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Comparison of SARS-CoV-2 N gene real-time RT-PCR targets and commercially available mastermixes

Julianne R. Brown [a,*], Denise M. O'Sullivan [b], Divya Shah [a], Laura Atkinson [a], Rui P.A. Pereira [b], Alexandra S. Whale [b], Eloise J. Busby [b], Jim F. Huggett [b,c], Kathryn Harris [a]

[a] Microbiology, Virology and Infection Prevention and Control, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom
[b] National Measurement Laboratory at LGC, Teddington, United Kingdom
[c] School of Biosciences & Medicine, Faculty of Health & Medical Sciences, University of Surrey, United Kingdom

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ABSTRACT

Background: This study aimed to evaluate the impact of four different reverse transcription quantitative PCR (RT-qPCR) master mixes on the performance of SARS-CoV-2 diagnostic PCRs using three primer/probe assays targeting the N gene (A, B and C). The dynamic range and lowest detected quantity was determined using a SARS-CoV-2 partial N gene RNA transcript dilution series (100,000–1 copy/µl) and verified using 72 nose and throat swabs, 29 of which tested positive for SARS-CoV-2 RNA.

Results: Assay C consistently detected the lowest quantity of partial N gene RNA transcript with all mastermixes. The Takara One Step PrimeScript™ III RT-PCR Kit mastermix enabled all primer pairs to detect the entire dynamic range evaluated, with the Qiagen Quantifast and Thermofisher TaqPath 1-Step kits also performing well. Sequences from all three primer/probe sets tested in this study (assay A, B and C) have 100 % homology to ≥97 % of the of SARS-CoV-2 sequences available up to 31st December 2020 (n = 291,483 sequences).

Conclusions: This work demonstrates that specific assays (in this case assay C) can perform well in terms of dynamic range and lowest detected quantity regardless of the mastermix used. However we also show that, by choosing the most appropriate mastermix, poorer performing primer pairs are also able to detect all of the template dilutions investigated. This work increases the potential options when choosing assays for SARS-CoV-2 diagnosis and provides solutions to enable them to work with optimal analytical sensitivity.

1. Introduction

The 2019/2020 pandemic of SARS-CoV-2 infection has resulted in the need for rapid and global implementation of diagnostic testing. The gold standard method for diagnosis of SARS-CoV-2 infection is real-time reverse transcription polymerase chain reaction (RT-PCR) to detect viral RNA (WHO, 2021). In order to achieve accurate diagnosis of infection, the test must be able to detect viral RNA throughout the course of infection. This includes the pre-symptomatic phase of viral shedding, during which the viral load is rising, the symptomatic phase, when the outward symptoms of a SARS-CoV-2 infection are apparent, and the post-symptomatic phase, that can be less than a week after symptom onset, when the viral load is falling (Pan et al., 2020; Wölfel et al., 2020; Zou et al., 2020; Kim et al., 2020). Maximising the sensitivity of the RT-PCR diagnostic test is critical for detection throughout the course of infection.

In early 2020, as the virus was spreading rapidly around the world, few commercial RT-PCR kit assays were available that offered the full workflow from patient sample through to the test result (Pérez-López and Mir, 2020). This was further compounded with worldwide shortages of critical reagents needed for the RT-PCR tests as countries hurried to establish their testing regimes, as well as contamination of many of the oligonucleotide manufacturing facilities that enable specific detection of SARS-CoV-2 (Wang et al., 2020; Sadowski and Bogutz, 2020). Consequently, most of the early UK diagnostic efforts relied solely on clinical laboratories establishing their own laboratory developed tests (LDTs) while the commercial solutions were launched and their production was scaled up. A further advantage of the development of LDTs was the flexibility to choose from the reagents that were currently available, target different viral regions and explore different strategies for primer

* Corresponding author at: Level 4 Camelia Botnar Labs, Great Ormond Street Hospital for Children NHS Foundation Trust, Great Ormond Street, London, WC1N 3JH, United Kingdom.
E-mail address: julianne.brown@gosh.nhs.uk (J.R. Brown).

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and probe design.

This study aimed to evaluate four commercial one-step real-time RT-PCR mastermix options that were available in the UK in early 2020 for use in the clinical LDT at Great Ormond Street Hospital. Three published SARS-CoV-2 assays, that targeted different regions of the nucleocapsid protein (N) gene, were used to evaluate the mastermix performance in terms of the linearity of the tested concentration range, sensitivity, PCR efficiency and lowest detection quantity (LDQ). The LDT was then used to measure the presence of SARS-CoV-2 in 72 clinical samples from patients presenting with symptoms of SARS-CoV-2 infection.

2. Materials and methods

2.1. Test material

2.1.1. N gene transcript

An RNA transcript of the SARS-CoV-2 nucleocapsid (N) gene was synthesised by *in vitro* transcription of a linearised plasmid containing the entire N gene. Full length transcripts were confirmed using the 2100 Bioanalyzer system (Agilent) and the RNA concentration was estimated using the Qubit Fluorometer (ThermoFisher). The RNA was diluted to approximately 1 million copies/μL in nuclease-free water and stored in aliquots at −80 °C. A six-point 10-fold serial dilution series of the N gene transcript was prepared from 100,000–1 copy/μl in nuclease-free water. Full details of the production and characterisation of the transcript are provided in the supplementary methods.

2.1.2. Nose and throat swabs

Total nucleic acid was purified from 74 nose and/or throat swabs from suspected cases of SARS-CoV-2 infection at Great Ormond Street Hospital (GOSH). Briefly, dry flocked swabs were re-suspended with 600 or 1,200 μL nuclease-free water (for single or double swabs, respectively). Total nucleic acid was purified from 225 μL swab suspension fluid using the Hamilton Nimbus (with Kingfisher Presto) and Omega Biotek Mag-Bind Viral DNA/RNA kit. RNA was eluted in 100 μL elution buffer. Each 225 μL specimen was spiked with 1.1 μL Phocine Distemper Virus (PDV) cell culture isolate (cultured in vero cells) before the nucleic acid extraction to act as an internal positive control. PDV is an established qualitative control used in all clinical RT-PCR assays at GOSH to control for gross PCR inhibition or nucleic acid extraction failure (Bibby et al., 2011). Negative extraction controls were included alongside the specimen extractions that contained water in place of swab suspension fluid; these were also spiked with PDV.

2.2. In silico evaluation of primer/probe design

For the *in silico* evaluation of primer and probe coverage 311,147 SARS-CoV-2 genome sequences were retrieved from database Global Initiative on Sharing All Influenza Data (GISAID) (Shu and McCauley, 2017) (https://www.gisaid.org/) (accessed 31.12.2020). In addition, 60 taxonomically closely related human SARS-CoV genome sequences were obtained from NCBI data, using SARS-CoV reference sequence (GenBank accession number NC_004718.3). Reduced length (<27,000 bp) and low quality sequences (≥10 % unknown bases (Ns)) were removed. Upon quality filtering, a database with 291,483 SARS-CoV-2 and 61 SARS-CoV sequences was used to evaluate *in silico* the inclusivity of the assays used in this study.

2.3. RT-PCR testing

All RT-PCR experiments are reported using the MIQE guidlines (Bustin et al., 2009). Further details to those described here are presented in the supplementary information: Table S1.

The N gene transcript dilution series was tested with duplicate RT-PCR reactions using the four commercially available mastermixes (Table 1) and three primer and probe sets (Table 2). Assay A is a duplex reaction that targets the N gene and internal extraction control target PDV gene. Assays B and C are singleplex reactions targeting the N gene only. The nucleic acids extracted from swabs were tested using the mastermixes produced by Takara and Qiagen with Assay A only. For all Mastermix and assay combinations, no template control (NTC) reactions were used containing nuclease-free water in place of RNA.

All PCR reactions were performed in 25 μL reaction volumes with 7.5 μL of RNA added per reaction. Qiagen mastermix reactions were run on a 7500 Fast thermocycler (ThermoFisher) while all other mastermix reactions were run on a QuantStudio5 thermocycler (ThermoFisher) with manufacturer recommended fast cycling conditions and 45 cycles (Table 1).

The cycling programmes were run and the data collected using the 7500 Fast System software v1.5.1 or QuantStudio 5 Dx Software v1.0.2. The quantification cycle (Cq; generic term for cycle threshold, Ct and crossing point, Cp (Bustin et al., 2009)) values were obtained using a manual threshold method (threshold set mid-point through the exponential phase of amplification, as per manufacturer guidance and standard practice in clinical PCR assays at GOSH). A positive result was

Table 2
Details of primer and probes used for detection of SARS-CoV-2 RNA (sequences are detailed in supplementary data).

	Description	Reference	Final primer and probe concentration		
			Forward	Reverse	Probe (fluorophore)
A	N geneTaq-PDV duplex	N gene; (Grant et al., 2020) PDV; (Bibby et al., 2011)	0.4 μM 0.125 μM	0.6 μM 0.125 μM	0.3 μM (FAM) 0.125 μM (Cy3)
B	N1, single target	13	0.5 μM	0.5 μM	0.125 μM (FAM)
C	N2, single target	13	0.5 μM	0.5 μM	0.125 μM (FAM)

Table 1
Details of commercial mastermixes and thermocycler platforms used. All reactions were run in 96-plate format.

Mastermix full name	One Step PrimeScript™ III RT-PCR Kit	Quantifast Multiplex RT-PCR + R mastermix	TaqPath 1-Step RT-qPCR mastermix	Taqman Fast Virus 1-step mastermix
Mastermix manufacturer (product code) PCR Instrument (manufacturer)	Takara(RR600B) QuantStudio 5 Real-Time PCR System (ThermoFisher)	Qiagen(204956) 7500 Fast Real-Time PCR System for N gene transcript; QuantStudio 5 Real-Time PCR System for swabs (ThermoFisher)	ThermosFisher(A15299) QuantStudio 5 Real-Time PCR System (ThermoFisher)	ThermosFisher(4444432) QuantStudio 5 Real-Time PCR System (ThermoFisher)
PCR cycling conditions 1 cycle of:	52 °C, 5 minutes	50 °C, 20 minutes	25 °C, 2 min 50 °C, 15 minutes	50 °C, 5 minutes
45 cycles of:	95 °C, 10 s 95 °C, 5 s 60 °C, 30 s	95 °C, 5 minutes 95 °C, 15 s 60 °C, 30 s	95 °C, 2 minutes 95 °C, 3 s 60 °C, 30 s	95 °C, 20 s 95 °C, 3 s 60 °C, 30 s
Total cycling time	56 minutes	84 minutes	69 minutes	55 minutes

defined as amplification detected above the threshold within 45 cycles. The data was exported for further analysis in MS Excel/GraphPad Prism v9.0.0. Images of the amplification curves were taken directly from the analysis software.

The LDQ was defined as the lowest dilution at which both duplicate reactions were detected. Where only one of the duplicates was detected, the LDQ was estimated to be between that and the consistently detected dilution. Where both duplicate reactions for the lowest dilution were amplified the LDQ was estimated to be less than the lowest dilution tested (described as <7.5 copies per reaction).

The PCR efficiencies ($E = -1 + 10^{(-1/\mathbf{slope})}$) were calculated based on the slope of the linear regression between the Cq value and the log (copies per reaction) for the concentrations that were above the LDQ. The linear correlation was based on the R (Pan et al., 2020) value of the data.

3. Results

3.1. In silico evaluation of primer/probe design

The primer and probe sequences from the three assays tested in this study were aligned with 291,483 SARS-CoV-2 sequences available up to 31st December 2020. All three primer/probe sets (assay A, B and C) have 100 % homology to a very high percentage of the sequenced strains of SARS-CoV-2 (≥97 %), including clinically relevant variants 501Y.V2 (B.1.351) and 501Y.V3 (P1). After exclusion of sequences with ambiguous bases, assays A, B and C had one mismatch to 8,842, 9493 and 8,736 SARS-CoV-2 sequences, respectively; and two or more mismatches to 19, 211 and 182 SARS-CoV-2 sequences, respectively (see Supplementary Results). Regarding variant 501Y.V1 (B.1.1.7), similar coverage was observed for assays B and C. Yet 501Y.V1 sequences present a C28977 T substitution, causing one mismatch on the 5′end of the Assay A forward primer.

3.2. Evaluation of the different commercial one-step RT-PCR mastermixes

The N gene transcript dilution series was used to establish the LDQ and assay efficiency of the three assays (A, B and C) with the four commercial mastermixes. Amplification curves for each dilution series can be seen in Supplementary data (Supplementary Figure S1–S4). The Takara One Step PrimeScript™ III RT-PCR Kit mastermix was the most sensitive, achieving the lowest LDQ of ≤7.5 copies per reaction for all assays compared to the other mastermixes tested in this study. Conversely the TaqMan Fast Virus 1-step Mastermix consistently gave the highest LDQ results (7.5–750 copies/reaction) across all assays tested (Table 3).

Good linearity was observed for all mastermix and assay combinations (R2 > 0.9784, median R2 = 0.9940) (Supplementary Figure S5). The assay efficiencies ranged from 84.5%–102.9% with the assay having a bigger impact on the efficiency than the mastermix (Table 3).

3.3. Detection of SARS-CoV-2 in nose and throat swabs

Following validation for the three assays and four mastermixes, the best performing Takara mastermix and the Qiagen mastermix were selected for swab analysis; the Qiagen mastermix was chosen due to availability at the time and use in other existing clinical RT-PCR assays at GOSH. Assay A was used for this part of the study as it had been validated for use in a duplex reaction with PDV assay that detects the internal extraction control that was spiked into each of the swabs prior to extraction.

The NTC reactions were examined for presence of amplification; no amplification was observed in any reactions. The PDV internal extraction control in Assay A was expected to give a Cq value of approximately 28 based on the experimental volumes used and historical data from the GOSH clinical laboratory; these values were observed for 72/74 extracted swabs (mean observed Cq value 28, range 26–30) (Supplementary Figure S6). Two swabs had undetectable PDV suggesting extraction failure; these were excluded from further analysis.

Of the 72 swabs tested using the Takara and Qiagen mastermixes, 67/72 had concordant SARS-CoV-2 RT-PCR results, of which 29 swabs were positive with both mastermixes and 38 were negative with both mastermixes (Table 4).

Of the discordant results, seven swabs were positive with the Takara

Table 3
Cq values from the RT-PCR reactions evaluating the four mastermixes with three N gene assays.

N gene transcript copies/µl (copies/reaction)		Takara		Qiagen Quantifast		Fast Virus		Taqpath	
Assay A	100,000 (750,000)	22.4	22.4	22.6	22.6	23.4	23.4	21.4	21.4
	10,000 (75,000)	26.5	26.2	26.3	26.2	27.1	27.3	25.2	24.9
	1000 (7500)	29.1	29	30.2	30.2	29.8	29.5	28.5	28.4
	100 (750)	32.6	32.9	33.4	33.7	33.3	33.4	31.7	31.7
	10 (75)	36.2	37.5	39	36.5	39.3	38.8	35.7	34.8
	1 (7.5)	39.5	40.2	ND	ND	ND	ND	38.1	ND
LDQ (copies/µl)		*1*		*10*		*10*		*1–10*	
LDQ (copies/reaction)		*7.5*		*75*		*75*		*7.5*	
Slope / R^2 / efficiency		*−3.5 / 0.995* / 93.1 %		*−3.8 / 0.988 / 84.5%*		*−3.7 / 0.978 / 85.0%*		*−3.4 / 0.998* / 97.6 %	
Assay B	100,000	21.7	21.7	21	21	22.8	22.8	20.7	20.8
	10,000	25.5	25.4	24.6	24.5	26.6	26.5	24.2	24.2
	1000	28.3	28.4	28.5	27.9	29.3	29	27	27.1
	100	31	31.7	31.1	31.8	33.1	32.9	29.9	31
	10	35.4	35.6	36.8	34.8	ND	37	34.4	33.4
	1	39.3	41.4	40.1	ND	ND	ND	ND	37.4
LDQ (copies/µl)		*1*		*1–10*		*10–100*		*1–10*	
LDQ (copies/reaction)		*7.5*		*7.5–75*		*75–750*		*7.5–75*	
Slope / R^2 / efficiency		*−3.6 / 0.986* / 89.5 %		*−3.8 / 0.989 / 83.6%*		*−3.5 / 0.995* / 95.1 %		*−3.3 / 0.996* / 99.8 %	
Assay C	100,000	21.1	21.2	20.2	20.2	21.8	21.8	20.2	20.2
	10,000	25	25	23.8	23.7	25.8	25.4	23.7	23.8
	1000	28	27.7	26.8	27.1	28.8	28.6	26	27
	100	31.5	31.5	30.9	30.7	32.5	32.5	30.7	30.3
	10	34.8	35	34.7	35.7	35.4	35.3	33.7	34.6
	1	38.1	38.7	37.6	36.2	38.9	ND	37.3	38.1
LDQ (copies/µl)		*<1*		*<1*		*1–10*		*<1*	
LDQ (copies/reaction)		*<7.5*		*<7.5*		*7.5–75*		*<7.5*	
Slope / R^2 / efficiency		*−3.4 / 0.999* / 95.9 %		*−3.5 / 0.989 / 94.5%*		*−3.4 / 0.998* / 97.5 %		*−3.5 / 0.995* / 92.8 %	

Key: ND, not detected; LDQ, lowest detected quantity.

mastermix but negative with the Qiagen mastermix, with a median Cq value of 40.9 (range 38.5–41.9). There were no specimens that were positive with the Qiagen mastermix but negative with Takara mastermix. The background fluorescence of the amplification plot was considerably lower with Takara mastermix compared to Qiagen Mastermix (Supplementary Figure S6).

4. Discussion

4.1. In silico evaluation of primer/probe design

There is 100 % homology for the forward primer and probe of Assay A to the closely related Betacoronavirus SARS-CoV, however cross-amplification is not expected due to four mismatches in the reverse primer. In assays B and C, cross-amplification of SARS-CoV is not expected due to multiple mismatches in forward and reverse primers and probe (see Supplementary Results). In addition, *in silico* evaluation predicted, for all assays, no cross-reactivity with other human-relevant coronaviruses (229E, HKU1, MERS-CoV, NL63 and OC43), as well as other major respiratory human pathogens (data not shown).

Although possible cross-reactivity with SARS-CoV is not of clinical concern in the UK at this time, further *in vitro* confirmatory testing is required to verify that the mismatches in Assay A (reverse primer) (Grant et al., 2020) are sufficient to prevent non-specific amplification as it may be of clinical concern in the future. Finally, whilst not predicted to have a detrimental effect, additional *in vitro* testing is needed to assess the effect of assay A forward primer (Grant et al., 2020) 5′ end mismatch on the detection of increasingly predominant 501Y.V1 variant.

4.2. Evaluation of the different commercial one-step RT-PCR mastermixes

The observed linearity for all mastermix and assay cmbinations suggests that the design of the assay and its specificity is a more important factor than the mastermix used as Assay C consistently performed well in terms of LDQ and efficiency across all four mastermixes, including when it was used with the most poorly performing mastermix (TaqMan Fast Virus mastermix) (Supplementary Figure 5c).

These findings demonstrate that the mastermix used can impact the LDQ by more than ten-fold which may affect the ability to detect low level positive clinical specimens, nevertheless demonstrate that the overall performance of the reaction is good across a wide range of viral concentrations. This facilitates the adaption of LDTs to accommodate changes in reagent availability should there be restrictions in the products available in the future.

The LDQ is a good indication of the likely analytical sensitivity of the PCR. This measure differs from the limit of detection (LOD) which is a more comprehensive statistical evaluation of the methodological performance (International Organization for Standardization, 2019). Further work is required to determine the LOD which was beyond the scope of this study as LDQ is sufficient for validation of a clinical assay in the UK (International Organization for Standardization, 2012).

4.3. Detection of SARS-CoV-2 in nose and throat swabs

The additional detection of SARS-CoV-2 in seven swabs using Takara Mastermix, compared to Qiagen, is unsurprising as the LDQ assessment using the N gene transcript indicated that the Takara mastermix had better sensitivity than the Qiagen mastermix. Indeed, the seven discordant samples had high Cq values indicating that these samples were below the LDQ of the Qiagen mastermix (Supplementary Figure S6c).

5. Conclusions

Based on the data generated in this study we recommend that for optimum detection of SARS-CoV-2 RNA from nose and throat swabs

Table 4
Concordance in SARS-CoV2 PCR results for 72 swabs tested using Qiagen Quantifast Multiplex RT-PCR + R mastermix and Takara One Step PrimeScript™ III RT-PCR Kit mastermix, and in-house N geneTaq-PDV real-time RT-PCR assay. Results expressed as number of specimens.

	Positive by Takara	Negative by Takara
Positive by Qiagen	29	0
Negative by Qiagen	7*	36

* Median Cq 40.9 (range 38.5–41.9).

Assay C (CDC N2 primers and probe) (CDC, 2021) is used with any of the following mastermixes assessed in this study; Takara, Qiagen and TaqPath. Alternatively, we recommend the N geneTaq (assay A) (Grant et al., 2020) or the N1 (Assay B) (CDC, 2021) assays are used with Takara mastermix.

The data presented here provides evidence for the recommendation of mastermixes for detection of SARS-CoV-2 RNA targeting the N gene and suggests adequate alternatives in the event of supply chain issues.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114215.

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