Application Note

A test panel for SARS-CoV-2 & winter viruses using multiplex probe 1-step RT-qPCR

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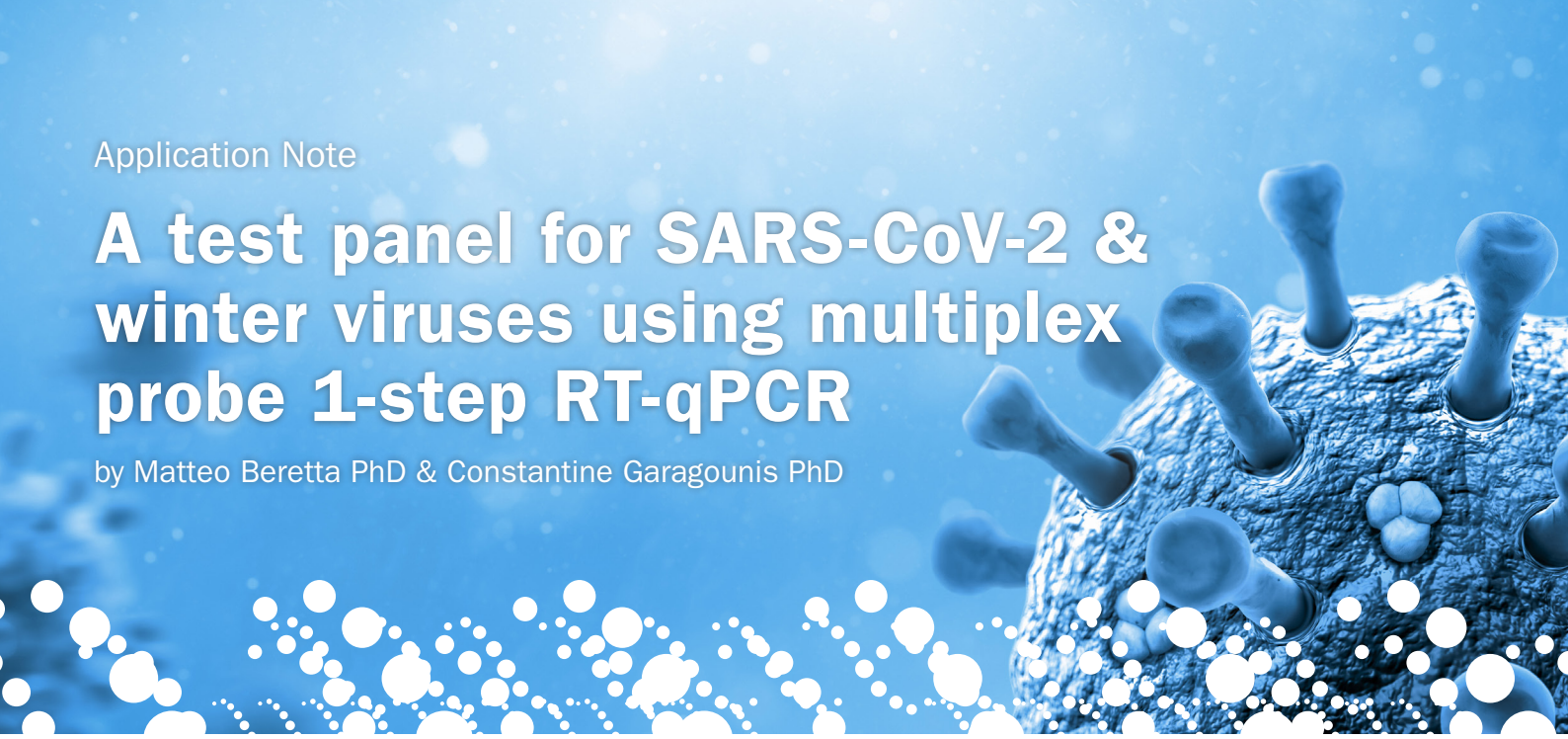

Introduction

Winter viruses are an annually recurring problem. Coupled with the novel SARS-CoV-2 virus they become a challenge for diagnosis. Thus reliable detection of these pathogens is critical for appropriate therapeutic and protective isolation measures to be taken in a timely manner. The gold standard for viral strain identification is whole viral genome sequencing or Sanger sequencing of key viral genes for specific strains. However, sequencing approaches are time consuming and laborious. Rapid screening by RT-qPCR is therefore the preferred method and can be followed up by sequencing when necessary for full diagnosis. RT-qPCR remains a benchmark technique for nucleic acid target detection and quantification, enabling reliable analysis of multiple samples and targets in a very short amount of time. Results from a typical 40 cycle RT-qPCR run can be available in less than 3hrs, factoring in 15-30min of plate setup time and near instant result reporting and analysis in modern instruments.

Several RT-qPCR protocols can be used to detect the various winter viruses. However, one of the best approaches is to use multiplex RT-qPCR to detect common pathogenic viral strains. This is because multiplexing enables testing for multiple targets simultaneously while making efficient use of time, reagents, and equipment. It is therefore ideal in any high-capacity diagnostic workflow. Multiplex RT-qPCR is carried out by combining primer pairs and specific probes for every target to be detected, along with the sample, in a single reaction mix. Each probe is labelled with a distinct fluorophore and therefore registers a unique signal during thermocycling. Thus, a single sample can be assayed for multiple targets in one reaction.

This application note describes a reliable and rapid (~ 1.5hrs) multiplex assay for a winter panel comprising common winter viruses: Influenza A virus, Influenza B virus, human Respiratory Syncytial Virus A2, and the novel SARS-CoV-2 virus. We envision the use of such winter panels to be commonplace even after the current pandemic and have therefore designed this assay to enable streamlined diagnostic testing of these common, recurring pathogens.

Method

At PCR Biosystems we have established a protocol for detection of a winter virus panel to include SARS-CoV-2. To this end we have used primer and probe sequences identified and verified for their diagnostic validity in peer reviewed journal reports[1] and in the WHO (World Health Organisation/Charité-Berlin protocol) and the CDC (Centers for Disease Control and Prevention in Atlanta, GA, USA) guidelines[2], listed in Table 1. We also used our newly developed qPCRBIO Probe 1-Step Virus Detect RT-qPCR master mix in this protocol. Test templates were synthetic RNA viral sequences and the target sequences for each virus were: the *Matrix protein M1* gene of the Influenza A virus, the *Hemagglutinin* gene of the Influenza B virus, the *Matrix protein* gene of human Respiratory Syncytial Virus A2, and the *E-gene* of the SARS-CoV-2 virus (Table 1). After assaying different conditions, we present the optimised multiplex RT-qPCR protocol for detection of these viruses.

Name	Dye-Sequence	Concentration	Template	Target
InfA-F	GACCRATCCTGTCACCTCTGAC	400 nM	Influenza virus A, A/Virginia/ ATCC1/2009 (H1N1). ATCC-VR-1736DQ. Lot 70029949.	*Matrix Protein M1* gene[1]
InfA-R	AGGGCATTYTGGACAAAKCGTCTA	400 nM		
InfA-P	**HEX**-TGCAGTCCTCGCTCACTGGGCACG-**BHQ1**	200 nM		
InfB-F	AAATACGGTGGATTAAATAAAAGCAA	400 nM	Influenza virus B, B/ Wisconsin/1/2020 BX-41A. ATCC-VR-1885DQ. Lot 70034872.	*Hemagglutinin* gene[1]
InfB-R	CCAGCAATAGCTCCGAAGAAA	400 nM		
InfB-P	**Cy5**-CACCCATATTGGGCAATTTCCTATGGC-**BHQ2**	200 nM		
RSV-F	GCAAATATGGAAACATACGTGAACA	400 nM	Respiratory Syncytial Virus, ATCC-VR-1540DQ, Lot 70027617	*Matrix Protein* gene[1]
RSV-R	GCACCCATATTGTWAGTGATGCA	400 nM		
RSV-P	**Tex615**-CTTCACGAAGGCTCCACATACACAGCWG-**BHQ2**	200 nM		
E_Sarbeco-F	ACAGGTACGTTAATAGTTAATAGCGT	400 nM	SARS-CoV-2, ATCC-VR-3276SD.	*Envelope (E)* gene[2]
E_Sarbeco-R	ATATTGCAGCAGTACGCACACA	400 nM		
E_Sarbeco-B	**FAM**-ACACTAGCCATCCTTACTGCGCTTCG-**BHQ1**	200 nM		

Table 1: Primers, probes and targets used

Primer and template preparation

For each target, a primer set was prepared by mixing 400µL 10µM primers (F + R) to 200µL 10µM probe (This is equivalent to adding 0.8µL 10µM primers F + R and 0.4µL of 10µM probe to each 20µL reaction mix).

Templates were prepared in a 5-point, 10-fold dilution series ranging from 40,000 copies to 4 copies per sample. No template control samples (NTCs) were included for each target. Four technical replicates were run for each sample.

Practical Note: Template stocks were added together in a tube to have a mix with 8000 copies/µL for each viral genome.

Reaction setup

Reactions were set up using a Qiagen QIAgility robot in a final volume of 20µL to include: 4.2µL Milli-Q water, 5µL 4x qPCRBIO Probe 1-Step Virus Detect, 1µL 20x UltraScript RTase, 1.2µL x 4 primers/probe mix (described above) and 5µL of pooled RNA sample. Table 2 summarizes the final concentration of the components added.

Cycling conditions

Thermocycling was carried out on a Bio-Rad CFX96 Touch™ qPCR machine with the cycling parameters outlined in Table 3. Fluorescence measurements were acquired at each cycle.

Results

All targets were successfully detected (Figure 1 and Table 4) before 40 cycles, even at their lowest concentration (4 copies per 20µL reaction) with no product detected in the NTCs. Efficiency for all targets was between 90-110%, with an R^2 value above 98% in all cases. The mean ± SD target Ct values for all targets were 24.13 ± 1.02 and 37.30 ± 1.18 at their highest (40,000 copies per reaction) and lowest concentration (4 copies per reaction), respectively.

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)	0.4µL	200nM
RNA template	5µL	Variable
PCR grade dH2O	4.52µL	–

Table 2: Reaction setup and composition

Cycles	Temperature	Time	Notes
1	45°C	20 minutes	Reverse transcription
1	95°C	3 minutes	Polymerase activation and RTase inactivation
50	95°C 60°C	15 seconds 30 seconds	Denaturation Annealing/Extension

Table 3: Cycling conditions

Discussion

Our approach has proven to be successful in detecting very low amounts of viral targets in fourplex RT-qPCR reactions (4 copies per 20µL reaction). This offers a rapid and reliable means of testing for three common winter viruses and the novel SARS-CoV-2 in a single test tube. While a positive result for nucleic acid targets does not necessarily reflect the presence of viable viral particles, RT-qPCR approaches remain the benchmark method for pathogen detection and quantification and are an integral part of many clinical and research diagnostic procedures. These results also highlight the power and efficiency of multiplex RT-qPCR, and in this context, it is crucial to state that additional targets can be included in

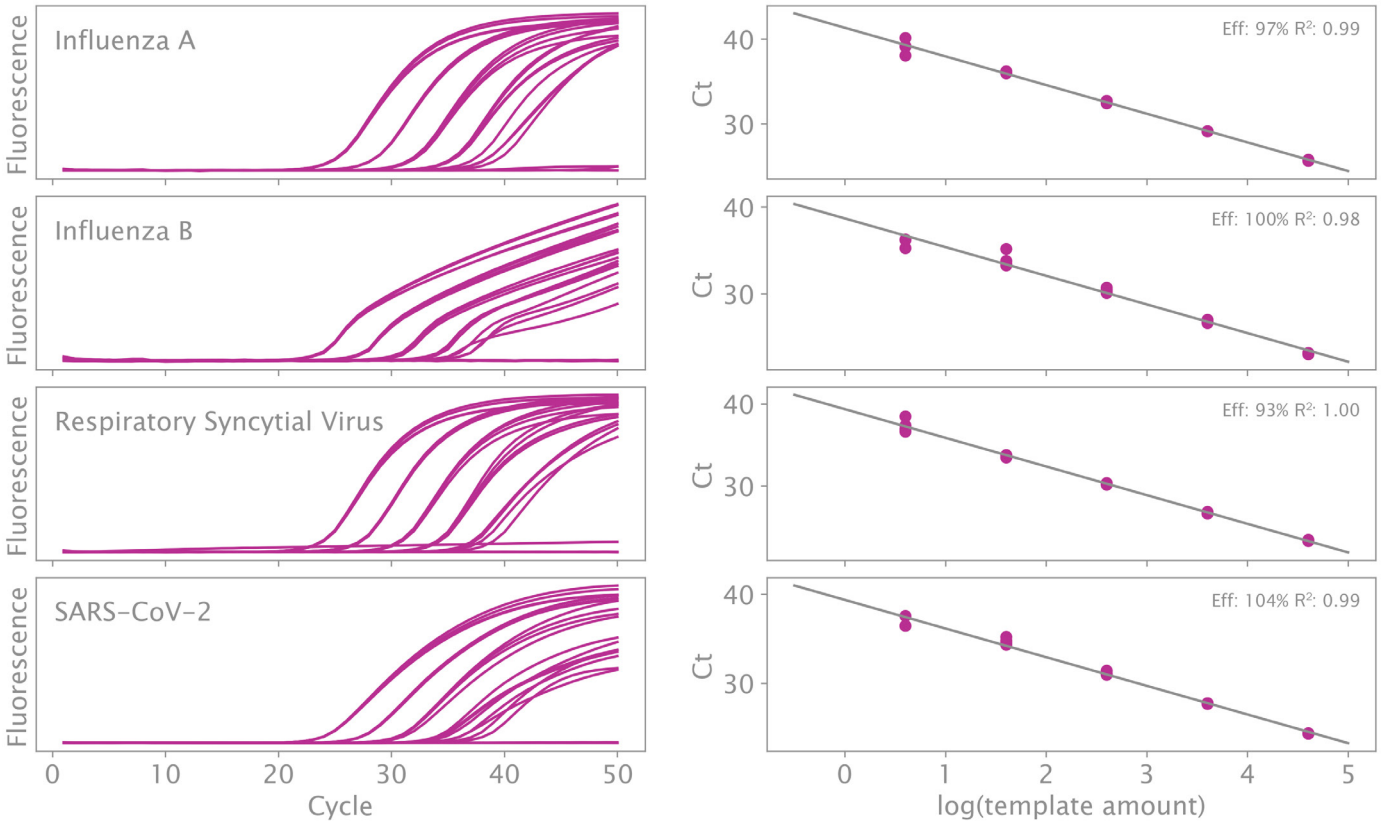

Figure 1: Fourplex detection of Influenza A, Influenza B, Respiratory syncytial, and SARS-CoV-2 viruses

Four-plex amplification of Influenza A, Influenza B, Respiratory syncytial, and SARS-CoV-2 viruses genes using qPCRBIO Probe 1-Step Virus Detect in quadruplicate. Amplification curves are shown on the left and efficiency on the right. 5 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. The total reaction volume was 20µL. The InfA probe was labelled with Hex, the InfB probe with Cy5, the RSV probe with Texas Red, and the SARS-CoV-2 E-gene probe labeled with FAM. Cycling conditions were reverse transcription at 45˚C for 10 min, denaturation at 95˚C 3 min and 50 cycles of amplification at 95˚C 15s, 60˚C 30s.

this panel. Ten-plex RT-qPCR assays for pathogenic virus detection have been successful[1]. Additionally, other targets, including probes designed for specific SARS-CoV-2 variants, can substitute those we chose in the current application.

At the time of writing, and despite progress with vaccinations against COVID-19 and employment of other preventive measures, the constant appearance of variants of concern for SARS-CoV-2 pose a stumbling block to our exit from the pandemic. Thus, screening patients for different coronavirus variants, in addition to other common viruses, becomes a critical challenge. The ECDC (European Centers for Disease Prevention and Control) guidelines require whole viral genome sequencing or at least full Sanger sequencing of the S-gene for SARS-CoV-2 variant identification[3]. However, because these approaches are laborious and time consuming, pre-screening samples for SARS-CoV-2 by RT-qPCR is also recommended. RT-qPCR reduces the total number of samples that need to be sequenced and offers a cheaper, quicker, high-throughput, preliminary approach to coronavirus variant identification. A few different approaches in addition to RT-qPCR can be used to pre-screen for specific variants, but in all cases these approaches must be followed up by sequencing for a confirmed result[3].

The RT-qPCR approaches recommended by the ECDC for preliminary variant screening include[3]:

- SARS-CoV-2 S-gene drop-out or target failure, caused by mutation or loss of the primer binding region, in multiplex RT-qPCR targeting multiple SARS-CoV-2 genes.
- Probe-based RT-qPCR with distinct probes and controls for each target variant SNP

Both these assays can be conducted in accordance with this application note, provided appropriate primers and probes are chosen.

Our qPCRBIO Probe 1-Step Virus Detect allows the rapid setup of new multiplex assays and is designed to be reliable under a range of template amounts, sample types, and target nucleotide compositions, in order to eliminate the need for laborious multiplex assay optimisation.

	HEX - Influenza A			Cy5 - Influenza B			Texas 615 - Respiratory Syncytial Virus			FAM - SARS-CoV-2		
RNA copies	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n	Mean Ct	SD Ct	n
40,000	25.71	0.07	4	23.12	0.08	4	23.32	0.06	4	24.36	0.03	4
4,000	29.15	0.01	4	26.82	0.16	4	26.71	0.10	4	27.72	0.05	4
400	32.62	0.14	4	30.41	0.26	4	30.26	0.09	4	31.11	0.20	4
40	36.09	0.10	4	33.92	0.86	4	33.63	0.15	4	34.72	0.36	4
4	39.14	0.86	4	35.91	0.57	3	37.34	0.81	4	36.81	0.64	3
NTC	N.D.	N.A.	N.A.	N.D.	N.A.	N.A.	N.D.	N.A.	N.A.	N.D.	N.A.	N.A.

Table 4: Mean Ct and standard deviation values for all samples in the qRT-PCR described in Figure 1.

Product use

PCR Biosystems products, including qPCRBIO Probe 1-Step Virus Detect, alone do not provide diagnostic results and are supplied for research use only. However, all products are manufactured under an ISO 13485-compliant management sytem and are suitable for use as components in molecular diagnostic assays, where applicable country laws allow and after clinical validation of an assay itself.

If you would like to discuss which products are best suited to your application or need further technical advice on how to use qPCRBIO Probe 1-Step Virus Detect for SARS-CoV-2 variant testing, contact our team of experts at technical@pcrbio.com.

References

1. Pretorius *et al.*, Respiratory Viral Coinfections Identified by a 10-Plex Real-Time Reverse-Transcription Polymerase Chain Reaction Assay in Patients Hospitalized With Severe Acute Respiratory Illness—South Africa, 2009–2010, The Journal of Infectious Diseases, Volume 206, Issue suppl_1, 15 December 2012, Pages S159–S165.

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