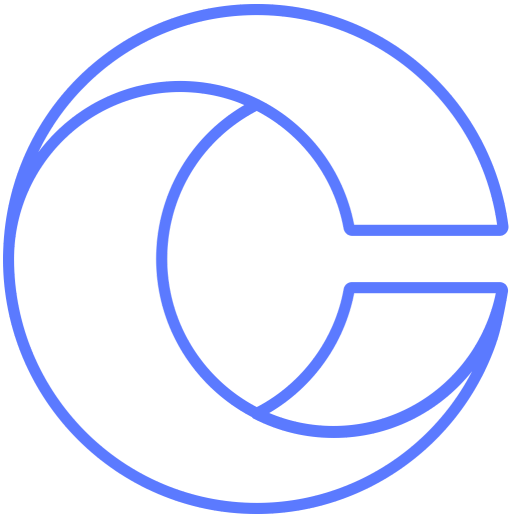
Chronomics Protocol Guide v2.0.0

SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit



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-20°C Store at -20°C

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IVD For In Vitro Diagnostic Use

CHRONOMICS

Intended use

The SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit is intended to be used for the detection of SARS-CoV-2 genomic RNA extracted from various biological specimens, including saliva. The inhibitor tolerant qRT-PCR mix provided in the kit is a one-step solution designed for amplification of the SARS-CoV-2 RNA targets and does not contain an internal reference dye. Included in the kit is a multiplex of primer / probe assays which target the nucleocapsid gene (assay N1) and the envelope gene (assay E) of SARS-CoV-2; and the human RPP30 gene (assay RP) used as internal RNA extraction control. Two plasmid controls are also included in the kit to confirm functionality of the assays and the qRT-PCR reaction: the RPP30 Negative Control and the combined 2019-nCoV nucleocapsid gene and 2019-nCoV envelope gene Positive Control. The kit also contains ROX reference dye at 10x concentration for use with real-time quantitative PCR (qPCR) instruments that require it.

Precautionary statements

This product should be handled only by those persons who have been trained in laboratory techniques and it should be used in accordance with the principles of good laboratory practice.

Avoid contact with eyes, skin and clothing. Avoid inhalation and ingestion. See MSDS at www.chronomics.com for more details.

Kit contents

Reagent	Volume (1,000 rxns)	Volume (10,000 rxns)	Storage
SaliVIR qRT-PCR Master Mix	4 x 1.5 ml	2 x 30 ml	- 20°C
Multiplex primer / probe mix	2 x 1.5 ml	1 x 30 ml	- 20°C
RPP30 Negative Control	1 x 0.5 ml	2 x 1 ml	- 20°C
2019-nCoV nucleocapsid and envelope genes Positive Control	1 x 0.5 ml	2 x 1 ml	- 20°C
ROX Reference Dye (10x)	1 x 240 µl	2 x 1.2 ml	+4°C

Required equipment

- Class II Biological safety cabinet
- Single and/or multichannel pipettes (10, 100, 200, 1000 µl)
- PCR-clean filtered tips
- 1.5 / 2 ml cold block (or access to ice)
- 96 well cold block (or access to ice)
- qPCR instrument (4 colour)
- 96 well plate and optical seal compatible with qPCR instrument
- 96 well plate compatible vortexer
- 96 well plate compatible minifuge or centrifuge

Additional user supplied consumables

- Molecular biology grade water

Storage and handling

Upon receipt, store all reagents at -20°C. All reagents (with the exception of the ROX Reference Dye) are stable at -20°C for 12 months.

Thaw the SaliVIR qRT-PCR Master Mix on ice and keep on ice at all times. Thaw the multiplex primer / probe mix at room temperature in the dark and then keep on ice throughout the qRT-PCR setup preparation. After thawing, ensure that all reagents are mixed briefly by vortexing and then spun down. Avoid repeated freeze / thawing whenever possible.

Handling of plasmid controls

The RPP30 Negative Control and 2019-nCoV nucleocapsid and envelope genes Positive control consist of plasmids containing the human RPP30 gene; and the nucleocapsid and envelope genes from SARS-CoV-2 respectively and are provided at a concentration of 200 copies/µl. When thawed for the first time, it is recommended to aliquot out the entire amount of both controls in single use aliquots in order to minimize freeze / thaw cycles. For each qRT-PCR run, 5 µl of each control are used (i.e. 5 µl per reaction = 1000 copies per reaction).

qRT-PCR setup procedure

Table 1 outlines the required volumes of sample / control and reagents needed to set up the qRT-PCR reaction. Each qRT-PCR run should include the No Template Control (i.e. molecular biology grade water), the RPP30 Negative Control and the 2019-nCoV nucleocapsid and envelope genes Positive Control. All procedures should be carried out in a sterile environment, ideally a Class II biosafety cabinet. Thaw reagents as described above and setup the reaction on ice (or on cold block).

If ROX Reference Dye is required, follow Table 2 for instruments requiring low ROX and Table 3 for instruments requiring high ROX.

Table 1. qRT-PCR reaction mix setup volumes for a single reaction. When preparing a mix for multiple reactions, include a 5% overage for each reagent.

Reagent	Volume
SaliVIR qRT-PCR Master Mix	5 µL
Multiplex primer / probe mix	2.5 µL
Template*	10 µL
Water*	2.5 µL
Total	**20 µL**

* As specified in the 'Handling of plasmid controls' section, the reactions for the Negative and Positive plasmid controls will have 5 µL of the control template (and, therefore, 7.5 µL of water).

Table 2. qRT-PCR reaction mix setup volumes for a single reaction with low ROX. When preparing a mix for multiple reactions, include a 5% overage for each reagent.

Reagent	Volume
SaliVIR qRT-PCR Master Mix	5 µL
Multiplex primer / probe mix	2.5 µL
Template*	10 µL
ROX Reference Dye (10x)	0.2 µL
Water*	2.3 µL
Total	**20 µL**

* As specified in the 'Handling of plasmid controls' section, the reactions for the Negative and Positive plasmid controls will have 5 µL of the control template (and, therefore, 7.3 µL of water).

Table 3. qRT-PCR reaction mix setup volumes for a single reaction with high ROX. When preparing a mix for multiple reactions, include a 5% overage for each reagent.

Reagent	Volume
SaliVIR qRT-PCR Master Mix	5 µL
Multiplex primer / probe mix	2.5 µL
Template*	10 µL
ROX Reference Dye (10x)	2 µL
Water*	0.5 µL
Total	**20 µL**

* As specified in the 'Handling of plasmid controls' section, the reactions for the Negative and Positive plasmid controls will have 5 µL of the control template (and, therefore, 5.5 µL of water).

NOTE: the volume of water and template can be adjusted to include more template in the reaction mix as required. After setting up the qRT-PCR plate, ensure that the reaction mixes inside the wells are properly mixed by vortexing the plate and then spinning it down.

qRT-PCR setup and cycling conditions

Set up the qPCR instrument using manufacturers guidelines. Where possible, choose the Quantitation by Comparative Ct (ΔΔCt) method with TaqMan or "Other" reagents (do not add a melt curve option). For qPCR instruments with "FAST" blocks, select the Standard ramp speed. Select the FAM filter for the N1 target, the HEX / VIC filter for the RP target and the Cy5 filter for the E target. If possible, select NFQ-MGB as quencher; or alternatively leave this field empty. Do not select a dye as quencher (such as TAMRA). If required, select ROX as reference dye. Select Program cycling conditions as shown in Table 4 below. Set reaction volume to 20 µl.

Table 4. qRT-PCR Program cycling conditions

Step	Cycles	Temperature	Time
1	1	50 °C	10 min
2	1	95 °C	2 min
3	45	95 °C	5 s
4		62 °C	30 s

NOTE: fluorescence acquisition is performed at step 4.

qRT-PCR data analysis and interpretation

Please note data analysis may vary between qPCR machines and thresholds must be determined empirically by the end user or laboratory. We recommend setting the Baseline start cycle at 5 and the end cycle at 15; and the threshold at 200 RFU or 0.02 ΔCt.

As per CDC guidelines, Ct values that fall below the 40 cycles threshold are considered positive signals. Refer to Table 5 below for interpretation of results from control and patient samples.

Table 5. Interpretation of results from control and patient samples

Sample	RP Result	N1 Result	E Result
RPP30 Negative Control	+	-	-
2019-nCoV nucleocapsid and envelope genes Positive Control	-	+	+
Positive patient sample	+	+	+
	-	+	+
Negative patient sample	+	-	-
Inconclusive patient sample	+	+	-
	+	-	+
	-	+	-
	-	-	+
Failed patient sample	-	-	-

Kit specification and performance

Application	Qualitative PCR test for detection of SARS-CoV-2 nucleocapsid (N) and envelope (E) genes
Type of detection	Ribonucleic acid (RNA) of SARS-CoV-2
Sample type	Saliva
qRT-PCR Limit of Detection (LoD)	$1x10^0$ copies
End-to-end* Limit of Detection (LoD)	750 copies/ml
Analytical specificity^	100%

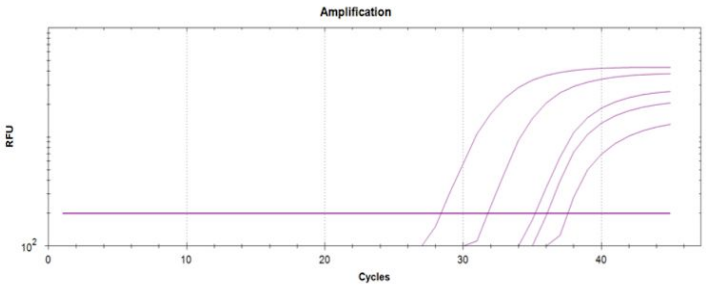
*Used in combination with the SaliVIR OME Collect kit and the SaliVIR Bead Xtract Viral RNA/DNA Kit.
^The SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit comprises only primers and probes designed by the CDC from the CDC 2019-Novel Coronavirus (2019-CoV) Real-Time RT-PCR Diagnostic Panel (assays N1 and RP) and by Cortman et al. from the Charité-Berlin WHO protocol (assay E) without any changes. Analytical specificity (cross-reactivity) of these diagnostic panels has been previously established.

qRT-PCR Limit of Detection (LoD) testing

To understand the limit of detection (LoD) for the SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit we undertook the following experiment to calculate the minimum copies of viral template RNA which can be detected in the qRT-PCR reaction.

Synthetic SARS-CoV-2 RNA (ORF, E, N; ATCC® VR- 3276SD™) was used in a serial dilution experiment (Table 6) to establish a limit of detection of SaliVIR qRT-PCR Master Mix using E and N1 primer and probe mixes on a BioRad CFX96 qPCR instrument.

E assay



N1 Assay

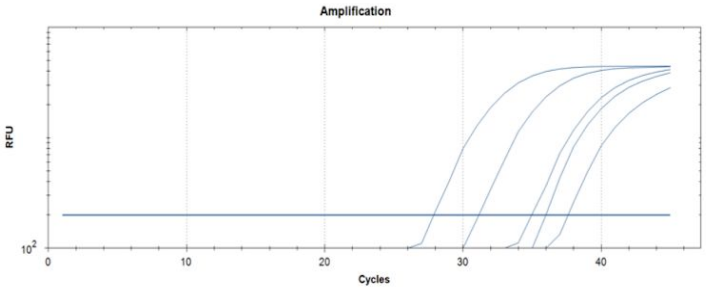


Table 6. Summary of qRT-PCR LoD testing results for the SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit.

Total copies	E assay			N1 assay		
	Positive replicates	Mean Ct	SD Ct	Positive replicates	Mean Ct	SD Ct
1000	100% (3/3)	28.25	0.10	100% (3/3)	27.78	0.14
100	100% (3/3)	31.49	0.22	100% (3/3)	30.95	0.22
10	100% (3/3)	35.12	0.11	100% (3/3)	34.48	0.43
5	100% (3/3)	36.09	0.42	100% (3/3)	35.94	0.77
1	100% (3/3)	37.05	0.60	100% (3/3)	37.27	0.52
0	0% (0/3)	NaN	NaN	0% (0/3)	NaN	NaN

End-to-end Limit of Detection (LoD) testing

The end-to-end LoD was determined by spiking heat-inactivated SARS-CoV-2 (ATCC® VR-1986HK™) into negative saliva matrices collected using the SaliVIR OME Collect kit. A 2-3X dilution series at viral load concentrations equivalent to 100,000 copies/ml to 250 copies/ml was performed (with four replicates per concentration, Table 7). The samples containing the RNA spike were subsequently extracted using the SaliVIR Bead Xtract Viral RNA/DNA kit and eluted in 100µl of elution buffer. 10µl of elution were processed according to SaliVIR COVID19 Rapid Multiplex qRT-PCR kit protocol in a BioRad CFX96 qPCR instrument.

Table 7. Summary of end-to-end LoD testing results for the SaliVIR Rapid Multiplex COVID19 vRNA Test using the SaliVIR OME Collect kit.

Concentration of heat-inactivated SARS-CoV-2 (copies/ml)	E assay			N1 assay		
	Positive replicates	Mean Ct	SD Ct	Positive replicates	Mean Ct	SD Ct
100,000	100% (4/4)	27.4629	0.0994	100% (4/4)	25.6513	0.0205
50,000	100% (4/4)	28.5101	0.1168	100% (4/4)	26.6564	0.1043
20,000	100% (4/4)	30.0263	0.3468	100% (4/4)	28.1600	0.1047
10,000	100% (4/4)	30.6094	0.4766	100% (4/4)	28.8211	0.2725
5,000	100% (4/4)	31.7376	0.4179	100% (4/4)	30.0309	0.1552
2,500	100% (4/4)	33.0549	0.1456	100% (4/4)	31.2977	0.2840
1,000	100% (4/4)	35.0624	0.7102	100% (4/4)	33.6436	1.1563
500	100% (4/4)	35.0674	0.2392	100% (4/4)	33.6343	0.6839
250	100% (4/4)	36.3935	0.5589	100% (4/4)	34.5248	1.1946
0	0% (0/4)	NaN	NaN	0% (0/4)	NaN	NaN

In order to confirm the final end-to-end LoD concentration, 20 replicates were tested, following the same protocol, at 1000 copies/ml, 750 copies/ml and 500 copies/ml respectively (Table 8). These experiments confirmed that the end-to-end LoD of the SaliVIR Rapid Multiplex COVID19 vRNA Test is 750 copies/ml.

Table 8. Summary of results to confirm the end-to-end LoD for the SaliVIR Rapid Multiplex COVID19 vRNA Test using the SaliVIR Collect OME kit.

Concentration of heat-inactivated SARS-CoV-2 (copies/ml)	E assay			N1 assay		
	Positive replicates	Mean Ct	SD Ct	Positive replicates	Mean Ct	SD Ct
1,000	100% (20/20)	35.2975	0.4749	100% (20/20)	32.4864	0.3996
750	100% (20/20)	36.0127	0.6511	100% (20/20)	33.5971	0.5478
500	40% (8/20)	35.5145	6.5222	85% (17/20)	34.7627	1.4976