and new master mixes:

		Concentration (positive replicates)							
Primer/Probes	RT-PCR mix	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
IDT (Cy5)	**NEB Luna 2x**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
Eurofins	GS Biomark 2x	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3

The lowest concentration at which 100% of replicates were positive for the new GS Biomark 2x Master Mix was within 2X of the previously authorized NEB Luna 2x Master Mix when tested side-by-side, indicating comparable analytical performance.

Additional Primer/Probe mix

The SalivaNow assay consists of a pre-mixed, ready to use mixture of the CDC-N1 and RNaseP primers and probes. For bridging of the SalivaNow SARS-CoV-2 assay, samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. All samples were tested in the standard SalivaDirect assay using the Thermo Proteinase K then in RT-qPCR with both the NEB Luna 2x RT-qPCR kit and the TaqPath One Step kit in the CFX96 Touch. Results were compared to the standard SalivaDirect assay using the Eurofins primer/probe sequences, the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit also in the CFX 96 Touch. The table below lists the positivity rates for each concentration when tested using validated and new primer/probe vendors:

		Concentration (positive replicates)							
Primer/Probes	RT-PCR mix	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
Eurofins	**NEB Luna 2x**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
SalivaNow	NEB Luna 2x	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
SalivaNow	TaqPath One Step	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3

A bridging study was performed to validate the pre-mixed GS Biomark N1 RP Primer/Probe Mix. A 2-fold dilution series was tested in triplicate with the new Primer/Probe Mix in parallel with previously validated primers and probes to establish equivalent performance. Samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. All samples were tested through the standard SalivaDirect workflow using the Thermo Proteinase K and tested in RT-qPCR using either the 2x NEB Luna RT-PCR mix or the GS Biomark 2X Inhibitaq Multiplex HotStart Master Mix (with Direct RT Mix) with the new pre-mixed GS N1 RP Primer/Probe Mix in the CFX96 Touch. Results were compared to the standard SalivaDirect assay using the previously validated IDT Primers, the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit also in the CFX96 Touch. The table below lists the positivity rates for each concentration when tested using validated and new reagents:

		Concentration (positive replicates)							
Primer/Probes	RT-PCR mix	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
IDT (Cy5)	**NEB Luna 2x**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
GS Biomark (Mix)	NEB Luna 2x	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
GS Biomark (Mix)	GS Biomark 2x	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3

The lowest concentration at which 100% of replicates were positive for the new GS Biomark primer/probe mix was within 2X of the previously authorized IDT primers and probes when tested side-by-side, indicating comparable analytical performance.

Additional RNAseP probe

For the SalivaDirect RT-qPCR assay to be compatible with the ABI PRISM 7000 and ABI StepOne, the Cy5 fluorophore on the RNAseP probe had to be exchanged to a HEX fluorophore. For this bridging study to validate the use of a HEX fluorophore on the RNAseP probe, samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. All samples were tested using the Thermo Proteinase K with the NEB Luna 2x RT-qPCR kit. The samples on the previously validated CFX96 Touch were tested with the RNAseP probe labelled with Cy5 and the samples on the ABI PRISM 7000 and ABI StepOne were tested with the RNAse probe labelled with HEX. The table below lists the positivity rates for each concentration when tested using validated and new thermocyclers:

	Concentration (positive replicates)							
	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
CFX96 Touch RP-Cy5	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI PRISM 7000 RP-HEX	3/3	3/3	3/3	3/3	2/3	3/3	2/3	0/3
ABI StepOne, RP-HEX	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3

Bridging Studies for Additional Instruments

Bridging studies were performed to validate additional thermocyclers. A 2-fold dilution series was tested in triplicate with each new thermocycler in parallel with a previously validated thermocycler to establish equivalent performance. The previously validated thermocycler is highlighted in bold for each study. Samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 100, 50, 25, 12, 6, 3, and 1.5 copies/µL. All samples were tested using the Thermo Proteinase K with the NEB Luna RT-qPCR kit. The previously validated thermocyclers were tested with the 2x NEB Luna RT-PCR mix, while the new thermocyclers were tested with either the 2x (for 96-well and 384-well instruments) or 4x (for 384-well instruments) RT-PCR mix. The table below lists the positivity rates for each concentration when tested using validated and new thermocyclers:

	Concentration (positive replicates)							
	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
Bridging Study 1								
ABI 7500 Dx Fast	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 5	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3
Bridging Study 2								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 6	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3
Bridging Study 3								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 7	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3
Bridging study 4								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 5, 384 well (NEB Luna 2x)	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3
ABI QuantStudio 5, 384 well (NEB Luna 4x)	3/3	3/3	3/3	3/3	3/3	2/3	0/3	0/3

Bridging study 5								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 6, 384 well (NEB Luna 2x)	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3
Bridging study 6								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**0/3**
ABI QuantStudio 7 Pro, 384 well (NEB Luna 2x)	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3
ABI QuantStudio 7 Pro, 384 well (NEB Luna 4x)	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3
Bridging study 7								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 7 Flex, 384 well (NEB Luna 4x)	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3
Bridging study 8								
Bio-Rad CFX96 Touch	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**0/3**
ABI QuantStudio 12K Flex, 384 well (NEB Luna 4x)	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3
Bridging study 9								
ABI 7500 Dx Fast	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**0/3**
ABI QuantStudio Dx, 96 well	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Bridging study 10								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Roche Cobas Z480	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Bridging study 11								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI PRISM 7000	3/3	3/3	3/3	3/3	3/3	2/3	2/3	0/3
Bridging study 12								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI StepOne	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3
Bridging study 13								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI QuantStudio 7 Flex	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Bridging study 14								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Analytik Jena qTower	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Bridging study 15								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Roche LightCycler 480	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3
Bridging study 16								
Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI StepOne Plus	3/3	3/3	3/3	3/3	3/3	0/3	0/3	0/3
Bridging study 17								

Bio-Rad CFX96 Touch	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
CHAI Open qPCR	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3

The lowest concentration at which 100% of replicates were positive for the new thermocyclers was within 2X of the validated thermocycler when tested side-by-side, indicating comparable analytical performance.

The bridging studies for the QuantStudio 5 (384) and QuantStudio 7 (384) thermocyclers also included testing with the Bio-Rad Reliance and TaqPath One Step RT-PCR reaction mixtures previously validated for the 96-well thermocyclers. These results also demonstrated comparable analytical performance for these reaction mixes when used on the 384-well instruments compared to the previously validated thermocycler (highlighted in bold):

		Concentration (positive replicates)							
	RT-PCR Mix	100 copies/µL	50 copies/µL	25 copies/µL	12 copies/µL	6 copies/µL	3 copies/µL	1.5 copies/µL	0 copies/µL
Bio-Rad CFX96 Touch	**NEB Luna 2x**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**2/3**	**0/3**
ABI QuantStudio 5, 384 well	Bio-Rad Reliance	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
Bio-Rad CFX96 Touch	**NEB Luna 2x**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**	**0/3**
ABI QuantStudio 7 Pro, 384 well	Bio-Rad Reliance	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3
ABI QuantStudio 7 Pro, 384 well	TaqPath One Step	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3

Validation of the Hamilton Automated Protocol (Appendix C in the Instructions for Use)

An LoD finding study was conducted by testing gamma irradiated SARS-CoV-2 virus (BEI) spiked into saliva negative for SARS-CoV-2 at concentrations of 100, 50, 25, 12, 6, 3 and 1.5 copies/µl. Samples were tested in triplicate following Workflow Three (heat pre-treatment of 95°C for 30 minutes) followed by RT-qPCR testing in the 384-well format QuantStudio 5 with the NEB Luna 2x RT-PCR mix and the Cy5 labelled RP probe. Following, 20 replicates at 0.5x, 1x, and 2x of the preliminary LoD (6 copies/µl) were also tested in the same workflow. Results for the Hamilton automated protocol are summarized below:

	Concentration (positive replicates)		
	12 copies/µL	6 copies/µL	3 copies/µL
Automated protocol	20/20	19/20	16/20

The LoD of the SalivaDirect Assay using the Hamilton automated protocol was confirmed to be 6 copies/µl.

In addition, a trial of the Hamilton automated protocol was also conducted using 10 negative and 10 contrived positive samples with 10^6 copies/mL of gamma-irradiated SARS-CoV-2 virus, loaded next to each other in a Matrix tube rack in alternating positions. This was to simulate a worst-case scenario for potential sample cross-contamination. All negative samples remained negative for SARS-CoV-2 N1 RNA.

Validation of the Tecan Fluent 780 Automated Protocol (Appendix D in the Instructions for Use)

An LoD finding study was conducted by testing AccuPlex SARS-CoV-2 Full Genome from SeraCare Life Sciences, Inc. spiked into saliva negative for SARS-CoV-2 at concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 copies/µl. Samples were tested in triplicate using both the proposed automated sample extraction protocol as well as the manual extraction following the standard SalivaDirect protocol (Workflow One: proteinase K and heat inactivation). Lysed saliva samples were tested as per the SalivaDirect IFU RT-qPCR protocol in ABI QuantStudio 7 Pro with the NEB Luna 2x RT-PCR mix probe. Following, 20 replicates at 0.5x, 1x, and 2x of the preliminary LoD (6 copies/µl) were also transferred and tested with the same extraction protocols. Results for the Tecan Fluent 780 automated protocol are summarized below:

	Concentration (positive replicates)		
	12 copies/µL	6 copies/µL	3 copies/µL
Automated protocol	20/20	20/20	16/20
Manual Protocol (standard)	20/20	20/20	19/20

The LoD of the SalivaDirect Assay using the Tecan Fluent 780 automated protocol was confirmed to be 6 copies/µl.

In addition, a trial of the Tecan Fluent 780 automated protocol was also conducted using 10 negative and 10 contrived positive samples with 10^6 copies/mL of gamma-irradiated SARS-CoV-2 virus, loaded next to each other in a Matrix tube rack in alternating positions. This was to simulate a worst-case scenario for potential sample cross-contamination. All negative samples remained negative for SARS-CoV-2 N1 RNA.

Validation of the Tecan Fluent 480 Assisted RT-qPCR Preparation (Appendix E in the Instructions for Use)

For both 96-well and 384-well RT-qPCR plates, an LoD finding study was conducted by testing AccuPlex SARS-CoV-2 Full Genome from SeraCare Life Sciences, Inc. spiked into saliva negative for SARS-CoV-2 at concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 copies/µl. Samples

were tested in triplicate following the standard SalivaDirect protocol (Workflow One: proteinase K and heat inactivation) followed by RT-qPCR testing in the 96-well and 384-well formats in ABI QuantStudio 7 Pro with the NEB Luna 2x RT-PCR mix probe. Following, 20 replicates at 0.5x, 1x, and 2x of the preliminary LoD (6 copies/µl) were also tested in the same workflow for both well formats. Results for the Tecan Fluent 480 RT-qPCR preparation of both the 96-well and 384-wll PCR preparation are summarized below:

	Concentration (positive replicates)		
	12 copies/µL	6 copies/µL	3 copies/µL
96-well format (automated)	20/20	20/20	20/20
96-well format (manual)	20/20	20/20	20/20
384-well format (automated)	20/20	20/20	16/20
384-well format (manual)	20/20	20/20	18/20

The LoD of the SalivaDirect Assay using the Tecan Fluent 480 RT-qPCR preparation was confirmed to be 3 copies/µl for the 96-well format and 6 copies/µl for the 384-well format.

In addition, a trial of the Tecan Fluent 480 RT-qPCR preparation was also conducted using 10 negative and 10 contrived positive samples with 10^6 copies/mL of gamma-irradiated SARS-CoV-2 virus. These samples were run using both the manual and automated protocols for the 96-well and 384-well formats, with an alternating order of positive-negative samples. This was to simulate a worst-case scenario for potential sample cross-contamination. All negative samples remained negative for SARS-CoV-2 N1 RNA.

Bridging Studies for Pre-Treatment Heat Step

An LoD confirmation study was performed to validate pre-treatment heat steps. Samples were prepared by spiking positive saliva from a confirmed COVID-19 healthcare worker with a known concentration (3.7×10^4 copies/µL) into saliva collected from healthcare workers who tested negative for SARS-CoV-2. The following concentrations were tested: 6, 3, and 1.5 copies/µL, each with 20 individual replicates. All samples were tested with or without the Thermo Proteinase K and heat inactivation step. Following, all lysates were tested by the standard SalivaDirect RT-qPCR protocol with the NEB Luna kit on the CFX96 Touch PCR instrument:

Pre-Treatment Heat step prior to SalivaDirect protocol without the addition of Proteinase K and heat inactivation step

	Concentration (positive replicates)		
	6 copies/µL	3 copies/µL	1.5 copies/µL
65°C for 15 minutes	20/20	20/20	18/20

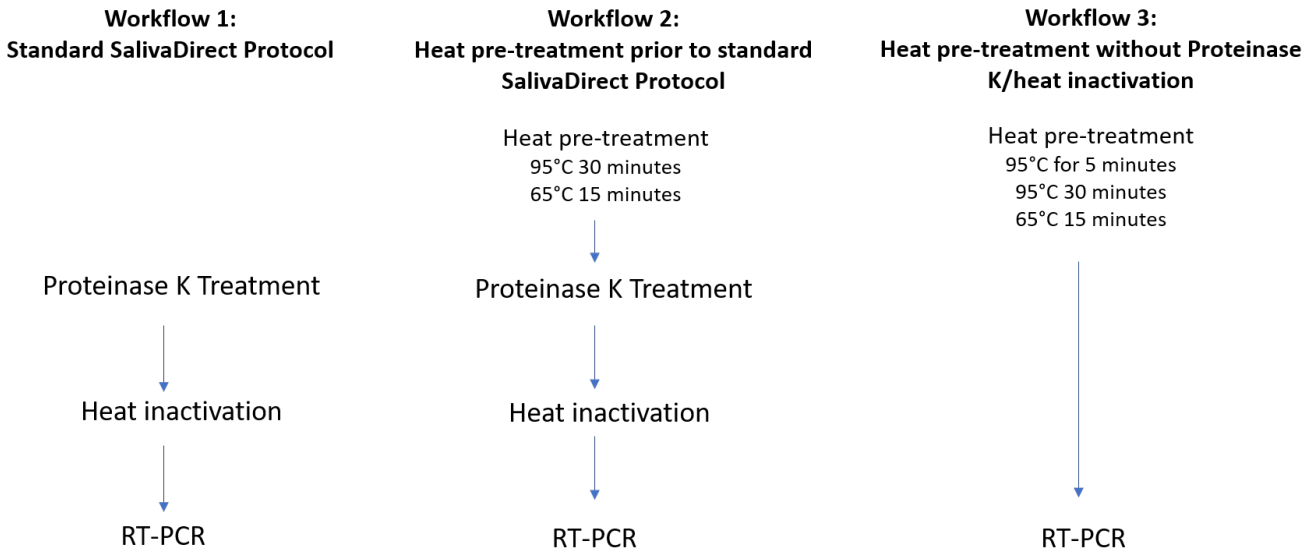
95°C for 5 minutes	20/20	19/20	18/20
95°C for 30 minutes	20/20	15/20	14/20

The LoD when utilizing a Pre-treatment heat step at the above conditions without the Proteinase K and heat inactivation step confirms to be 3-6 copies/µl, which is comparable to the standard SalivaDirect protocol.

Pre-Treatment Heat step prior to standard SalivaDirect protocol with Proteinase K and heat inactivation step

	Concentration (positive replicates)		
	6 copies/µL	3 copies/µL	1.5 copies/µL
65°C for 15 minutes	20/20	17/20	15/20
95°C for 30 minutes	20/20	16/20	19/20

The LoD when utilizing a Pre-treatment heat step at the above conditions prior to the standard SalivaDirect protocol with the Proteinase K and heat inactivation confirms to be 6 copies/µl, which is comparable to the standard SalivaDirect protocol. Below is an illustrative summary of the workflows including the heat pre-treatment steps:



2. Analytical Inclusivity/Cross Reactivity

The sequences for the N1 primers and probe used in this assay are identical to the primer/probe sequences used in the FDA authorized CDC SARS-CoV-2 assay. Please refer to EUA200001/A004 for an updated in silico analysis of the primers/probes used with the CDC assay.

In addition, SalivaDirect was tested on 52 saliva specimens collected from adults during the 2018/2019 and 2019/2020 (pre-COVID19) autumn/winter influenza seasons. Out of the 52 specimens tested, 51 resulted as negative, and one resulted as invalid (both N1 and RP were not detected).

3. Clinical Evaluation

Individual Sample Testing

Performance in a population suspected of COVID-19

Performance of SalivaDirect was compared to the authorized ThermoFisher Scientific TaqPath RT-PCR COVID-19 combo kit by testing paired nasopharyngeal and saliva samples. Nasopharyngeal swabs and saliva were collected from inpatients and healthcare workers in the Yale-New Haven Hospital. Saliva was collected in sterile urine cups or 5 mL tubes without addition of any preservatives.

For the preliminary selection of specimens, specimens were tested with a modified version of the US CDC assay. Based on these results, a total of 67 NP/saliva pairs were tested for the current study, with 37 being NP positive and 30 being NP negative by the modified CDC assay. These NP and saliva specimens were subsequently tested in parallel with the EUA-authorized TaqPath COVID-19 combo kit (on NP specimens) and SalivaDirect (on saliva specimens). The ThermoFisher Scientific TaqPath COVID-19 combo kit combines RNA extraction using the MagMax Viral/Pathogen Nucleic Acid Isolation Kit with a multiplex RT-PCR diagnostic assay targeting 3 regions of the SARS-CoV-2 genome. For SalivaDirect testing, the ThermoFisher Scientific proteinase K, ThermoFisher Scientific TaqPath RT-PCR kit, and Bio-Rad CFX96 Touch instrument were utilized.

Out of the 37 NP specimens that originally tested positive by the modified CDC assay, 34 tested positive with the TaqPath COVID-19 Combo Kit and three tested negative. The TaqPath results from these 34 specimens were used as the comparator for the SalivaDirect when evaluating positive percent agreement (PPA). All 30 NP specimens that were negative by the original modified CDC assay also tested negative by the TaqPath assay. The results from these 30 specimens plus the three TaqPath negative NP specimens described above were used as the comparator for the SalivaDirect when evaluating negative percent agreement (NPA). The results from this paired study are described below:

Qualitative outcome of parallel testing of paired nasopharyngeal swabs and saliva with SalivaDirect and the ThermoFisher Scientific TaqPath COVID-19 combo kit.

		TaqPath RT-PCR COVID-19	
		Nasopharyngeal swab	
		Positive	Negative
SalivaDirect Saliva	Positive	32	3
	Negative	2	30
Total		34	33
Positive agreement = 94.1% (32/34) Negative agreement = 90.9% (30/33)			

Out of the 34 individuals with nasopharyngeal swab specimens that tested positive by the TaqPath COVID-19 kit, 32 had saliva specimens that were positive by the SalivaDirect, yielding a PPA of 94.1%. Out of the 33 individuals with negative NP swab specimens by the TaqPath assay, 30 had saliva specimens that were negative by SalivaDirect, generating an NPA of 90.9%. There were three individuals who tested positive by SalivaDirect on saliva specimens but negative by TaqPath on NP specimens. It should be noted that these 3 individuals previously tested weakly positive with the modified CDC assay.

As an additional analysis, the results from the SalivaDirect on saliva specimens were compared to the results from the modified CDC assay on the paired NP specimens. This modified CDC assay used the 2019-nCoV_N1, 2019-nCoV_N2, and RP primer-probe sets with the NEB Luna Universal Probe One-Step RT-qPCR kit on the Bio-Rad CFX96 Touch. The SalivaDirect results were concordant with 94.6% (35/37) of the NP positive results and 100% of the NP negative results, as shown below:

		Modified CDC RT-PCR	
		Nasopharyngeal swab	
		Positive	Negative
SalivaDirect Saliva	Positive	35	0
	Negative	2	30
Total		37	30
Positive agreement = 94.6% (35/37) Negative agreement = 100% (30/30)			

Performance in an Asymptomatic Screening Population

To validate the SalivaDirect test for SARS-CoV-2 detection in a screening population, paired nasopharyngeal and saliva samples were collected from asymptomatic individuals enrolled in a routine SARS-CoV-2 testing program. Paired nasopharyngeal and saliva samples were collected at the same sampling moment from 20 consecutive individuals who tested positive and 100

consecutive individuals who tested negative. Paired samples were collected on the same day as initial sample collection and diagnosis while all individuals were still asymptomatic. Saliva samples were tested using the SalivaDirect test with the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit on the QuantStudio 7 Pro PCR instrument while nasopharyngeal swab specimens were tested using an FDA EUA-authorized high sensitivity testing platform for SARS-CoV-2 detection. A total of 45% of the nasopharyngeal swab specimens tested were low positives according to the EUA authorized comparator assay. Results between the two sample types were 100% concordant:

		EUA-authorized comparator	
		Nasopharyngeal swab	
		Positive	Negative
SalivaDirect Saliva	Positive	20	0
	Negative	0	100
Total		20	100
Positive agreement = 100% (20/20) (95% CI: 83.89%, 100%) Negative agreement = 100% (100/100) (95% CI: 96.3%, 100%)			

These results indicate acceptable performance of the SalivaDirect assay in an asymptomatic screening population.

Pooled Sample Testing

Deidentified saliva samples of known Ct value (as previously tested using the SalivaDirect test with the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit on the CFX96 Touch) were pooled with four saliva samples from asymptomatic individuals (which previously tested negative for the SARS-CoV-2 N1 target when tested using the SalivaDirect test with the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit on the CFX96 Touch) and tested in a pooling validation study using workflow 1.

A total of 50 µL of each individual saliva sample (1x positive, 4x negative) was pooled together then run through the standard SalivaDirect protocol (Workflow 1, above). These pooled samples were run alongside the individual samples to evaluate the positive percent agreement between pooled and individual results.

Ten of the positive individual results were derived from low positive samples with Ct values that were within 0-2.5 Ct of the observed mean Ct at the established LOD when using the Thermo Proteinase K and the NEB Luna 2x RT-qPCR kit and testing on the CFX96 Touch. Ct-distribution of the 22 samples tested through the standard SalivaDirect protocol was as follows:

Ct range*	Pooled Testing Workflow A, number (%) of 22 samples
20.0-29.9	7 (32%)
30.0-34.9	5 (23%)
35.0-40.0	10 (45%)

*samples <40 Ct are considered positive on the CFX96 Touch
*mean Ct for the CFX96 instrument when determining LOD for analytical sensitivity using this set of reagents was 36.7

The SalivaDirect pooled protocol resulted in a positive percent agreement of 86.4% (19/22, 95% CI: 66.67%, 95.25%) compared to the individual testing results using the standard SalivaDirect protocol (Workflow 1). The three positive samples that yielded false negative results upon pooled testing had individual Ct values of 37.7, 38, and 39.43.

Overall, a theoretical Ct shift of $Log_2(n)$ can be estimated for most RT-PCR tests due to the dilution of positive samples when pooled with negative samples. This means that for pools of n=5, a Ct shift of 2.3 would be expected. In this study, regression analysis of the wet testing results indicated that a Ct value shift of 1.99 was observed upon 5-sample pooling, confirming a slight loss of assay sensitivity. To assess the clinical impact of this loss in sensitivity with pooled testing, an *in silico* analysis was conducted using historical data. In this analysis, a Ct shift of 1.99 was applied to the individual positive results from this historical dataset to determine the percent of positive results that would remain positive upon 5-sample pooling.

A total of 613 historical positive results from six different high complexity CLIA labs (representing four different geographical locations) designated to run the SalivaDirect test were used in this analysis. When a Ct shift of 1.99 was applied to the Ct values obtained from these samples, the percent of samples returning a Ct value under the cut-off for individual sample testing of 45 was evaluated. Out of the 613 results, all samples would have Ct values remaining under the cutoff of 45 after applying this shift. This corresponds to a PPA of 100% (613/613, 95% CI: 99.38%, 100.0%) between pooled and individual testing.

4. Human Usability Study for SalivaDirect Unsupervised Collection Kit

A total of 30 participants between the ages of 20 and 80 years who represented a range of racial and educational backgrounds were enrolled in this study. Study demographics are presented below:

Category	**n (%)**
Sex	
Male	11 (37)
Female	19 (63)
Age	
18-29	7 (23)
30-39	16 (53)

Category	**n (%)**
40-49	4 (13)
50-59	0 (0)
60-69	1 (3)
70+	2 (7)
Education	
High School/GED	2 (7)
Bachelors	7 (23)
Masters	10 (33)
PhD/MD	11 (37)
Race	
Black/African American	4 (13)
Hispanic/Latino	4 (13)
Asian/South Asian	6 (20)
White	15 (50)
Native American	1 (3)

Individuals who had previously provided a saliva sample, who had relevant, career-level laboratory experience, or who were experiencing symptoms of respiratory infection were excluded from enrollment. Once informed consent was provided, participants received a collection kit containing (1) the four devices for obtaining a saliva specimen, (2) corresponding collection instructions, (3) a biohazard bag, and (4) five alcohol wipes. Participants self-collected four saliva samples consecutively and in a randomized order. Members of the study team observed these collections via a video platform. The observer turned off the camera and audio on their device for the duration of the four collections. Both the observer and the participant completed a survey about their experience following each collection, scoring responses on a scale of 1 (strongly disagree) to 5 (strongly agree). All of the samples (n = 120) were tested for SARS-CoV-2 using SalivaDirect. A laboratory survey assessing the sample quality was completed by the technician during testing.

In 100% of the observed collections, study participants appeared confident in their ability to complete the collection correctly. The majority of participants (93%) understood the importance of following the instructions carefully to avoid incorrect test results, and during only two collections (1.67%), participants appeared to not adequately follow these instructions for proper sample collection. Results for the questions in the observer survey are summarized below:

	Collection device feed-back (1 = strongly disagree, 5 = strongly agree)	**Straw**	**Pipette Tip**	**Funnel**	**Bulb Pipette**
1	Did the study participant read the instructions?	4.93	4.93	5.00	4.97
2	Did the study participant appear confident in their ability to follow the instructions?	4.20	4.30	4.30	4.40
3	Did the study participant properly wash their hands before and after sample collection?	4.20	4.27	4.37	4.60
4	Did the study participant appear to properly follow instructions for sample collection set up?	4.57	4.60	4.63	4.53
5	Did the study participant appear to properly follow instructions for adequate sample collection?	4.63	4.30	4.67	4.43
8	Did the study participant securely fasten the collection tube?	4.90	4.97	4.90	5.00
9	Did the study participant clean down the outside of the sample tube following collection?	4.93	4.97	4.77	4.97
10	Did the study participant properly store their sample in the biohazard bag after collection?	4.07	4.10	4.30	4.13
11	Did the study participant appear to struggle with any particular step? If so, explain which.	1.46	1.79	1.54	1.96

The secondary objective was to compare the quality of samples collected using each device. True saliva, which naturally pools in the mouth, can be easily handled in the laboratory. In contrast, saliva samples that are improperly collected may be problematic. It was found that every sample could be tested for SARS-CoV-2 with SalivaDirect. The internal control, RNaseP was detected in 100% of the samples collected with each of the devices, indicating an adequate specimen was collected. Laboratory survey responses confirmed that 100% of the samples were easy to pipette and of sufficient volume. Slight discoloration was noted in 18 samples (15%) and food particles were observed in 20 samples (5 participants, 16.7%), but this did not affect test results. No sample tested positive for SARS-CoV-2. Results for the questions in the laboratory survey are summarized below:

Lab questions (1 = strongly disagree, 5 = strongly agree)	Straw	Pipette Tip	Funnel	Bulb Pipette	Average
The sample was of sufficient volume (200-500 ul)	4.97	5	5	5	4.99
The sample was easy to pipette	4.87	4.87	4.87	4.87	4.87
The sample was normal, true saliva	4.87	4.87	4.87	4.87	4.87
The sample was free from food particles	4.76	4.76	4.76	4.76	4.76
The sample was not unusually discolored	4.80	4.87	4.83	4.80	4.83

Lab questions (1 = strongly disagree, 5 = strongly agree)	Straw	Pipette Tip	Funnel	Bulb Pipette	Average
The sample tested positive for human RNAse P	5.00	5.00	5.00	5.00	5.00
The sample tested positive for SARS-CoV-2	0	0	0	0	0
If the sample tested positive for SARS-CoV-2, this was reported back to the study participant	NA	NA	NA	NA	NA

The results from this study demonstrate that users are able to comprehend the instructions for the four different saliva collection devices as well as collect an adequate specimen for SARS-CoV-2 testing with the SalivaDirect.

FDA SARS-CoV-2 Reference Panel Testing

The evaluation of sensitivity and MERS-CoV cross-reactivity was performed using reference material (T1), blinded samples and a standard protocol provided by the FDA. The study included a range finding study and a confirmatory study for LoD. Blinded sample testing was used to establish specificity and to confirm the LoD. For the study, the ThermoFisher Scientific proteinase K, ThermoFisher Scientific TaqPath RT-PCR kit, and Bio-Rad CFX96 Touch instrument were utilized. The results are summarized in the following Table.

Summary of LoD Confirmation Result using the FDA SARS-CoV-2 Reference Panel

Reference Materials Provided by FDA	**Specimen Type**	**Product LoD**	**Cross- Reactivity**
SARS-CoV-2	Saliva	$1.8x10^4$ NDU/mL	N/A
MERS-CoV		N/A	ND

NDU/mL = RNA NAAT detectable units/mL

N/A: Not applicable

ND: Not detected

LIMITATIONS

- Negative results for SARS-CoV-2 RNA from saliva should be confirmed by testing of an alternative specimen type if clinically indicated.
- The performance of this test was established based on the evaluation of a limited number of clinical specimens. Clinical performance has not been established in all circulating variants but is anticipated to be reflective of the prevalent variants in circulation at the time and location of the clinical evaluation. Performance at the time of testing may vary depending

on the variants circulating, including newly emerging strains of SARS-CoV-2 and their prevalence, which change over time.

WARNINGS

- This product has not been FDA cleared or approved, but has been authorized for emergency use by FDA under an EUA for use by authorized laboratories;
- This product has been authorized only for the detection of nucleic acid from SARS-CoV-2, not for any other viruses or pathogens; and
- The emergency use of this product is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostics for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Federal Food, Drug and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the declaration is terminated or authorization is revoked sooner.