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

Content

1. Product details

The ProbeSure™ COVID-19 One Step RT-PCR Multiplex kit utilises one assay to detect three targets. The assay targets the nucleocapsid genes N1 (labelled with FAM) and N2 (labelled with HEX), as well as the RNase P control (labelled with ATTO 647). The plate reader or real-time instrument used must be capable of fluorescence detection of FAM, HEX, ATTO 647 and ROX (passive reference). Using the Multiplex kit, each RNA sample can be analysed in just one PCR plate well, i.e., N1, N2 and RNase P are all detected in the same reaction.

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-1001-3	2x ProbeSure™ COVID-19 One Step RT-PCR	Multiplex	1	N1 N2 RNase P	FAM HEX ATTO 647	3	1 mL	100	20 uL	Low (25 nM)
COV-2001-3	2x ProbeSure™ COVID-19 One Step RT-PCR	Multiplex	1	N1 N2 RNase P	FAM HEX ATTO 647	3	1 mL	100	20 uL	High (500 nM)
COV-1010-3	2x ProbeSure™ COVID-19 One Step RT-PCR	Multiplex	1	N1 N2 RNase P	FAM HEX ATTO 647	3	10 mL	1000	20 uL	Low (25 nM)
COV-2010-3	2x ProbeSure™ COVID-19 One Step RT-PCR	Multiplex	1	N1 N2 RNase P	FAM HEX ATTO 647	3	10 mL	1000	20 uL	High (500 nM)

2. Description

ProbeSure™ COVID-19 One Step RT-PCR Multiplex 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 and an internal RNase P control assay. The three assays are combined into one multiplex assay, N1 + N2 + RNase P which is amplified and detected in one reaction. 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)
- 10x Multiplex Assay Mix (Purple lid) for N Gene targets N1 (reports with FAM), N2 (reports with HEX) and RNase P control (reports with ATTO 647)

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 Duplex + RNase P 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 the 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

a. Reaction Assembly

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
10X Primer/Probe Mix for N1 + N2 + RNase P	2	0.5
Water	*Variable*	*Variable*
RNA [1]	*Up to 8.0*	*Up to 2.0*
TOTAL	**20.0**	**5.0**

***Table 1*. Reagent volume for assembly of reaction mix. Total volumes indicated are recommended for the associated PCR plate type. Include positive, negative, and no-template controls.** [1] **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 Control, or Positive Control 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	Nº. Cycles
1	Reverse Transcription	50˚C	10 min	1
2	Enzyme activation	95˚C	10 min	1
3	Template denaturation	95˚C	15 secs	45
	Annealing and extension	60˚C	30 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, HEX, ATTO 647 and ROX.

f. Interpretation of data

Data analysis should be performed with the software of the real-time PCR instrument according to the manufacturer's instructions.

Samples should be considered positive for each assay if the cycle threshold (C_t) value is equal to or below 34 for FAM and 35 for HEX. If the Ct value is greater than indicated, the sample should be considered negative (see *Table 3*). Please note that

Ct values might vary with QPCR instrument being used for the testing. These Ct values were validated on ABI 7900.

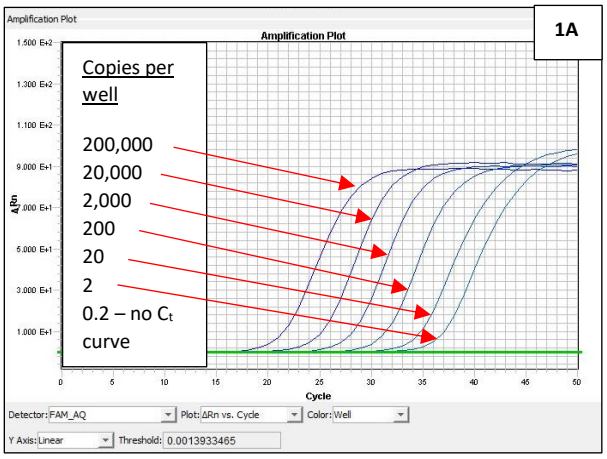
N1	N2	RNase P	Result
+	+	+	**COVID-19 positive**
-	+	+	COVID-19 negative
+	-	+	COVID-19 negative
-	-	+	COVID-19 negative
-	-	-	Reaction failed
+	+	-	False positive
+	-	-	False positive
-	+	-	False positive

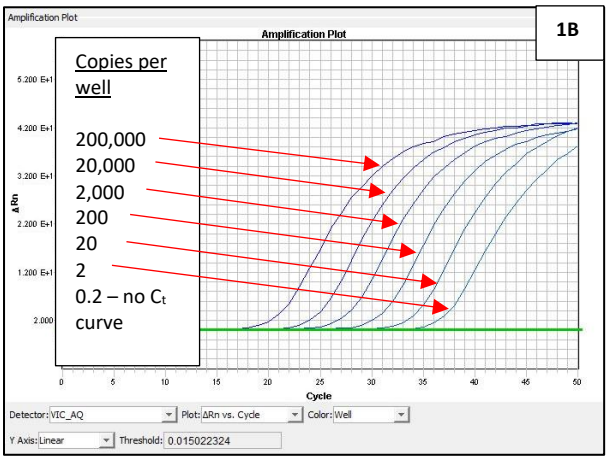
***Table 3*. Analysis of the results of the ProbeSure™ COVID-19 one step RT-PCR Multiplex kit.**

The data should be assessed as described in *Table 3*. For a sample to be positive for COVID-19 it must amplify with all three assays. If a sample amplifies only with the RNase P control assay, but not with assays 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 only one or other of the N1 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.

g. Kit performance

Figures 1A and B show the performance of the ProbeSure™ COVID-19 One Step RT-PCR kit with a range of RNA concentrations. The Figures demonstrate that the kit can be used with a wide dynamic range of RNA sample concentrations, even down to very low copy numbers.





***Figures 1 A & B*. Real-time PCR analysis showing FAM values (1A) and HEX values (1B) for synthetic RNA dilution samples with ProbeSure™ COVID-19 One Step RT-PCR Master Mix. Synthetic RNA controls are shown for 10,000, 1,000, 100, 10, 1 and 0.1 copies per µL. The data were generated using 5µL reactions in a 384-well PCR plate, containing 2µL of diluted RNA sample per well.**

10. Ordering information

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

11. Licence information

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