



ProbeSure™ COVID -19
One Step RT-PCR Kit -
Singleplex
User Guide

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1. Product details

The ProbeSure™ COVID-19 One Step RT-PCR Singleplex kit utilises three separate assays, all labelled with FAM, to detect the nucleocapsid genes N1 and N2 and the RNase P control. The plate reader or real-time instrument used must be capable of fluorescence detection of FAM and ROX (passive reference). Using the Singleplex kit, each RNA sample to be analysed is assayed in three PCR plate wells, i.e., one reaction for N1, one reaction for N2 and one reaction for RNase P.

Part number	Master mix	Assay configuration	Number of assays supplied with kit	Assays in kit	Fluorescent Label	Number of assays per well	Mix volume	Reactions	Reaction volume	ROX level
COV-1001-1	2x ProbeSure™ COVID-19 One Step RT-PCR	Singleplex	3	N1	FAM	1	1 mL	100	20 µL	Low (25 nM)
				N2	FAM					
				RNase P	FAM					
COV-2001-1	2x ProbeSure™ COVID-19 One Step RT-PCR	Singleplex	3	N1	FAM	1	1 mL	100	20 µL	High (500 nM)
				N2	FAM					
				RNase P	FAM					
COV-1010-1	2x ProbeSure™ COVID-19 One Step RT-PCR	Singleplex	3	N1	FAM	1	10 mL	1000	20 µL	Low (25 nM)
				N2	FAM					
				RNase P	FAM					
COV-2010-1	2x ProbeSure™ COVID-19 One Step RT-PCR	Singleplex	3	N1	FAM	1	10 mL	1000	20 µL	High (500 nM)
				N2	FAM					
				RNase P	FAM					

2. Description

ProbeSure™ COVID-19 One Step RT-PCR Singleplex Kit is intended for the qualitative detection of RNA of the novel Coronavirus (SARS-CoV-2) in human respiratory samples. The kit contains assays for two target sequences in the nucleocapsid (N) gene region and an internal RNase P control assay. The three assays are amplified and detected in separate reactions. The kit is specifically formulated for endpoint fluorescent detection but works well in real-time detection as well.

3. Storage and shelf life

- All components must be stored at -20 °C upon arrival.
- Repeated freeze / thaw cycles of reagents should be avoided since this might affect the performance of the kit. Reagents should be frozen in aliquots if they are to be used intermittently.
- Contents can be stored at 4°C for short term storage (and protected from light), whilst carrying out the work.

4. Safety warnings and precautions

This product should be handled only by trained laboratory personnel. It is advisable to wear suitable personal protective equipment (PPE) when using the product. In case of contact with skin or eyes, wash immediately with water.

5. Kit components

- ProbeSure™ one step RT-PCR Master Mix (2x)
- 20x Assay (Blue lid) for N Gene target N1 (reports with FAM)
- 20x Assay (Yellow lid) for N Gene target N2 (reports with FAM)
- 20x Control Assay (Orange lid) for RNase P (reports with FAM)

Not included in the kit

- PCR-grade water
- Template RNA
- Positive / negative controls
- PCR plate and optically clear seal

6. ROX compatibility

ProbeSure™ COVID-19 One Step RT-PCR Kit is supplied with ROX as a passive reference dye. Please ensure compatibility between the ROX level of the master mix and the qPCR machine. Should you require further assistance about ROX level selection, please contact your reader manufacturer or 3CR Bioscience.

7. Primer and probe design

All primers and probes used to make N1, N2 and RNase P assays in this kit are CDC approved for COVID-19 detection and no similarity with other SARS/Coronavirus strains have been reported.

8. Controls

To increase confidence in the data, control samples should be used on the PCR plate in addition to the test samples. Negative controls (no-template controls, NTCs) consisting of the same buffer used to hydrate the RNA samples or PCR grade water, should be dispensed into few wells of the PCR plate. Positive and negative COVID-19 controls can also be used if available.

When viewing the genotyping data, NTCs should show no amplification and remain at the origin of the cluster plot (see *Figure 1*), giving confidence that any amplification observed is real. Any amplification observed in the NTC wells would indicate contamination or non-specific amplification. The positive control samples should cluster in the expected regions for their genotype.

9. Genotyping procedure

Ensure the components are completely thawed; mix and centrifuge briefly prior to use. The enzymes used in ProbeSure™ One Step RT-PCR Master Mix are modified such that they are completely inactive at ambient temperature. Such inactivation allows bench top reaction assembly without leading to primer-dimer issues and other side reactions. The enzymes are reactivated during the initial stage of thermal cycling.

A total reaction mix should always be made to eliminate well-to-well variation of component concentrations. Assemble the total reaction mix using *Table 1* as a guide. Scale all components except the template according to the number of reactions to be performed. Include 5% overage to account for variations in pipetting.

	96-well plate	384-well plate
2x ProbeSure-RT Master Mix	10.0	2.5
20X Primer/Probe Mix for N1 or N2 or RNase P ¹	1	0.25
Water	Variable	Variable
RNA ²	Up to 9.0	Up to 2.25
TOTAL	20.0	5.0

Table 1. Reagent volumes for assembly of reaction mix. Total volumes indicated are recommended for the associated PCR plate type. Include positive, negative, and no-template controls. ¹ A separate total reaction mix must be assembled for the N1, N2 and RNase P assays. ² Add the maximum volume of RNA possible to maximise RNA copy number in the reaction; this volume should be determined experimentally as some crude extraction methods may introduce PCR inhibitors.

b. Total Reaction mix Dispensing

The total reaction mix must now be dispensed into the PCR plate wells. Use a liquid handling system that is appropriate to the scale of the work. Once the total reaction mix has been dispensed, the PCR plate must be centrifuged to ensure all components are at the bottom of the wells.

c. Arraying template RNA

Use a liquid handling system that is appropriate to the number of RNA samples to array. RNA samples can also be arrayed manually if working with a small number of samples and large reaction

volumes. Add either sample RNA, negative controls, or positive controls to each well of the reaction plate in accordance with *Table 1*. To maximise detection of low copy number samples, as much RNA as possible should be added to each reaction, providing the samples do not contain inhibitory substances. A small number of no-template controls (NTCs) should be included on each plate; NTCs should consist of the same buffer used to hydrate the RNA samples or PCR grade water. Seal the plate with an optically clear seal. The plate should then be centrifuged briefly to locate all liquid at the bottom of the wells.

d. Thermal cycling

Place the plate on a thermal cycler or qPCR instrument and carry out the thermal cycling step. The recommended thermal cycling protocol is shown in *Table 2*.

Step	Description	Temp	Time	N°. Cycles
1	Reverse Transcription	50°C	10 min	1
2	Enzyme activation	95°C	10 min	1
3	Template denaturation	95°C	15 secs	50
	Annealing and extension	60°C	60 secs	

Table 2. Thermal cycling conditions for ProbeSure™ COVID-19 RT-PCR

e. Fluorescent signal detection

After thermal cycling is complete, detect the fluorescent signal in a FRET-capable plate reader or qPCR machine in endpoint mode. Alternatively, if assessing the data in real-time, detect fluorescence during thermal cycling. Record fluorescence for FAM and ROX.

f. Interpretation of data

Data can be viewed and analysed as a standard real-time reaction. Endpoint data analysis and interpretation can also be carried out using cluster analysis software or alternatively in Excel. Using the Singleplex kit, the data must be viewed as three

separate cluster plots: one for N1 (FAM), one for N2 (FAM) and one for RNase P (FAM) control, respectively A, B and C in *Figure 1*.

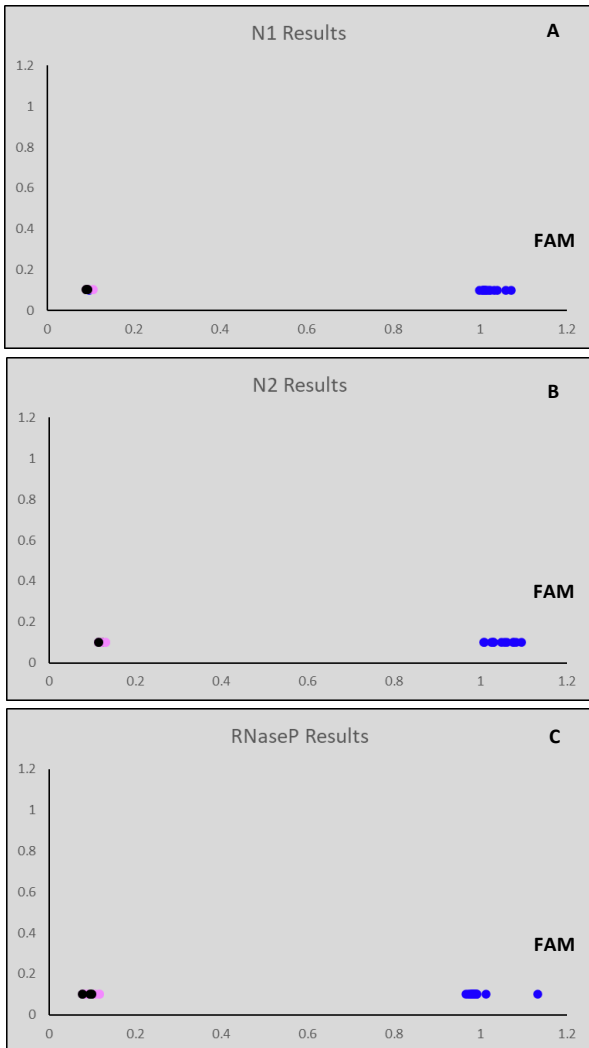


Figure 1. Example endpoint cluster plot data for ProbeSure™ COVID-19 one step RT-PCR Singleplex kit, showing assays N1, N2 and RNase P.

g. Example cluster plots

The kit has been designed such that all positive samples amplify to a similar level of endpoint fluorescence. This way the positive samples cluster tightly together when viewed graphically and are easy to distinguish from samples not showing amplification.

The blue clusters in *Figure 1* A-C represent PCR amplification and are therefore positive for the assay used. The samples marked black are no-template controls and those marked pink are samples that failed with the assay used; both are

negative. The data should be assessed as described in *Table 3*.

N1	N2	RNase P	Result
+	+	+	COVID-19 positive
-	+	+	COVID-19 negative
+	-	+	COVID-19 negative
-	-	+	COVID-19 negative
-	-	-	Reaction failed
+	+	-	False positive
+	-	-	False positive
-	+	-	False positive

Table 3. Analysis of the results of the cluster plots of the three singleplex assays of the ProbeSure™ COVID-19 one step RT-PCR Singleplex kit.

For a sample to be deemed COVID-19 positive it must amplify with all three assays (i.e., blue on all endpoint plots in *Figure 1*, A-C). If a sample amplifies only with the RNase P control assay, but not with N1 and N2, it would indicate that the RNA isolation, reverse transcription and PCR steps have been successful but that no viral RNA has been detected, demonstrating that the sample is negative for COVID-19. If the sample is positive for RNase P and positive for one or other of the N gene assays, it would still be deemed negative for COVID-19 in accordance with current CDC guidelines requiring that both N gene assays must amplify. Amplification of the no-template controls indicates the presence of contamination.

10. Ordering information

For ordering details, please visit www.3crbio.com/products/ordering

11. Licence information

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