Application Note

Multiplex 1-step RT-qPCR assay for accurate and sensitive detection of SARS-CoV-2

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Introduction

The emergence and rapid spread of coronavirus disease 2019 (COVID-19) represents a major health threat to countries and populations around the world. The outbreak, originating in Wuhan, China, in December 2019 was declared a pandemic by the World Health Organisation (WHO) on 11th March 2020. At the time of writing there have been 51,718,344 reported cases and 1,276,527 deaths globally.[1]

The causative agent of COVID-19 is the highly contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; previously 2019-nCoV), a betacoronavirus believed to have originated in bats. The virus is transmitted between humans mainly by direct contact with respiratory droplets.

Coronaviruses are enveloped, single-stranded, positive-sense RNA viruses that infect humans and animals. The SARS-CoV-2 virus (see figure 1) infects human cells via spike protein binding to ACE2 transmembrane receptors which are widely expressed in lung, heart, kidney, blood vessels and gastrointestinal tissue.

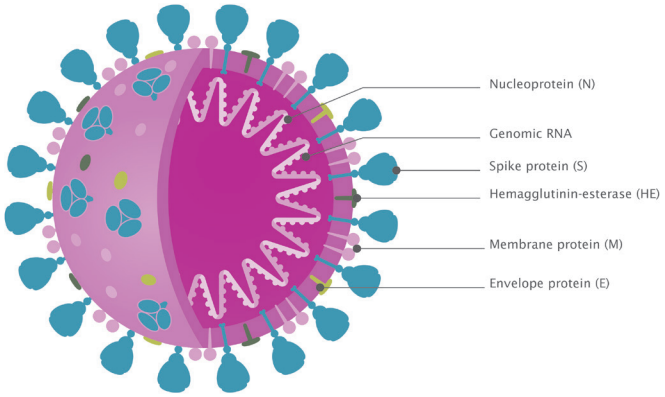

Figure 1. Structure of SARS-CoV-2

The SARS-CoV-2 genome consists of 29,903 nucleotides.[2] The first two thirds comprise two open reading frames, ORF1a and ORF1b, which encode 16 nonstructural proteins (NSPs) that assist in replicating and proofreading the viral genome (see figure 2). NSP12 encodes the highly conserved RNA-dependent RNA polymerase (RdRp). The remaining third of the genome comprises genes for the four structural proteins: the spike (S) protein, membrane (M) protein, envelope (E) protein, and nucleocapsid (N) protein, and also eight genes for accessory proteins that inhibit host defences.

The emergence of new viral diseases such as COVID-19 highlights the need for rapid methods to detect and identify target viruses at scale. Mass testing, together with strong and effective tracking and tracing, is key to controlling the pandemic. In terms of testing, patient nasopharyngeal swab sampling in combination with RT-qPCR-based detection in a specialised clinical containment lab is considered the gold standard.

Multiplex testing

RT-qPCR-based detection of SARS-CoV-2 can be performed in singleplex, where a single target sequence is amplified in a single well, or in multiplex, where multiple targets are amplified simultaneously using multiple primer pairs in combination with probes labeled with spectrally distinct fluorophores.

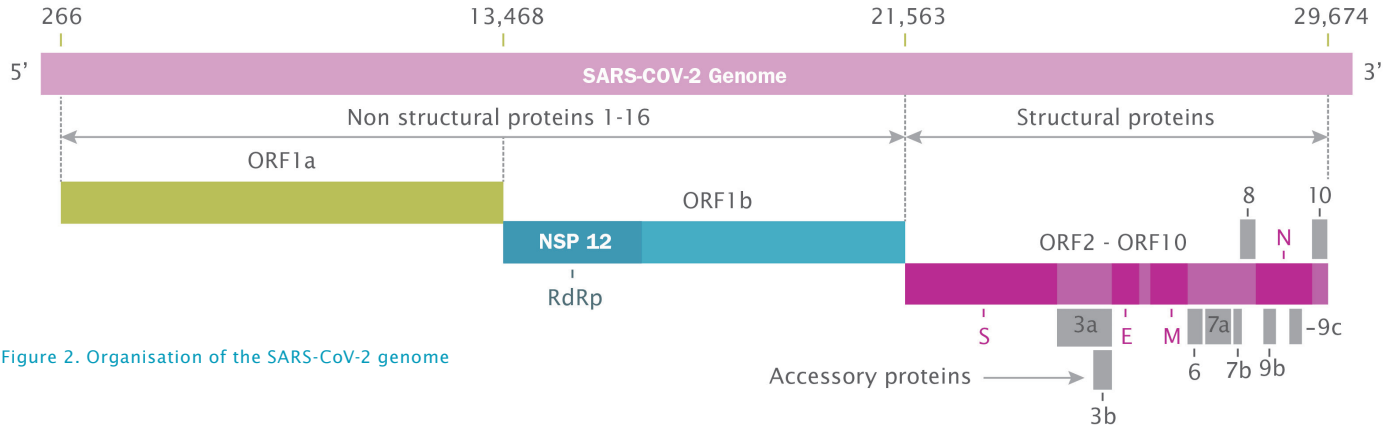

Figure 2. Organisation of the SARS-CoV-2 genome

SARS-CoV-2 testing during the colder months will likely form part of a multiplexed respiratory panel test designed to distinguish SARS-CoV-2 from other common seasonal viruses such as Influenza A, Influenza B and Respiratory Syncytial Virus (RSV). Multiplex assays have the potential to save a considerable amount of time, effort and reagent use, however they are far harder to optimise, given the increased number of targets in one RT-qPCR reaction.

This application note details a rapid and sensitive multiplex RT-qPCR assay for SARS-CoV-2 detection, and is designed to contribute to, and support efforts in developing, effective high-throughput diagnostic testing for COVID-19.

Method

PCR Biosystems have recently introduced qPCRBIO Probe 1-Step Virus Detect for detection of viral RNA by 1-step RT-qPCR using sequence-specific probes. The kit has been optimised with a high-concentration 4x mix and UltraScript Reverse Transcriptase. We describe experimental conditions for the qualitative detection of SARS-CoV-2 in multiplex using synthetic viral RNA as template and target sequences outlined in the WHO/Charité-Berlin protocol[3] and Centers for Disease Control and Prevention (CDC, Atlanta, USA) protocol[4]. To save scientists time and resources we investigated the RT-qPCR variables of reverse transcription time and temperature, as well as concentrations of primers and probes. Herein we outline an optimised experimental setup to get the SARS-CoV-2 multiplex assay working well first time.

Target sequences

We assayed four target sequences (see table 1): the RdRp and E gene target described by the WHO/Charité-Berlin; the N2 target described by the CDC; and the human extraction control RPP30. RPP30 encodes the RNAse P subunit P30 and is often present in diagnostic kits. Primers and probes were sourced from Biolegio and Integrated DNA Technologies.

RNA template

Viral genome (ATCC, catalogue number VR-3276SD) dilutions were performed with a Qiagen QIAgility robot using PCR grade dH_20. Five sequential ten-fold RNA virus dilutions were performed in quadruplicate, starting with 40,000 copies in 5µL, going down to four copies in 5µL. No template controls (NTCs) were also included in quadruplicate.

Target gene	Description	Assay	Sequence (5'-3')	Concentration (nM)
RdRp	RdRP_SARSr-F2	WHO/Charité	GTGARATGGTCATGTGTGGCGG	400
	RdRP_SARSr-R1		CARATGTTAAASACACTATTAGCATA	400
	RdRP_SARSr-P2		TexasRed-CAGGTGGAACCTCATCAGGAGATGC-BHQ2	160
E	E_Sarbeco_F1	WHO/Charité	ACAGGTACGTTAATAGTTAATAGCGT	400
	E_Sarbeco_R2		ATATTGCAGCAGTACGCACACA	400
	E_Sarbeco_P1		FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1	160
N (sequence 2)	2019-nCoV_N2-F	CDC	TTACAAACATTGGCCGCAAA	400
	2019-nCoV_N2-R		GCGCGACATTCCGAAGAA	400
	2019-nCoV_N2-P		Cy5-ACAATTTGCCCCCAGCGCTTCAG-BHQ2	160
RNase P	RPP30 F	Extraction Control	AGATTTGGACCTGCGAGCG	400
	RPP30 R		GAGCGGCTGTCTCCACAAGT	400
	RPP30 P		HEX-TTCTGACCTGAAGGCTCTGCGCG-BHQ1	160

Table 1. SARS-CoV-2 and human RNase P primer and probe sequences

Human lung RNA (ThermoFisher, catalogue number AM7968) dilutions were performed with a Qiagen QIAgility robot using PCR grade dH_20 together with viral RNA. Five sequential ten-fold human lung RNA dilutions were performed in quadruplicate, starting with 250ng in 5µL, going down to 25pg in 5µL. NTCs were also included In quadruplicate.

Reaction setup

The PCR master mix was prepared with a Qiagen QIAgility robot as reported in table 2, with a final reaction volume of 20µL.

Reagent	Volume	Final conc.
20x UltraScript RTase	1µL	1x
4x PCRBIO Probe 1-Step Virus Detect	5µL	1x
Primer mix (10µM)	0.8µL	400nM
Probe (10µM)	0.32µL	160nM
RNA template	5µL	Variable
PCR grade dH_2O	4.52µL	–

Table 2. Reaction setup

The four sets of primer pairs (F+R) were mixed to obtain a final concentration of 10µM for each oligonucleotide (e.g. 10µL for each of the eight stocks of primers at 100µM + 20µL PCR grade dH_2O). Similarly, the four probes were mixed to obtain a final concentration of 10µM each (e.g. 10µL for each of the four stocks of probes at 100µM + 60µL PCR grade dH_2O).

Cycling conditions

PCR cycling was carried out using a Bio-Rad CFX96 Touch™. The optimal RT-qPCR programme is shown in table 3.

Cycles	Temperature	Time	Notes
1	55°C	10 minutes	Reverse transcription
1	95°C	3 minutes	Polymerase activation and RTase inactivation
50	95°C 58°C	15 seconds 30 seconds	Denaturation Anneal/extension

Table 3. Cycling conditions

Results

All dilutions of SARS-CoV-2 viral target were detected in this fourplex RT-qPCR programme with high efficiency values (see figure 3 and table 4). The average first Ct was 24.74 for 40,000 copies and average final Ct was 39.16 for four viral copies. qPCRBIO Probe 1-Step Virus Detect can be used to detect SARS-CoV-2 targets in multiplex, and with high specificity and sensitivity, tested down to four copies per 20µL reaction (0.8 copies per µL).

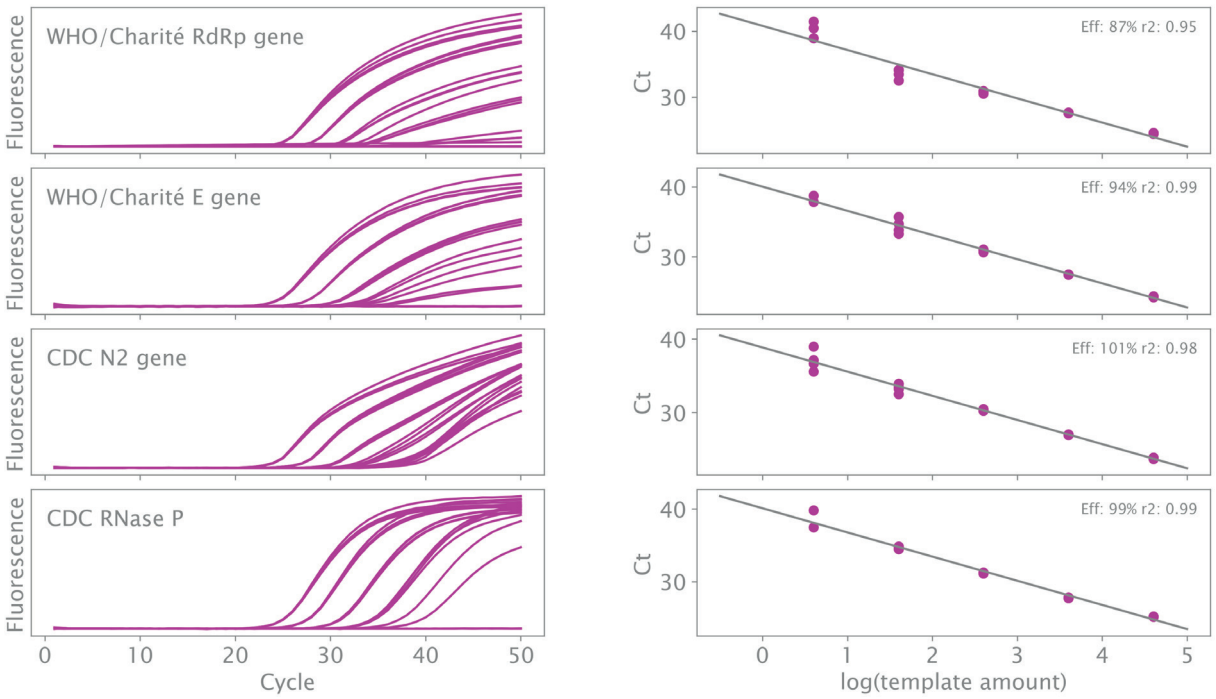

Figure 3. Multiplex detection of SARS-CoV-2 nucleic acid using qPCRBIO Probe 1-Step Virus Detect

Fourplex amplification of SARS-CoV-2 RdRp, E and N genes and human RNase P gene using qPCRBIO Probe 1-Step Virus Detect in quadruplicate. Amplification curves are shown on the left and efficiency on the right. Five serial dilutions of synthetic viral RNA template were used, corresponding to 40,000, 4,000, 400, 40 and 4 copies of the SARS-CoV-2 genome. Five serial dilutions of human lung RNA template were used in viral genome solution, from 250ng to 25pg. The total reaction volume was 20µL. RdRp-P2 probe was labelled with Texas Red, E gene probe with FAM, N2 probe with Cy5 and RNase P probe with HEX. Cycling conditions used are reported in table 3.

	RdRp gene (Texas Red)			E gene (FAM)			N2 (Cy5)			RNase P (HEX)		
RNA copies	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n
40,000	24.25	0.05	4	25.43	0.04	4	24.65	0.08	4	24.63	0.07	4
4,000	27.36	0.04	4	28.07	0.04	4	27.78	0.02	4	27.72	0.07	4
400	30.78	0.14	4	31.44	0.09	4	31.22	0.12	4	30.89	0.17	4
40	33.87	0.62	4	34.99	0.17	4	34.84	1.10	4	33.68	0.76	4
4	37.87	1.27	4	38.99	1.65	2	39.10	0.72	2	40.67	1.37	3
NTC	37.96	0.62	4	N/A	0.00	0	N/A	0.00	0	N/A	0.00	0

Table 4. Mean Ct and standard deviation values

The table shows mean Ct and standard deviation (SD Ct) values achieved in the amplification of SARS-CoV-2 RdRp, E and N genes and human RNase P gene using qPCRBIO Probe 1-Step Virus Detect. n indicates the number of replicates (out of four) giving a positive amplification.

Discussion

RT-qPCR tests when performed correctly are extremely sensitive and specific and provide a gold standard for diagnosis of COVID-19 by the detection of SARS-Cov-2 genomic material. Though it should be noted that a "positive" PCR result reflects only the detection of viral RNA and does not necessarily indicate the presence of viable virus. The sensitivity of this assay is such that very low amounts of viral RNA can be detected from a patient. Detecting these low positive patients as early as possible is crucial in order to curtail the spread of the virus.

Multiplex testing will save valuable time with regard to sample processing because multiple respiratory virus targets can be tested in one qPCR reaction. Multiplex testing gives more information to the clinician regarding each patient sample. Optimising a multiplex RT-qPCR reaction can be difficult and cost valuable time, so in this application note we detail RT-qPCR parameters and mastermix suggestions that will lead to a sensitive result. The sensitivity of RT-qPCR will be affected by exterior factors such as effective nasopharyngeal swab sampling, the temperature of transit of the virus in viral inactivation buffer and subsequent quality of viral sample for the test. This application note describes only the sensitivity of qPCRBIO Probe 1-Step Virus Detect with respect to detecting synthetic RNA and not extracted patient swabs.

Pooling patient samples, an idea put forward by the FDA in June 2020[5], will be useful in screening asymptomatic patients, and has value because it will disregard large numbers of negative patient samples quickly. Further testing and investigation can then be focussed only on the pooled samples that tested positive.

At the time of writing, R values are once again on the rise. It is unknown whether this virus will persist in the population forever, or if people will receive repeated vaccinations, as is the case for seasonal flu. But in the absence of a vaccination, fast and efficient diagnosis is the key to controlling the spread of this virus as well as reducing the global social and economic impact over the coming months and years.

Product use

qPCRBIO Probe 1-Step Virus Detect alone does not provide any diagnostic result and is supplied for Research Use Only. It is suitable for use as a component of molecular diagnostic assays, where applicable country laws allow.

References

1. COVID-19 Dashboard by the Centre for Systems Science and Engineering at Johns Hopkins University accessed 11th November 2020 (https://coronavirus.jhu.edu/map.html)

2. Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome (https://www.ncbi.nlm.nih.gov/nuccore/MN908947)

3. Diagnostic detection of 2019-nCoV by real-time RT-PCR (https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf)

4. Research Use Only 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primers and Probes (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html)

5. Coronavirus (COVID-19) Update: Facilitating Diagnostic Test Availability for Asymptomatic Testing and Sample Pooling, (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-facilitating-diagnostic-test-availability-asymptomatic-testing-and)

Ordering information

Cat. no.	Product name	Pack size	Presentation
PB25.51-01	qPCRBIO Probe 1-Step Virus Detect Lo-ROX	200 x 20µL rxns	[1 x 1mL mix] & [1 x 200µL UltraScript]
PB25.51-03		600 x 20µL rxns	[3 x 1mL mix] & [1 x 600µL UltraScript]
PB25.51-05		1000 x 20µL rxns	[1 x 5mL mix] & [1 x 1mL UltraScript]
PB25.51-50		10000 x 20µL rxns	[1 x 50mL mix] & [2 x 5mL UltraScript]
PB25.51-500		50000 x 20µL rxns	[1 x 500mL mix] & [1 x 100mL UltraScript]
PB25.52-01	qPCRBIO Probe 1-Step Virus Detect Hi-ROX	200 x 20µL rxns	[1 x 1mL mix] & [1 x 200µL UltraScript]
PB25.52-03		600 x 20µL rxns	[3 x 1mL mix] & [x 600µL UltraScript]
PB25.52-05		1000 x 20µL rxns	[1 x 5mL mix] & [1 x 1mL UltraScript]
PB25.52-50		10000 x 20µL rxns	[1 x 50mL mix] & [2 x 5mL UltraScript]
PB25.52-500		50000 x 20µL rxns	[1 x 500mL mix] & [1 x 100mL UltraScript]
PB25.53-01	qPCRBIO Probe 1-Step Virus Detect No-ROX	200 x 20µL rxns	[1 x 1mL mix] & [1 x 200µL UltraScript]
PB25.53-03		600 x 20µL rxns	[3 x 1mL mix] & [x 600µL UltraScript]
PB25.53-05		1000 x 20µL rxns	[1 x 5mL mix] & [1 x 1mL UltraScript]
PB25.53-50		10000 x 20µL rxns	[1 x 50mL mix] & [2 x 5mL UltraScript]
PB25.53-500		50000 x 20µL rxns	[1 x 500mL mix] & [1 x 100mL UltraScript]
PB25.54-01	qPCRBIO Probe 1-Step Virus Detect Separate-ROX	200 x 20µL rxns	[1 x 1mL mix] & [1 x 200µL ROX] & [1 x 200µL UltraScript]
PB25.54-03		600 x 20µL rxns	[3 x 1mL mix] & [1 x 200µL ROX] & [1 x 600µL UltraScript]
PB25.54-05		1000 x 20µL rxns	[1 x 5mL mix] & [1 x 200µL ROX] & [1 x 1mL UltraScript]

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