Application note

RapiDxFire 1-step RT-qPCR System for SARS-CoV-2 detection

Introduction

The recent emergence of Novel Coronavirus 2019 (Severe Acute Respiratory Syndrome Coronavirus 2: SARS-CoV-2) has demonstrated the global need for diversification in the use of molecular biology reagents and the design of reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) workflows to detect the virus in clinical samples. This type of testing workflow can allow for rapid infection containment, minimising the impact of disease pathology on the wider population, as well as limiting pressure on national healthcare systems. During this time of crisis, supply chains have become constrained, and having alternative sources of critical assay components is highly valuable.

Here we describe the combining of two existing, well-characterised amplification reagents from LGC, Biosearch Technologies™, EpiScript™ RNase H- Reverse Transcriptase and RapiDxFire™ qPCR 5X Master Mix GF for use in the detection and characterisation of SARS-CoV-2, in a 1-step RT-qPCR system.

EpiScript is a recombinant Moloney murine leukaemia virus (MMLV) RT with greatly reduced RNase H activity that allows it to be highly efficient at temperatures up to 55 °C, unlike native MMLV-derived reverse transcriptases. The optimised reverse transcription protocol can start from as little as 50 pg of total RNA, ideal for sensitive real-time RT-PCR analysis and other applications.

RapiDxFire qPCR Master Mix can be combined with gene-specific primers and hydrolysis probes for immediate use in high-throughput laboratory developed tests (LDT). Provided at a 5X concentration without passive reference dye, this flexible master mix allows more volume for the sample and up to 5 targets for detection at one time.

The following datum describes the use of EpiScript RT and RapiDxFire qPCR Master Mix in conjunction with Biosearch Technologies' 2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2, specific for the

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nucleocapsid N1 and N2 2019-nCoV targets and human RNase P. Here we show analytical sensitivity with limit of detection (LoD), the dynamic range of the RT-qPCR system, and stability of the reaction components under various storage and handling conditions.

Methods

SARS-CoV-2 testing

All data were generated using synthetic SARS-CoV-2 RNA Control 2 (Twist Bioscience, #MN908947.3) diluted in Tris-EDTA buffer with carrier RNA (TE: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 ng/µL carrier RNA) in order to deliver the appropriate copies per reaction. Each target level was tested with 20 replicates for limit of detection (LoD) evaluations and 6 replicates for dynamic range determinations.

For LoD testing, no template controls (NTCs) were included in Biosearch Technologies and competitor comparisons. For dynamic range testing, NTCs were included in all runs. Centers for Disease Control and Prevention recommendations (CDC, 2020) were used to resuspend the 2019-nCoV CDC-qualified Probe and Primer Kits for SARS-CoV-2 in nuclease-free molecular-grade water. All reactions had a final volume of 20 µL (Table 1).

Stability testing

MS2 phage RNA was diluted down to various total copies per reaction, and tested using a FAM-labelled MS2-specific in-house assay on the BIO-RAD CFX96, with a 60 °C annealing temperature.

	RT-qPCR reaction setup		
	Biosearch Technologies	**Competitor A**	**Competitor B**
Reaction mix (final concentrations):	• 0.5 µL EpiScript • 4 µL RapiDxFire qPCR Master Mix • 1.5 µL SARS-CoV-2 primer/probe • 5 µL Twist control RNA or TE • For LoD testing, 0.13 µL Biosearch Technologies 15 µM SuperROX was also added.	• 1X Competitor A RT-qPCR Master Mix • 1.5 µL SARS-CoV-2 primer/probe • 5 µL Twist RNA control or TE	• 1X Competitor B qPCR Master Mix • 1X Competitor B Reverse Transcriptase Mix • 1.5 µL SARS-CoV-2 primer/probe • 5 µL Twist RNA control or TE
Thermal-cycling conditions*	• [1 cycle] 42 °C for 15 minutes • [1 cycle] 95 °C for 2 minutes • [45 cycles] 95 °C for 3 seconds, 55 °C for 30 seconds	• [1 cycle] 25 °C for 2 minutes • [1 cycle] 50 °C for 15 minutes • [1 cycle] 95 °C for 2 minutes • [45 cycles] 95 °C for 3 seconds, 55 °C for 30 seconds	• [1 cycle] 45 °C for 15 minutes • [1 cycle] 95 °C for 2 minutes • [45 cycles] 95 °C for 3 seconds, 55 °C for 30 seconds

*All RT-qPCRs were run on either an Applied Biosystems ViiA 7 or Bio-Rad CFX96 qPCR instrument.

Table 1. Comparison of RT-qPCR reaction setups from Biosearch Technologies and two competitors.

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Results and discussion

LoD determination

The LoD, as defined by the Minimum Information for Publication for Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin, *et al.*, 2009), is "the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure". Here we demonstrate the RapiDxFire 1-step RT-qPCR System has a LoD of 80 copies per reaction for the N1 assay, and 20 copies per reaction for the N2 assay, across 20 replicates for each copy number tested (Table 2).

The Twist synthetic SARS-CoV-2 RNA Control 2 is supplied at an approximate concentration of one million copies per microliter as quantified by mass using a fluorescence assay prior to final dilution. In an additional study, the RapiDxFire 1-Step RT-qPCR System for SARS-CoV-2 detection was found to have an LoD of 4 copies per 20 µL reaction volume. This study used the SeraCare Accuplex SARS-CoV-2 Reference Material as template with the 2019-nCoV N2 assay mix. The SeraCare Accuplex SARS-CoV-2 Reference Material is quantified by digital PCR, a more accurate quantification method than that reported for the Twist control material. See reference: sbeadex viral RNA purification kit and COVID-19.

Dynamic range and PCR efficiency

Reproducibility of replicates of a known target copy number across a range of Cq values is used to test the dynamic range across a span of five 10-fold serial dilutions. Figure 1 demonstrates the dynamic range of both the EpiScript/RapiDxFire qPCR Master Mix 1-step system for both the N1 and N2 targets, when compared with Competitor A and Competitor B reagents. The dynamic range of both target data sets is comparable, indicating high reproducibility and sensitivity from $1x10^6$ copies/reaction down to $1x10^2$ copies/reaction (Figure 1, Table 3, and Table 4).

Stability

To show that there is no loss in performance of the reagents over time or when repeatedly freeze-thawed, it is important to see robust stability data. Here we demonstrate targeting of

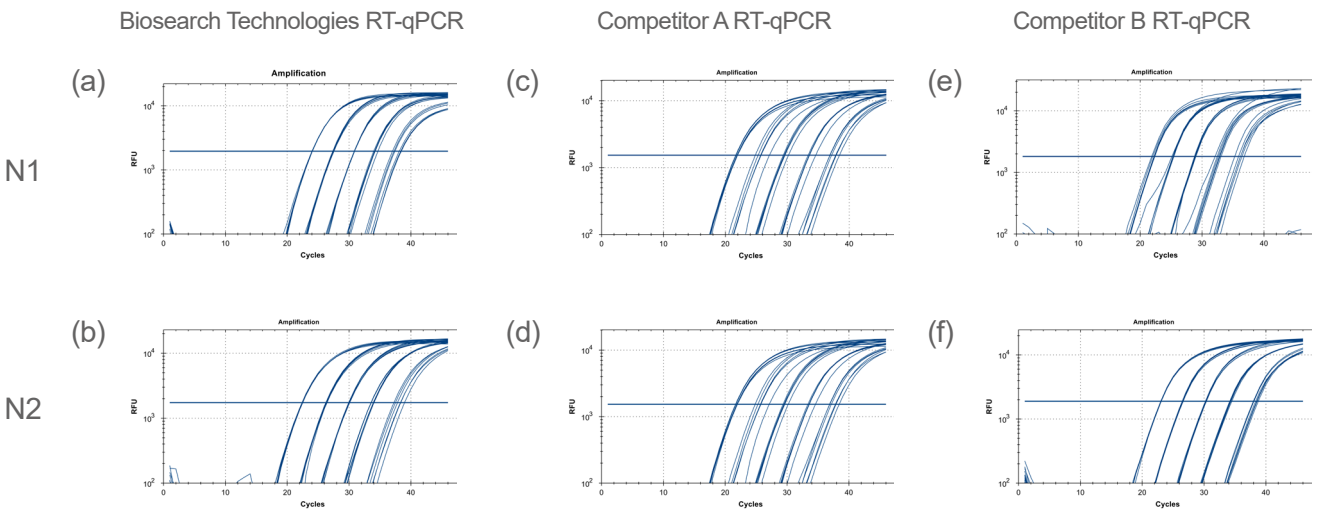

Figure 1. RT-qPCR using either [(a) and (b)] EpiScript and RapiDxFire qPCR Master Mix, [(c) and (d)] Competitor A reagents or [(e) and (f)] Competitor B reagents, for both the N1 and N2 targets. Target copy number was tested from $1x10^6$ copies/reaction in 10-fold serial dilution, down to $1x10^2$ copies/reaction.

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		Biosearch Technologies RT-qPCR			Competitor A RT-qPCR			Competitor B RT-qPCR		
Assay	Copies / reaction	+ / total	Cq average	Cq standard deviation	+ / total	Cq average	Cq standard deviation	+ / total	Cq average	Cq standard deviation
N1	10	12/20	38.6	0.9	20/20	36.5	0.8	15/20	38.3	0.9
	20	15/20	37.8	1.1	20/20	35.2	0.7	20/20	36.8	0.9
	40	18/20	37.0	1.2	19/20	34.7	1.0	20/20	36.1	1.0
	80	19/20	36.2	0.9	20/20	33.4	0.5	20/20	34.9	0.5
N2	10	13/20	38.2	0.7	19/20	38.5	0.8	14/20	38.0	0.7
	20	19/20	37.5	1.0	20/20	36.7	0.7	19/20	37.9	0.9
	40	20/20	36.7	0.9	20/20	35.6	0.7	20/20	36.3	0.5
	80	20/20	35.6	0.4	20/20	34.4	0.5	20/20	35.2	0.3

Table 2. Limit of detection for N1 and N2 assays. LoD defined as lowest target level at which the highlighted 19/20 replicates have Cq<40. + / total: Number positive replicates out of total number of replicates tested. Cq: Quantification cycle.

		Biosearch Technologies RT-qPCR		Competitor A RT-qPCR		Competitor B RT-qPCR	
Assay	Copies / reaction	Cq average	Cq standard deviation	Cq average	Cq standard deviation	Cq average	Cq standard deviation
N1	$1x10^6$	24.0	0.03	21.6	0.06	22.1	0.17
	$1x10^5$	27.4	0.06	24.9	0.09	25.3	0.10
	$1x10^4$	30.8	0.03	28.3	0.10	28.9	0.10
	$1x10^3$	34.2	0.20	31.9	0.11	32.6	0.35
	$1x10^2$	37.7	0.55	35.2	0.49	36.1	0.52
N2	$1x10^6$	22.4	0.04	21.8	0.14	23.0	0.03
	$1x10^5$	26.3	0.06	25.7	0.70	26.5	0.07
	$1x10^4$	30.0	0.06	29.6	0.28	30.2	0.06
	$1x10^3$	33.8	0.20	33.6	0.41	34.2	0.11
	$1x10^2$	38.0	0.58	37.5	0.57	38.3	0.29

Table 3. Average Cq values and standard deviations of replicates for dynamic range amplification plots illustrated in Figure 1, for both N1 and N2 targets.

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Assay	Standard curve calculation	Biosearch Technologies RT-qPCR	Competitor A RT-qPCR	Competitor B RT-qPCR
N1	PCR efficiency (%)	96.0	95.8	92.0
	R^2	0.997	0.997	0.996
N2	PCR efficiency (%)	81.3	79.6	82.2
	R^2	0.997	0.993	0.998

Table 4. PCR efficiency and R^2 values for standard curves generated from 10-fold serial dilutions ($1x10^6$ to $1x10^2$ copies/ reaction), for both N1 and N2 targets.

an MS2 RNA phage transcript following various storage or handling conditions, with no loss in sensitivity of detection.

Figure 2 shows the effect of preparing the RapiDxFire 1-step RT-qPCR System reaction mix at 2X concentration, and then storing the reagents for either 24 hours or 4 hours, at -20 °C, +25 °C or +4 °C. These reagents were then used to test 100 or 1,000 copies per reaction of MS2 phage RNA. As shown, there is no significant difference in the Cq obtained for any of the copy numbers or storage conditions.

Figure 3 illustrates the performance of subjecting the RapiDxFire 1-step RT-qPCR System, at 2X concentration, to 6 freeze-thaw cycles, followed by testing the reagents at 100 and 1,000 copies of the MS2 phage RNA. As shown, there is no significant effect on performance of the reagents that were treated to freeze-thaw cycles versus reagents that were fresh (no freeze-thaw cycles).

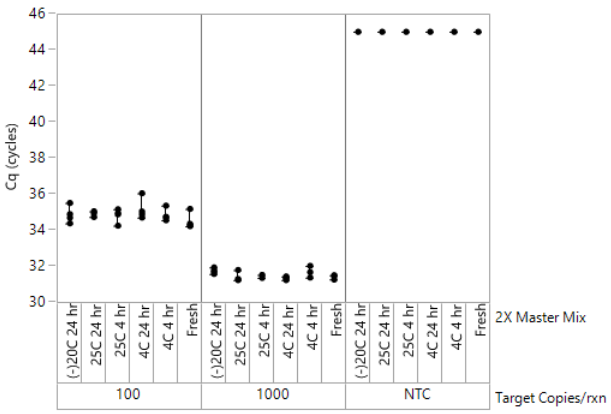

Figure 2: Performance of RapiDxFire 1-step RT-qPCR System, when stored under different conditions. Reagents were stored at 2X concentration at the indicated temperatures and times, and then tested using the MS2 phage RNA-specific assay.

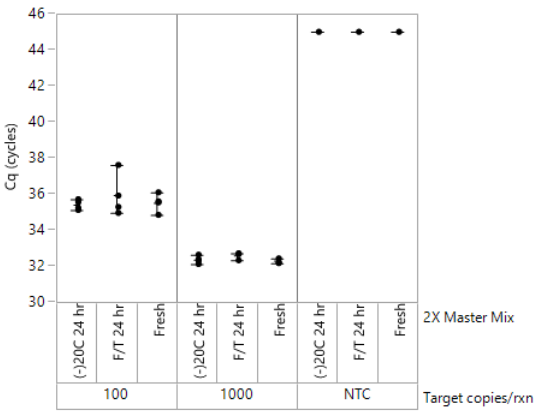

Figure 3: Performance of RapiDxFire 1-step RT-qPCR System, when subjected to 6 freeze-thaw cycles, and then tested using the MS2 phage RNA-specific assay.

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Summary

As demonstrated here, we have successfully combined two of our amplification reagents, EpiScript RNase H- Reverse Transcriptase and RapiDxFire qPCR 5X Master Mix GF into a 1-step system for the detection of SARS-CoV-2. We have shown that we can accurately define a reproducible analytical limit of detection below 100 copies per reaction, for both the CDC-validated N1 and N2 2019-nCoV-specific targets. In addition, the amplification reagents generate a consistent amplification efficiency over five 10-fold dilutions, down to 100 copies per reaction. We have also shown robustness in storage and handling of both reagents over various storage temperatures and multiple freeze-thaw cycles. This clearly demonstrates that when used in combination as a 1-step system, the EpiScript and RapiDxFire qPCR Master Mix can be used for SARS-CoV-2 analytical testing.

References

Bustin, S. A. et al., 2009. The MIQE Guidelines: Minimum Information for Publication for Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55(4), pp. 611-622.

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Available at: https://www.fda.gov/media/134922/download [Accessed March 2020].

CDC-released lots of Biosearch Technologies Primer-Probes available online at: https://www.cdc.gov/coronavirus/2019-ncov/downloads/List-of-Acceptable-Commercial-Primers-Probes.pdf

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