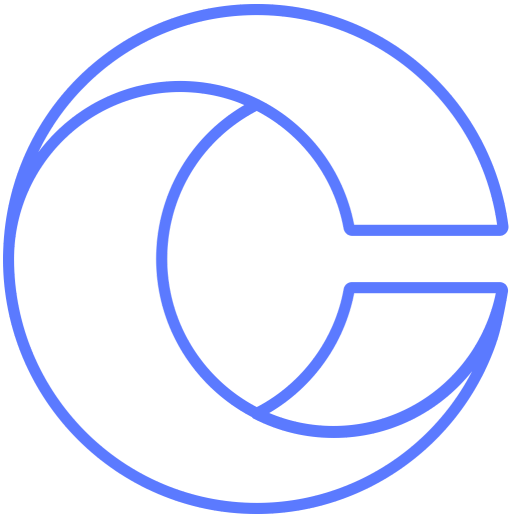
Chronomics Protocol Guide v1.0.1

SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit



Made in the UK. Chronomics Limited,
1 St James Court, Norwich, NR3 1RU, UK.

-20°C Store at -20°C

REF C1COV00700
C1COV00800

CE

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 and nasopharyngeal swabs stored in viral transport medium. 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	5 µL
Water	7.5 µL
Total	**20 µL**

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	5 µL
ROX Reference Dye (10x)	0.2 µL
Water	7.3 µL
Total	**20 µL**

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	5 µL
ROX Reference Dye (10x)	2 µL
Water	5.5 µL
Total	**20 µL**

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	Upper and lower respiratory tract specimens (such as saliva, sputum and nasopharyngeal swabs)
qRT-PCR Limit of Detection (LoD)	$1x10^0$ copies
End-to-end* Limit of Detection (LoD)	750 copies/ml
Analytical specificity^	100%

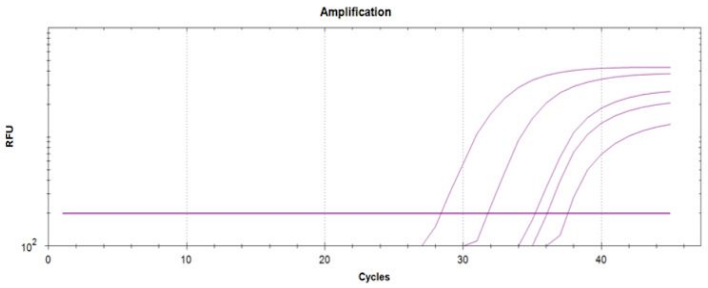
*Used in combination with the SaliVIR Collect Kit and the SaliVIR Bead Xtract Viral RNA/DNA Kit.
^The SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit comprises only primers and probes designed by the CDC from the CDC 2019-Novel Coronavirus (2019-CoV) Real-Time RT-PCR Diagnostic Panel (assays N1 and RP) and 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

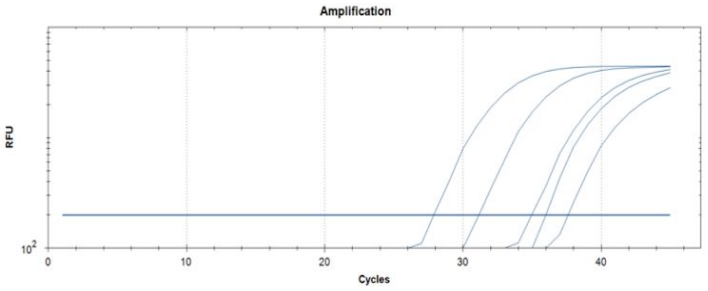


Table 6. Summary of qRT-PCR LoD testing results for the SaliVIR COVID19 Rapid Multiplex qRT-PCR Kit.

Total copies	E assay			N1 assay		
	Positive replicates	Mean Ct	StDev	Positive replicates	Mean Ct	StDev
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 SARS-CoV-2 RNA (ORF, E, N; ATCC® VR- 3276SD™) into negative saliva matrices collected using the SaliVIR Collect Kit. A 2-3X dilution series at viral load concentrations equivalent to 2,500 copies/ml to 200 copies/ml was performed (with three replicates per concentration, Table 7). The samples containing the RNA spike in were subsequently extracted using the SaliVIR Bead Xtract Viral RNA/DNA kit and eluted in 50µl of elution buffer. 5µl of elution were processed according to this 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.

Copies / ml	E assay			N1 assay		
	Positive replicates	Mean Ct	StDev	Positive replicates	Mean Ct	StDev
2500	100% (3/3)	35.04	1.01	100% (3/3)	35.41	0.82
1000	100% (3/3)	36.50	1.83	100% (3/3)	36.00	0.64
500	100% (3/3)	37.23	1.20	100% (3/3)	37.49	2.09
200	0% (0/3)	NaN	NaN	100% (3/3)	38.19	0.88
0	0% (0/3)	NaN	NaN	0% (0/3)	NaN	NaN

In order to confirm the final end-to-end LoD concentration, 20 replicates were tested, following the same protocol, at 750 copies/ml and 500 copies/ml respectively (Table 8). This study confirmed that the end-to-end LoD of the SaliVIR Rapid Multiplex COVID19 vRNA Test is 750 copies/ml (20/20 replicates positive for SARS-CoV-2).

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

Copies / ml	E assay			N1 assay		
	Positive replicates	Mean Ct	StDev	Positive replicates	Mean Ct	StDev
750	100% (20/20)	36.22	0.68	100% (20/20)	36.38	1.00
500	85% (17/20)	36.75	1.52	100% (20/20)	37.44	1.17