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Diagnostic Microbiology and Infectious Disease 101 (2021) 115458

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Extractionless nucleic acid detection: a high capacity solution to COVID-19 testing

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ARTICLE INFO

Article history:
Received 13 January 2021
Revised in revised form 12 May 2021
Accepted 13 June 2021
Available online 17 June 2021

Keywords:
Extractionless
COVID-19
Heat extraction
SARS-CoV-2
Roche
COBAS 4800
E-gene
N1-gene
N2-gene
Orf1ab gene
RdRp gene
Quantabio

ABSTRACT

We describe an extractionless real-time reverse transcriptase-PCR (rRT-PCR) protocol for SARS-CoV-2 nucleic acid detection using heat as an accurate cost-effective high-capacity solution to COVID-19 testing. We present the effect of temperature, transport media, rRT-PCR mastermixes and gene assays on SARS-CoV-2 gene amplification and limits of detection. Utilizing our heated methodology, our limits of detection were 12.5 and 1 genome copy/reaction for singleplex E- and N1-gene assays, respectively, and 1 genome copy/reaction by utilizing an E/N1 or Orf1ab/N1 multiplex assay combination. Using this approach, we detected up to 98% of COVID-19 positive patient samples analyzed in our various cohorts including a significant percentage of weak positives. Importantly, this extractionless approach will allow for >2-fold increase in testing capacity with existing instruments, circumvent the additional need for expensive extraction devices, provide the sensitivity needed for COVID-19 detection and significantly reduce the turn-around time of reporting COVID-19 test results.

1. Introduction

In many parts of the world, a second wave of infections has already started due to a return to work, increased social gatherings and decreased interest in following COVID-19 safety protocols. In addition, many parts of the world are now into flu season that will put many vulnerable populations in a position of increased susceptibility to COVID-19 infections. Many have predicted that we will see a continual increase in the number of COVID-19 cases in the fall/winter of 2020/2021 that will certainly put a burden on many health care systems (Tosi and Campi, 2020; Li et al., 2020). It is now widely recognized that we should no longer focus solely on the symptomatic patients but also monitor the asymptomatic population (Wiersinga et al., 2020). The asymptomatic and pre-symptomatic population can be carriers of coronavirus, show no clinical symptoms and can infect 3–5 bystanders without knowing it (Kimball et al., n. d.; Wycliffe et al., 2020). Most public health agencies have anywhere from a 4 to 8-day reporting time for COVID-19 testing which is not ideal if effective prevention of the spread of COVID-19, particularly from asymptomatic/pre-symptomatic individuals, is a priority (Kretzschmar et al., 2020). Thus, there is a need to accelerate public testing and achieve faster reporting turn-around-times.

Several platforms exist for COVID-19 with several commentaries and reviews recently written about these various platforms (Fang and Meng, 2020). The identification of COVID-19 in most diagnostic laboratories is based on genetic analysis for SARS-CoV-2 genes, and more commonly for the envelope protein (the E-gene), the nucleocapsid protein (the N-gene), the RNA dependent RNA polymerase (the RdRP gene) and the open reading frame 1ab protein (the Orf1ab gene). These genes have been utilized in singleplex and multiplex assays with varying analytical performance (Vogels et al., 2020). These salient parameters have promoted an increased number of

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# Equal contributions to this article.

https://doi.org/10.1016/j.diagmicrobio.2021.115458

reports on ways to increase testing capacity, increase accuracy of the genetic test, the exploration of other avenues of COVID-19 detection such as serology testing and rapid point of care testing for the detection of viral proteins (Espejo et al., 2020) and the design of extractionless approaches.

The design of an extractionless system is an attractive idea that suggests the need for only a rRT-PCR machine, reduction in the turnaround time and cost of testing, potential for increased capacity and an accessible approach for remote testing. Two extractionless approaches have been reported over the past several months: a heated approach (Pastorino et al., 2020; Pastorino et al., 2020; Lubke et al., 2020; Smyrlaki et al., 2020) and proteinase K dependent analysis of saliva (Wyllie et al., 2020; Ott et al., 2020). The approach utilizing saliva has been recently optimized to suggest that saliva specimens have similar sensitivity (and sometimes higher viral load) to nasopharyngeal swab specimens in the detection of SARS-CoV-2 in strong positive or hospitalized patients. One main attraction of the saliva based extractionless approach is that it is less invasive to the patient than nasopharyngeal or throat swabbing. However, a variability with this approach lies in the use of proteinase K, a protease that can potentially inhibit the rRT-PCR reaction if not properly inactivated, and the use of 95°C heating, a temperature known to degrade 80% of the initial viral RNA (Pastorino et al., 2020). Thus, with crude samples (such as saliva), compatibility with purified enzymes driving rRT-PCR and the potential denaturation of intact viral RNA with heating will affect the performance of the assay in detecting small amount of viral particles that accompany weak patient positives.

The other extractionless approach is the use of heat extraction of nucleic acid. Several reports have suggested the use of 56°C, 65°C and 95°C to inactivate *Coronavirus* and release its nucleic acid for detection (Pastorino et al., 2020; Lubke et al., 2020; Smyrlaki et al., 2020; Zou et al., 2020). These reports have shown 80%−100% detection rate based on the use of heated samples. However, because of the lack of uniformity among existing studies on the specific heated methodology used, including differing rRT-PCR assay combinations being utilized (Smyrlaki et al., 2020), lack of an appropriate internal control, lack of detailed analytical performance analysis, among other factors, it proved difficult to decipher which would lend to a robust extractionless COVID-19 diagnostic protocol and gene assays for our clinical laboratory to adopt. In this study, we addressed the challenges encountered with the use of heat extraction for genetic testing for *Coronavirus* that has not been addressed in previous studies. We also describe cost saving options to increase testing capacity without sacrificing analytical performance.

2. Material and methods

2.1. Composition of patient positive and negative samples

For this study, we were able to obtain patient positive and negative frozen swab samples from the Research Sample Repository at the Alberta Precision Laboratory as well as fresh patient positive and negative specimens from *DynaLIFE* Medical Labs COVID-19 testing program. All 510 clinical samples (255 confirmed positive and 255 confirmed negative) used in this study, were collected from April 2020 to October 2020 and either stored at −80°C or kept at 4°C until they were ready for analyses.

2.2. rRT-PCR Primers/probes/mastermixes

Primers (forward/reverse)/probes to E (WHO) (IDT, Coralville, Iowa, USA)(23), N1, N2 [both from SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay](21), RdRP and RNaseP genes were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa, USA) while the E-gene/Equine Arteritis Virus (EAV) primer/probes were obtained from Roche as part of the LightMix® Modular EAV RNA Extraction Control kit (Roche Diagnostics, Mississauga, Ontario, Canada). EAV is a positive-sense single-stranded RNA virus that is added to all clinical specimens prior to nucleic acid extraction to serve as an extraction and amplification control. ATCC VR-1986HK™, a heat-inactivated Sars-CoV-2 virus commercially available from ATCC, was obtained from its distributor Cedarlane Labs (Cedarlane Inc, Ontario, Canada) for determination of the limit of detection of the assay being developed and for comparison purposes (henceforth referred to as "SARS-COV-2 Heat Inactivated Virus"). Mastermixes studied included: Roche LightMix (Roche Diagnostics, Mississauga, Ontario, Canada), Quantabio UltraPlex 1-Step Toughmix (Quantabio Inc., Beverly, Massachusetts, USA) (henceforth referred to as "Quantabio mastermix"), Promega (Promega, Madison, Wisconsin, USA) and Agilent (Agilent Technologies Canada Inc., Mississauga, Ontario, Canada).

2.3. Sample preparations with Roche COBAS 4800 nucleic acid extraction

Specimens were vortexed gently for 1 minute, and 500 μL of each specimen was mixed with 500 μL of lysis buffer. This was followed by automatic nucleic acid extraction on Cobas® 4800 system (Roche Diagnostics, Mississauga, Ontario, Canada), which was performed according to the manufacturer's recommendations.

2.4. Sample preparations for direct rRT-PCR

Specimens were gently vortexed, 100 μL transferred to a 96 well rRT-PCR plate and inactivated at either 95°C for 3 minutes or 65°C for 15 minutes in the Roche z480 rRT-PCR machine. Alternatively, patient swab samples in transport media were inactivated by immersion in a water bath at 65°C for 30 minutes (validated in a separate study; data not shown). After heating, the samples were cooled down for 5−10 minutes and 5 μL used directly in rRT-PCR or were stored at −20°C for longer term storage.

2.5. Cobas 4800 Extraction

The Cobas 4800 is a fully automated instrument that allows extraction of nucleic acids from samples, followed by real-time polymerase chain reaction (PCR) amplification and detection. Prior to loading 96 samples onto the Cobas® 4800 system for extraction, MagNA Pure 96 external lysis buffer (Roche Diagnostics, Mississauga, Ontario, Canada) is added at a ratio of 1:1 and 400 μl is tested for each sample. Due to the lack of user-defined workflow for SARS-CoV-2 on the Cobas® 4800 system, the CT/NG user-defined workflow (under "test type" in the system's software) was selected instead following the manufacturer's recommendation. Once each extraction was completed, the original 96-well plate is discarded, but the corresponding "deep-well" plate containing the purified nucleic acids is conserved for the remaining steps in the SARS-CoV-2 amplification and detection process.

LightMix® SarbecoV E-gene plus EAV control assay is performed (Roche Diagnostics, Mississauga, Ontario, Canada), as described in Tib Molbiol document MDx 40-0776-96-V200422. Cobas 4800 software version 2.2.0.1509 was used for extraction and Cobas user-defined workflow (UDF) software version 2.0.1 was used for PCR.

2.6. rRT-PCR detection of SARS-CoV-2

Five microliters (5 μL) of the heated sample or 10 μL of the nucleic acid extracts from the Cobas® 4800 extraction was mixed with the LightMix mastermix according to manufacturer's recommendation to a total final pre-amplification volume of 20 μL. For E-gene/EAV amplification, 0.5 μL of the LightMix SarbecoV E-gene plus EAV primer/probes was utilized (Roche Diagnostics, Mississauga, Ontario, Canada); for N1 gene amplification, 1 μL of the N1-FAM

primer/probe (CDC sequence)(IDT, Coralville, Iowa, USA)(22) was utilized; and for the RdRp gene amplification, 3.2 μL of Rdrp-FAM primer/probe (WHO sequence) (IDT, Coralville, Iowa, USA) was utilized.

Thermal Cycling was performed in the LightCycler 480 as per steps described in Table 2. Cq from FAM (E, N1 or Orf1ab gene) and Cy5 (EAV or RNases P) channels were obtained.

Samples were considered "*positive*" when a signal was detected with a Cq < 40 and fluorescence ≥ 1.75 arbitrary units for any gene, provided the extraction control/internal control successfully amplified. A sample was considered "*negative*" when the Cq ≥ 40 and fluorescence of the E, N1 or Orf1ab gene was <1.75 arbitrary units (and thus not amplified), provided the extraction control/internal control successfully amplified. A sample was considered "*invalid*" when there was no amplification of the extraction control/internal control or when the fluorescence of the extraction control/internal control was <1 arbitrary unit. All negative results from positive patient cohorts were re-extracted using the Roche COBAS extraction to confirm the integrity of the sample. Any sample result falling outside of the above parameters would be subject to retesting on an FDA and Health Canada-approved commercial platform (i.e., Panther Fusion® SARS-CoV-2 Assay on the Hologic Panther System).

The rRT-PCR mastermix was prepared following the vendor's recommended instructions with primer/probe volumes as indicated above and in Table 1. For EAV addition, EAV was dissolved in 1000 μL RNase/DNase free water and 5 μL was added to the mastermix. Fifteen μl of the mastermix was then added to a well containing 5 μL of a clinical sample to bring the total volume to 20 μL for the rRT-PCR reaction. The use of less sample volume for the heat extraction approach as compared to the 10 μL normally used from an extracted sample allowed for more dilution of any interfering components that may be present in a sample and for better compatibility with the rRT-PCR reagents. For all rRT-PCRs, we utilized the European standard for running COVID-19 tests, the EURM-019 single stranded RNA (ssRNA) fragments of SARS-CoV-2 (Sigma-Aldrich). This standard can be diluted in RNase/DNase free water and utilized at 1:5000 as a positive control.

2.7. Determination of the limit of detection for E, N1 and RdRP genes between conventional extraction vs extractionless approach

ATCC VR-1986HK™ ("SARS-COV-2 Heat Inactivated Virus") is provided commercially at a concentration of 3.75×10^5 genome copies/μl. Sixteen serially diluted aliquots from this initial concentration were prepared and 6 (x 5 μL) of each dilution were assayed in parallel for each E, N1 and RdRP gene targets using the Cobas® 4800 system and the resulting Cq values were compared between those assayed with extraction vs those without (i.e., extractionless). Limit of detection was defined as a concentration whereby the virus was detected in >95% of its replicates with good sigmoidal curve and fluorescence >1 unit.

Table 1
Primers and probes used in for SARS-COV-2 rRT-PCR.

Name	Amplicon Length (bp)	Primer/Probe	Sequence (5' – 3)	Final concentration in one tube mix and rRT-PCR[a]	Catalog [b]
E(WHO)[c]	113	Forward	ACAGGTACGTTAATAGTTAATAGCGT	4 μM/400 nM	Ref (Corman et al., 2020).
FAM based probe		Reverse	ATATTGCAGCAGTACGCACACA	4 μM/400 nM	IDT, #10006804
		Probe	FAM-ACACTAGCA/ZEN/ TCCTTACTGCGCTTCG-IABkFQ	2 μM/200 nM	Use 2 μL of this working solution in rRT-PCR reaction
N1[b]	72	Forward	GAC CCC AAA ATC AGC GAA AT	13.4 μM/670 nM	Designed by US CDC (Zhang, 2021, 2021).
FAM based probe		Reverse	TCT GGT TAC TGC CAG TTG AAT CTG	13.4 μM/670 nM	Obtained from IDT, #10006830
		Probe	FAM-ACC CCG CAT /ZEN/TAC GTT TGG TGG ACC-IABkFQ	3.4 μM/170 nM	#10006831 #10006832 Use 1 μL of this working solution in rRT-PCR reaction
RdRP[b]	99	Forward-F2	GTGARATGGTCATGTGTGGCGG	500	IDT, #10006806
FAM based probe		Reverse-R1	CARATGTTAAASACACTATTAGCATA	500	Use 3.6 μL of this working solution in rRT-PCR reaction
		Probe-P2	FAM/CAGGTGGAA/ZEN/CCTCATCAG-GAGATGC/3IABkFQ/	125	
RNaseP	65	Forward	AGA TTT GGA CCT GCG AGC G	6.7 μM/670 nM	IDT, #10006827
Atto647 based probe		Reverse	GAG CGG CTG TCT CCA CAA GT	6.7 μM/670 nM	IDT, #10006828
		Probe	Atto647NN-TTC TGA CCT /TAO/ GAA GGC TCT GCG CG-IABRQSp	1.7 μM/170 nM	Obtained from IDT #10007061 Use 2.0 μL of this working solution in rRT-PCR reaction
E	Proprietary to Roche	Forward	Proprietary to Roche	Proprietary to Roche	Proprietary to Roche (FAM based probe)
EAV	Proprietary to Roche	Forward	Proprietary to Roche	Proprietary to Roche	Proprietary to Roche (Cy5 based probe)
Cy5 based probe		Reverse	Proprietary to Roche	Proprietary to Roche	(ivRNA EAV from TIB MOL BIOL)
		Probe	Proprietary to Roche	Proprietary to Roche	
Orf1ab	119	Forward	CCCTGTGGGTTTTACACTTAA	6.7 μM/335 nM	Custom Primer/Probe
FAM based probe		Reverse	ACGATTGTGCATCAGCTGA	6.7 μM/335 nM	Use 1 μL of this working solution in rRT-PCR reaction
		Probe	FAM/TTGCTGCTG/ZEN/CTTG ACA GAT T-IABkFQ	1.7 μM/85 nM	
N2	67	Forward	TTA CAA ACA TTG GCC GCA AA	10 μM/500 nM	Designed by US CDC (Zhang, 2021, 2021).
FAM based probe		Reverse	GCG CGA CAT TCC GAA GAA	10 μM/500 nM	Obtained from IDT
		Probe	FAM-ACA ATT TGC /ZEN/CCC CAG CGC TTC AG-IABkFQ	2.5 μM/125 nM	# 10006824, 10006825, 10006826 Use 1 mL of this working solution in rRT-PCR reaction

[a] Final concentration in rRT-PCR is based on a 20 μl final reaction volume for the rRT-PCR.
[b] W is A/T; R is G/A; M is A/C; S is G/C and all primers and probes are made up in 100 μM 1 X TE pH 7.5.
[c] Already pre-mixed. However, this pre-mixed working solution can be made from catalog# 10006889/10006891/10006893 in the ratio of 4 μM, 4 μM (primers) and 2 μM (probe).

2.8. Performance of the extractionless protocol on pooled clinical samples

Nasopharyngeal swabs collected in Yocon VTM (Yocon biotechnology Co. Ltd, Beijing, Beijing, China) were used for pooling experiment using E (WHO)/N1 multiplex with Quantabio mastermix. Twenty-nine known positive samples were pooled with known negative samples in 1 in 2 and 1 in 4 ratios and the resulting Cq values changes were analyzed. In a separate experiment seeking to evaluate the effect of sample pooling and viral load on resulting Cq values, known negative nasopharyngeal swabs were spiked with SARS-COV-2 Heat Inactivated Virus to make viral dilutions ranging from 1.0 copies/μL to 1600 copies/μL. These were then assayed directly and resulting Cq and fluorescence values were compared with those that were pooled with known negative samples in a 1-in-2 and 1-in-4 ratio.

2.9. Effect of various transport media

At the time of the development of this extractionless protocol, transport media approved for SARS-COV-2 were in dire shortage. In this study, using combined E (Roche) + N1 primers/probe multiplex on 9 patient samples, we verified various viral transport media that were available to us and suitable for collection and preservation of clinical specimens containing viruses, including Sars-CoV-2, *Chlamydia, Mycoplasma* or *Ureaplasma*. These included: (Tosi and Campi, 2020) Copan UTM (Copan Diagnostics Inc, Murrieta, CA, USA), (Li et al., 2020) SI-UTM (Synergy Innovation Inc, Libertyville, IL, USA), and (Wiersinga et al., 2020) Yokon VTM. We also verified the utility of (Tosi and Campi, 2020) Copan eSwab (Copan Diagnostics Inc, USA), which included a flocked swab with 1 mL of Liquid Amies in a plastic, screw cap tube, and (Li et al., 2020) sterile physiologic 0.9% saline (NaCl). 100 μL of each of the 9-patient positive sample were allocated to each of the above transport media in 3 preparation formats (no-dilution, 4x dilution, 10x dilution) and assayed on the Roche Cobas 4800 using our extractionless protocol, E/N1 multiplex and the Roche mastermix. The resulting Cq values were analyzed for any possible inhibitory effects introduced by each media.

The stability of SARS-CoV-2 RNA in transport media was investigated over 2 common states (fresh vs one freeze/thaw cycle) and over a total period of 7 days (same samples tested at 1, 2, 4 and 7 days). Eight patient positive samples were used for this section of the study.

2.10. Establishment of an internal control

In order to establish an internal control for our extractionless rRT-PCR assay, we investigated two methodologies involving the E-gene (Roche Diagnostics)/N1 multiplex ± EAV and the E (WHO)/N1 multiplex ± RNase P. The molecular assays were performed as aforementioned, and the effect on Cq values, fluorescence and the overall shape of the sigmoidal curves were analyzed without and with the addition of EAV or RNaseP primers (Integrated DNA Technologies, Coralville, Iowa, USA) into the respective multiplex.

2.11. Ethics statement

This work was completed as part of a quality assurance and quality improvement study conducted at *DynaLIFE* Medical Laboratory. All clinical samples utilized in this work were previously collected for the purpose of COVID-19 clinical diagnostic testing and were obtained in an anonymized format from the Research Sample Biorepository at *DynaLIFE* Medical Labs and at the Alberta Precision Laboratory. These samples were utilized in accordance with the Tri-Council Policy Statement - Ethical Conduct for Research Involving Humans.

2.12. Data analysis

All experiments were carried out at least three times and statistical analysis was carried out using the Student's T test analysis with two tailed analysis. Furthermore, for all applicable datasets, one-way ANOVA analysis was also carried out to evaluate significance with P value < 0.0001 unless otherwise stated. All analysis was conducted using GraphPad Prism 8.4.3 software.

3. Results

3.1. Establishment of appropriate temperature and gene assays for the extractionless approach

Utilizing SARS-COV-2 Heat Inactivated Virus-spiked COPAN UTM media with Roche LightMix, we were able to determine the limits of detection of three gene targets (E/N1/RdRP) utilized in our current COVID-19 testing protocol with extraction and compare it to rRT-PCR without extraction (Fig. 1a and table in Fig. 1).

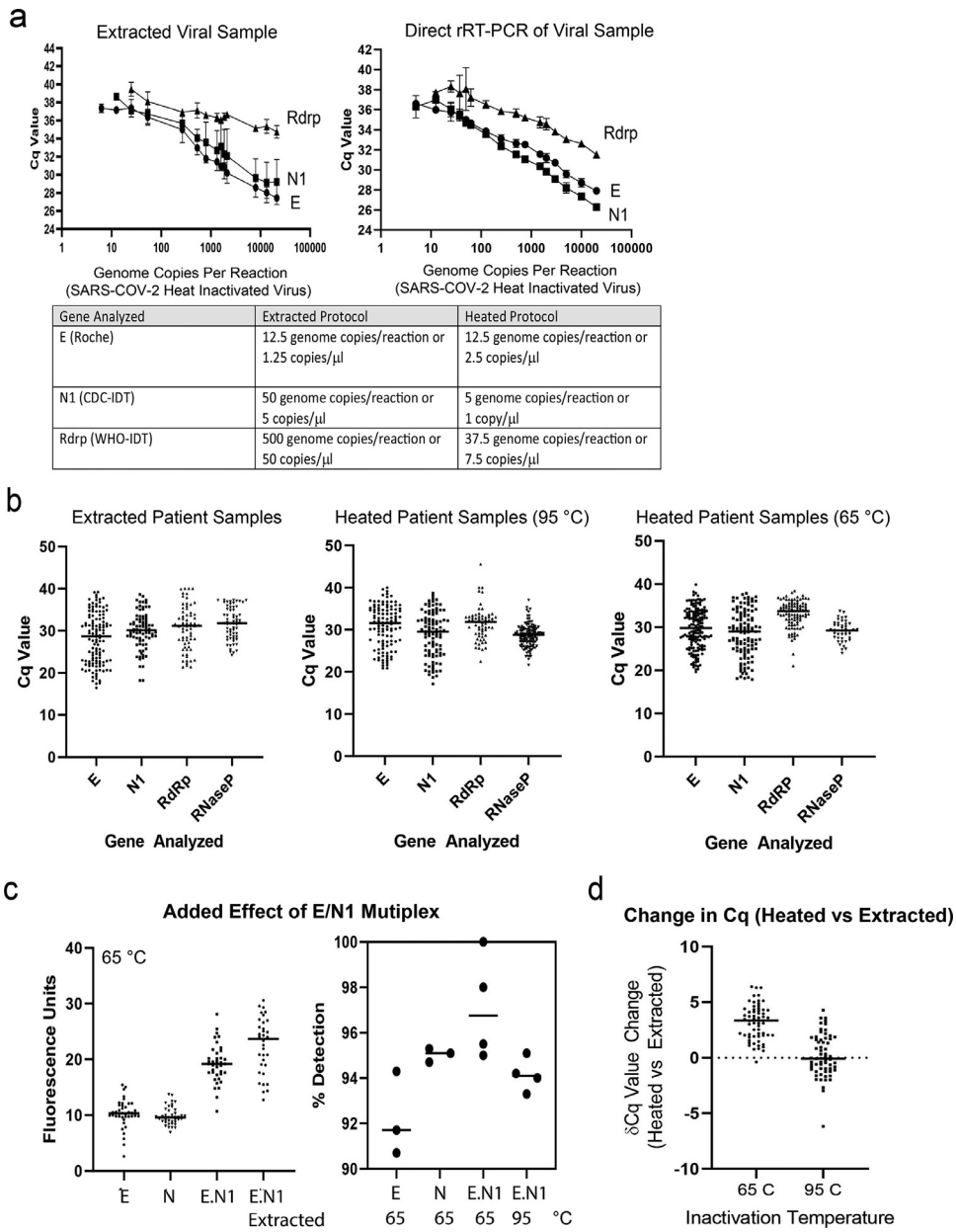
We then proceeded to explore the effect of heat and heating times on the ability to detect patient positive samples obtained from our local research sample biorepository and on the performance of the primers/probes used for the amplification of each individual gene (E, N1, RdRp, RNase P) with Roche LightMix. We analyzed the Cq values after pretreating patient positive samples with heat at 95°C (x 3 min) and at 65°C (x 15 min) and compared these with the Cq values from assaying the same samples using the traditional Roche 4800 extraction system. As noted, comparable Cq distributions can be observed between heat treated vs extracted patient positive samples (Fig. 1b). Among all the gene targets, RdRp singleplex assay consistently resulted in higher Cq values. We also noted that the use of higher temperature (i.e., 95°C), even if for a short duration (i.e., 3 min), exerted some detrimental effect on viral RNA resulting in higher Cq values being produced (Supplementary Figure S1a).

Next, we evaluated the strength of the resulting fluorescence signal relative to that obtained with the extraction method as well as the detection rate of the assay for patient positive samples. Utilizing singleplex assays (either E or N1 gene), we were able to attain a 90% – 95% detection rate, but with lower fluorescence values, when compared to that seen with Roche 4800 extraction method (Fig. 1c). Both issues of lower fluorescence and detection rates were resolved by utilizing a E/N1 multiplex, both employing the same FAM probe to result in cumulative fluorescence and boosting of the overall signal (Fig. 1c). Cq values appear to only differ by less than 5 units with either the 95°C or 65°C heating methodology (Fig. 1d).

3.2. The influence of rRT-PCR mastermixes and gene assays on percent detection, false positive rates, Cq and fluorescence values

We next explored optimizing our extractionless assay in order to attain a higher detection rate (particularly of weak positives), comparable Cq (to those obtained in Fig. 1) and higher fluorescence values. Most of the mastermixes utilized in this study (as assessed using primers/probes to N1 on spiked patient negative samples) performed well with extracted viral RNA (i.e., RNA that were purified during the extraction process) (Fig. 2a, left panel). However, with direct rRT-PCR of patient negative samples spiked with SARS-COV-2 Heat Inactivated Virus (i.e. crude unpurified sample), there appears to be less compatibility with mastermixes from Promega or Agilent (Fig. 2a, right panel) but more compatibility with those from Roche or Quantabio (Fig. 2a, right panel). Limits of detection were extremely robust at 0.2 to 1.0 copy/μL if utilizing the mastermixes from Quantabio and Roche, respectively (see appended table with Fig 2a).

We further looked at optimization with dual gene multiplexing using contrived clinical samples spiked with varying concentrations of SARS-COV-2 Heat Inactivated Virus. The use of the E/N1 (E-gene



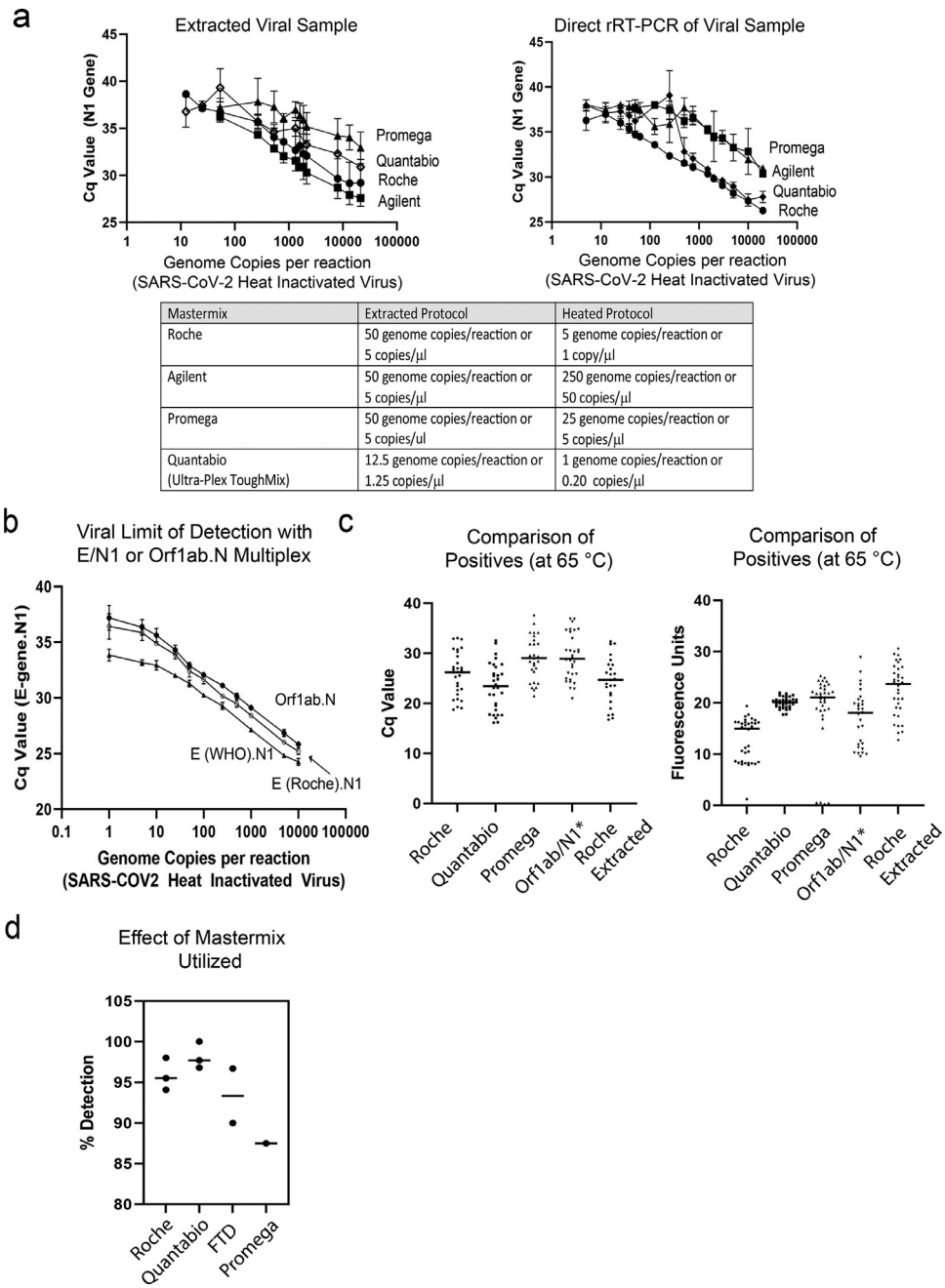
Gene Analyzed	Extracted Protocol	Heated Protocol
E (Roche)	12.5 genome copies/reaction or 1.25 copies/µl	12.5 genome copies/reaction or 2.5 copies/µl
N1 (CDC-IDT)	50 genome copies/reaction or 5 copies/µl	5 genome copies/reaction or 1 copy/µl
Rdrp (WHO-IDT)	500 genome copies/reaction or 50 copies/µl	37.5 genome copies/reaction or 7.5 copies/µl

Fig. 1. Comparisons between rRT-PCR results utilizing extracted or heat inactivated samples. (A) limits of detection for each indicated gene using the Roche Diagnostics LightMix mastermix. N = 6 for each data point. (B) Cq distribution for each gene assay from an extracted, 95°C or 65°C heated protocol. Each dot represents a patient sample. N = E (124/115/160); N1 (80/100/117); RdRP (66/60/100); and RNaseP (68/125/50). Numbers assigned to patient samples utilized for extracted, heated at 95°C and heated at 65°C, respectively. One-way Anova analysis suggested $P < 0.0001$ for differences between the data sets for all categories in this panel. (C) added value of using both FAM-labelled E and N1 gene. Left panel, fluorescence changes in using each gene assay. Each dot represents a patient sample, P value < 0.0001 between E/N1 vs E or N1 and n = 40 for each group of samples. Right panel, percent detection for each assay is indicated. Each dot represents a separate cohort of 70–80 samples for a total of 220 for each condition. () Cq difference between heated and extracted samples at 2 temperatures. Each dot represents a patient sample, P value < 0.0001 and n = 60 matched samples for each temperature.

from Roche Diagnostics), E (WHO)/N1 (E-gene primers/probe recommended by WHO) or Orf1ab/N (Fig. 2b) multiplexes resulted in a linear pattern of viral RNA detection to 1 copy/μL confirming the advantage of utilizing multiplexes and the sensitivity of the assay. E-gene from WHO was utilized to explore the outcome of using a different sequence to the same gene target.

We then proceeded to explore the effect of the various mastermixes on the detection of SARS-COV-2 in 35 patient positive samples utilizing the above dual gene multiplex setup. Analysis was carried out with the E/N1 multiplexes and the Orf1ab/N primer/probe set from Fast Track Diagnostics (FTD, a Siemens company) with patient positive samples. Testing of this set of patient positive samples using the various mastermixes revealed Cq values between 15 and 40 cycles for Roche, Quantabio (E/N1 multiplex) and Orf1ab/N1 mastermixes with a significantly higher Cq average for Promega mastermix (P value < 0.009) when compared to that from Roche mastermix (Fig. 2c). Utilizing the E-gene primer/probe designed by the WHO, we noted changes in Cq value and increased fluorescence closer to 30 fluorescence units for a number of samples (Supplementary Fig. S3a). More importantly, the percent detection was 97%–100% in 3 cohorts (Supplementary Fig. S3a) when compared to 95%–97% in Fig. 1c (middle panel) and Fig. 2d.

A detailed analysis of the detection rate of strong (Cq ≤ 33) or weak (Cq > 33) positives from our cohorts revealed that both the Roche and Quantabio mastermixes can be utilized to detect > 97% of strong positive samples (Fig. 3). However, the detection rate of weak



Mastermix	Extracted Protocol	Heated Protocol
Roche	50 genome copies/reaction or 5 copies/µl	5 genome copies/reaction or 1 copy/µl
Agilent	50 genome copies/reaction or 5 copies/µl	250 genome copies/reaction or 50 copies/µl
Promega	50 genome copies/reaction or 5 copies/ul	25 genome copies/reaction or 5 copies/µl
Quantabio (Ultra-Plex ToughMix)	12.5 genome copies/reaction or 1.25 copies/µl	1 genome copies/reaction or 0.20 copies/µl

Fig.2. The limitations of mastermix selection for the heated approach. (A) limits of viral detection for each using the N1 gene readout and the indicated mastermix and for extracted and heated samples. n = 6 for all data points except for the use of Promega mastermix (n = 3). (B) limits of viral detection for E-gene (Roche)/N1, E-gene (WHO). N1 and Orf1ab.N on patient positive samples utilizing the Roche Diagnostics mastermix. n -= 6 for each data point. (C) Cq (left panel) or fluorescence units (right panel) for the E/N1 multiplex (or as indicated) using the various mastermixes in patient positive patient samples. FTD, Fast Track Diagnostics, Ex, Roche COBAS extracted samples, n = 35 matched samples for all categories. *denotes use of only the primers/probes from the FTD EUA kit with the mastermix from Roche Diagnostics. (D) Summary of % detection utilizing the various mastermixes. Each dot represents a separate cohort of 70–80 samples for a total of >200 patient samples (except for FTD with 45 and Promega with 80. Please note for FTD, the entire rRT-PCR kit was used to document % detection for FTD. Samples for this panel were obtained from cohorts from the Alberta Precision Laboratories and *DynaLIFE* Medical Labs. For panels b-e, the E-gene utilized was from Roche Diagnostics.

positives is significantly lower using the heated approach and is much more robust utilizing the Quantabio mastermix (Fig. 3). FTD % detection of weak positives, on the other hand, was ~ 70% (data not shown).

Furthermore, altering our gene assay, Orf1ab in combination with N1 and Quantabio mastermix produced an average Cq closest to that of extracted samples with the fluorescence being much higher than that produced by the E/N1 multiplex (supplementary Fig. S3b). Orf1ab/N1 in combination with Roche mastermix, on the other hand, produced results comparable to that seen with the E/N1 multiplex. The increased fluorescence was more amplified with the use of the Quantabio mastermix with patient positive samples showing an average of > 30 fluorescence units and 4–5 Cq values lower than with the use of Roche Diagnostics mastermix
(Supplementary Fig. S3b).

Utilizing the Roche LightMix/Roche z480 software, we observed a 5%–10% production of computer positive calls in patient negative samples ("false positives", Supplementary Figure S4). This was particularly the case with the use of E/N1 multiplex/Roche mastermix

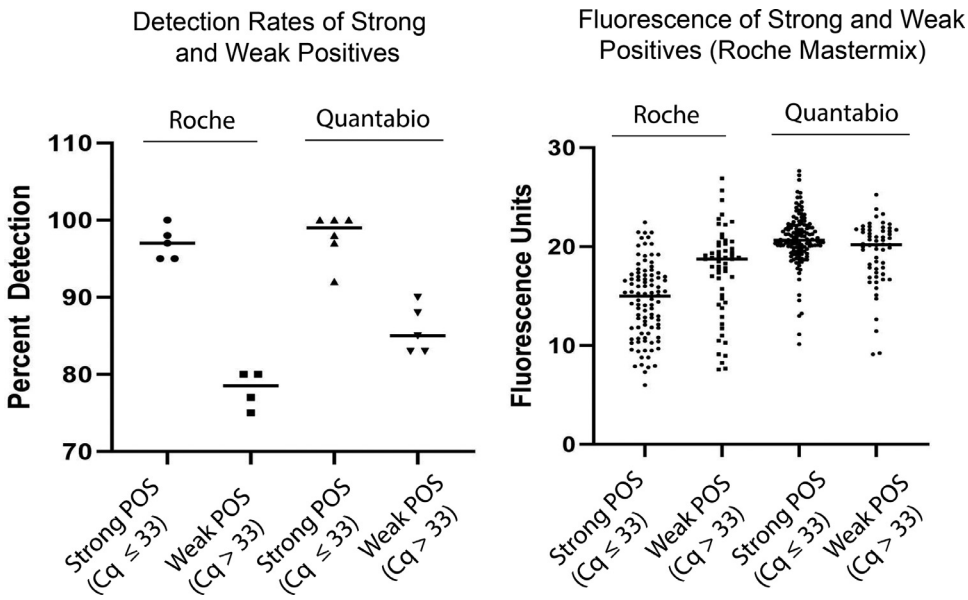


Fig. 3. Detection rate of strong and weak positives. Percent detection of strong and weak positives with the indicated mastermix and E/N1 multiplex. Left panel is percent detection with each dot representing a patient positive cohort of 40–50 samples for a total of 160–200 samples. Right panel, fluorescence distribution of strong and weak positives utilizing the Roche mastermix and E/N1 multiplex. For the Roche Diagnostics mastermix, n = 150 for both strong and weak positives. For Quantabio, n = 150 for strong positives and 65 for weak positives). The E-gene utilized was from Roche Diagnostics.

combination. Examples of false positives are shown including "straight line" plots producing a Cq value with fluorescence > 1 unit (Supplementary Figure S4). All "false positives" were confirmed negative by the extraction protocol prior to analysis by heat extraction and some were confirmed negative after heat extraction analysis on other in-house platforms, namely the Seegene or the Hologic Panther System (data not shown). It should be noted that the E-gene primer/probe set from Roche used for extraction and in all panels presented in this article (except for Fig. 5a or otherwise mentioned) have primers/probe for equine arteritis virus (EAV), which was introduced by Roche to serve as an internal extraction and rRT-PCR control. Significantly lower fluorescence of patient negatives (P value of < 0.0001) was observed with the use of either the Orf1ab.N multiplex/Roche mastermix (Supplementary Figure S1c), the E/N1 multiplex/Quantabio mastermix (Supplementary Figure S1c) or a different E-gene from WHO (with no EAV primers, Supplementary Fig. S3a) (data utilizing 65°C heated samples).

3.3. Performance of the extractionless protocol on pooled clinical samples

Using a range of clinical samples containing low, moderate and high viral loads (as determined by their Cq values), we observed that 1-in-2 and 1-in-4 dilutions with patient negative samples resulted in an average/median increase in Cq values of 1.00/0.99 and 1.91/2.00 respectively. The respective change in fluorescence values were -0.77/-0.60 and -1.52/-1/72 (Fig. 4a). Spiking patient negative nasopharyngeal samples with SARS-COV-2 Heat Inactivated Virus at known viral loads (ranging from 1.0 copies/μL to 1600 copies/μL) revealed a linear relationship between viral load and Cq value (Fig. 4b).

3.4. Effect of various transport media

Lastly, we explored the effect of various swab type and viral transport media (Copan UTM, SI UTM, Yokon VTM, eSwab, saline,) on the Cq and fluorescence values of patient positive samples. They did not affect the positive call of the 9 patient positive samples used in this part of the study but did change the Cq value by a few units when diluted by 4x or 10x (Fig. 4c). We only observed interference with another transport media we had later received, namely GDL Korea kit transport media (GDL Korea Co. Ltd, Anyang, Gyeonggi, South Korea), when utilized with the Roche Diagnostics mastermix but not with the Quantabio mastermix (data not shown).

Moreover, from our study on the stability of SARS-CoV-2 RNA in transport media, we observed that Cq values did not change significantly in all the conditions assayed (Supplementary Figure S1b).

3.5. Establishment of an internal control

Competition among the 3-primer set setup for the available amount of rRT-PCR reagents in each multiplex was noted, such that the least efficient reaction consistently lagged behind and produce a lower fluorescence than the rest. In the case of E/N1/EAV multiplex (Supplementary Figure S2), the EAV primer/probe set was the least efficient reaction producing a low fluorescence positivity. Signals for Cq and fluorescence became stronger in the presence of patient negatives as there was, in this case, no competition for rRT-PCR reagents in the absence of amplification reactions for E/N1 gene targets (Supplementary Figure S2, NEG panel in right bottom graph). Thus, with patient negative samples, EAV displayed a higher fluorescence and lower Cq value. On the other hand, E/N1 Cq values remained the same -regardless of the presence or absence of EAV.

Because of this issue with the use of EAV, we explored the use of the Human ribonuclease P (RNaseP) as an internal control. When utilizing the E/N1/RNaseP multiplex (Fig. 5), E/N1 Cq values decreased and fluorescence values increased on average when RNase P was present in the mix. RNaseP Cq and fluorescence did change slightly but not in a negative manner compared with that seen with EAV (compare fluorescence units of EAV and RNaseP -/+ E/N1 in Fig. 5 and Supplementary Figure S2). Similar results with changes in fluorescence values were observed with the use of the Quantabio mastermix (Supplementary Fig. S5b).

3.6. Additional multiplex rRT-PCR combinations for COVID-19 nucleic acid testing

In addition to the E/N1/RNaseP multiplex panel, there are several other genes that can be utilized to detect COVID-19. These include the Orf1ab, N2 and Rdrp targets. For this analysis, we used a selection

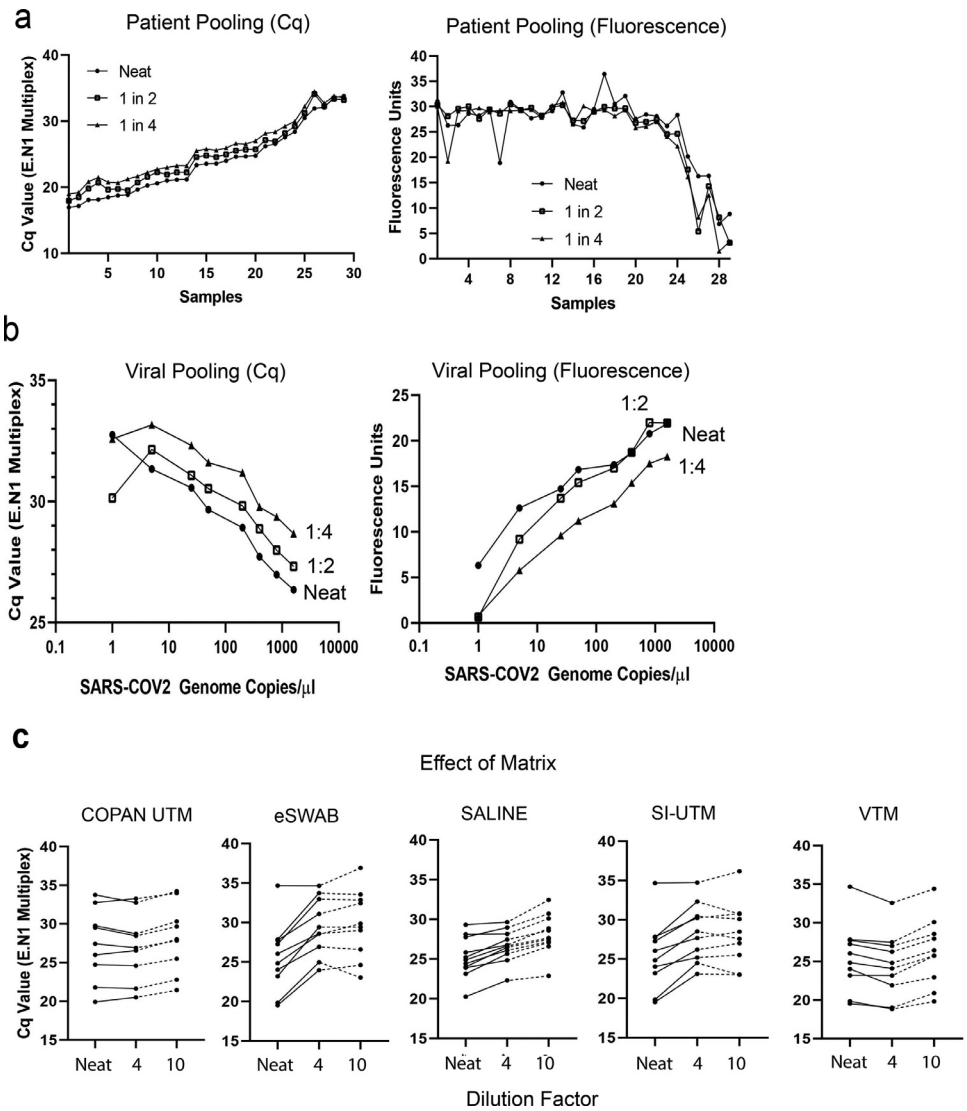


Fig. 4. The utilization of the heated approach for detection of pooled clinical samples. A,B The effect of (A) patient sample pooling or (B) SARS-COV-2 Heat Inactivated viral pooling on Cq and fluorescence values in the E/N1 rRT-PCR utilizing Quantabio mastermix as indicated. A total of 29 samples were utilized for the patient sample pooling experiment with a range of Cq values from <20 to >30 and n =1 for each viral point. (C) the effect of various viral transport medium/matrices on Cq values of heated samples at no dilution (neat), 4x or 10x as indicated. The Roche Diagnostics mastemix and n = 9 matched samples were utilized for these analyses.

of previously confirmed strong and weak patient positives samples and assayed them using Orf1ab/N1/RNaseP or Orf1ab/N2 multiplexes to determine their detection accuracy. Results show that both Orf1ab/N1/RNaseP and Orf1ab/N2 multiplexes have robust fluorescence signal and excellent diagnostic performance displaying > 98% analytical sensitivity (Supplementary Figure S7a through c). As shown in this table also, multiplex combination involving RdRp (i.e., RdRp/N2) performed poorly at 79% sensitivity.

4. Discussion

Since COVID-19 was declared a pandemic by the WHO in March 2020, there has been an exponential increase in the development of new diagnostic methods and devices for COVID-19 testing. While many are exploring and marketing rapid genetic and serology tests for COVID-19 (Espejo et al., 2020; Chau et al., 2020), identifying mutations to COVID-19 that may impact genetic detection and/or response to current COVID-19 therapies (Hartley et al., 2021; Ziegler et al., 2020), others are exploring ways to rapidly identify COVID-19 positive patients using the least costly methodology that is not only scalable but also able to maintain an acceptable performance sensitivity and accuracy. We have developed an optimized heated protocol without the need for nucleic acid extraction that is affordable, rapid and easily scalable without the need to procure more instrumentation.

4.1. Establishment of appropriate temperature and gene assays for the extractionless approach

Using the Roche LightMix mastermix and primer/probes to E/N1/RdRP genes, comparable, if not improved, limits of detection for SARS-COV-2 were seen between extractionless rRT-PCR and the traditional Roche 4800 extraction system (Fig. 1a, left panel). The E and N1 genes produced the lowest limit of detection at 2.5 and 1 copy per μL respectively.

Biosafety level 3 (BL3) facilities are not as readily available as are BL2 facilities in most places. As such, research around *Coronavirus* has relied on the use of heat to inactivate the virus so the latter could be safely handled in a BL2 facility. Although several reports have documented the inactivation of COVID−19 with heat at 56°C or 60°C for 15 – 30 minutes (Pastorino et al., 2020; Kampf et al., 2020), it was not known how high temperature

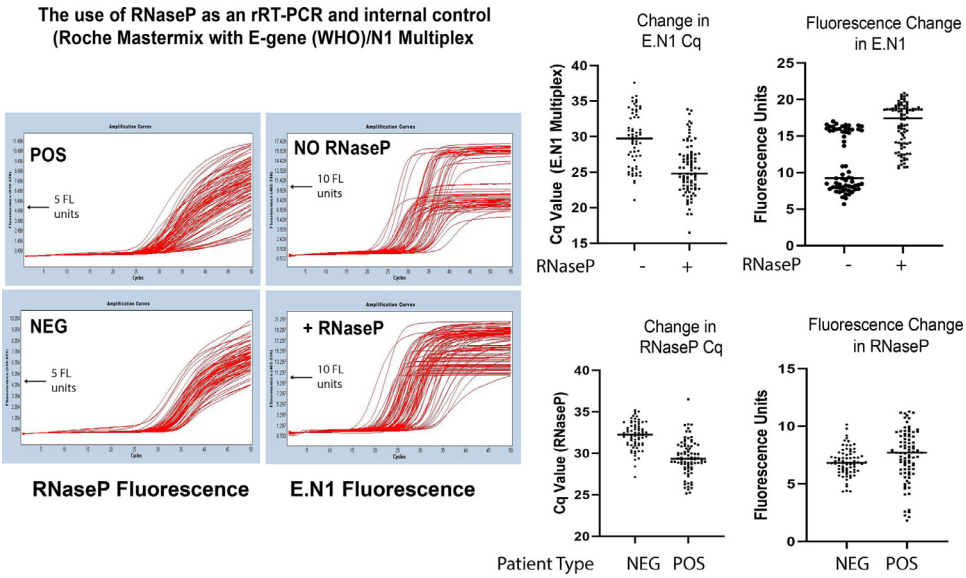


Fig. 5. The use of controls to monitor sample input and amplification of DNA. RNaseP (primer/probe combination from IDT)) was added to the rRT-PCR reaction mix and rRT-PCR carried out without or with RNaseP primer/probes and on positives and negatives. An example of such a profile run is shown on the left panel. Right panel, results of Cq and fluorescence for both E/N1 and RNaseP. *P* value is <0.005 for comparing the samples within a graph plot (n = 75 for all categories). For this rRT-PCR reaction, 1 μL of E-gene (WHO), 2 μL N1 gene and 2 μL of RNaseP was utilized in a 20 μL reaction with the Roche Diagnostics mastermix.

pretreatment of clinical samples would affect subsequent test performance. Performing direct extractionless rRT-PCR using E-gene singleplex assay, we noted that the resulting Cq values were the most optimal in the patient positive sample groups that had no heat pretreatment and those that had heat pretreatment at 65°C for either 15 minutes or 30 minutes. Cq value elevation was noted in the patient positive sample group that had heat pretreatment at 95°C for 3 minutes (Supplementary Figure S1a). For most primers/probes (except those targeting RdRp), Cq values for samples pretreated at 65°C seemed to be unaffected and most comparable with those assayed by the conventional system with extraction (Fig. 1b). Our observation correlates with that reported by Pastorino et al (2020) (Pastorino et al., 2020) and others that demonstrated that higher heat pretreatment of patient samples had a detrimental effect on resulting Cq values. In this process, we also noted that water bath and PCR amplificator heating was found to be equivalent (data not shown).

Singleplex assays that were run on our extractionless protocol gave results with lower fluorescence, which also translated into lower detection rates. By introducing the right multiplex in an additive manner (E/N1, vs in parallel channels that most are utilizing) and the right temperature to release and preserve the viral single stranded RNA (65 °C for 15 minutes), we were able to obtain robust detection of weak and strong positives. This strategy was particularly useful in boosting fluorescence signal, especially from weak positive samples (Fig. 1d), and consequently improved the detection rate of positives to greater than 95%. Furthermore, what can be concluded from Fig. 1d is that although primary N1 screening for COVID-19 testing is robust and reproducible by itself, it can be further enhanced when multiplexed with E (Fig. 2b–e) or Orf1ab (Supplementary Fig. S3 and S7) without any increase in false positivity (Fig. 2d and Supplementary Fig. S5). When used in combination with the primer/probe set from Fast Track Diagnostics or with our own custom formulation (Supplementary Fig. S7), the use of Orf1ab. N1 (or Orf1ab.N2) appears to also be a robust choice as a screening tool as it produced the highest fluorescence in patient positives and lowest fluorescence in patient negatives to suggest potentially a 0% false positivity rate (Fig. 2b, 2d, Supplementary Fig. S7 and Supplementary Fig. S3).

4.2. The influence of rRT-PCR mastermixes and gene assays on percent detection, false positive rates, Cq and fluorescence values

As suspected, with the application of a crude unpurified sample, different mastermixes (containing purified enzymes such as DNA polymerase and reverse transcriptase) performed differently and resulted in different limits of detection when exposed directly to SARS-COV-2 Heat Inactivated Virus (Fig. 2a). In our study, we observed that Quantabio and Roche mastermixes performed the best in this scenario out of all 4 mastermixes.

Moreover, as we experimented with different gene assay combinations in conjunction with Roche vs Quantabio mastermixes, we noted that Orf1ab/N1 multiplex in Quantabio mastermix produced the lowest Cq values and the highest fluorescence. Of the 4 mastermixes, use of the Quantabio mastermix resulted in the highest average detection rate (≥98%) of SARS-CoV-2 in clinical samples (particularly strong positives) while use of the Promega mastermix resulted in the lowest. Thus, finding the right combination of mastermix and multiplex will ultimately determine the analytical sensitivity of the extractionless assay.

If our detection rate of weak positives is 85% utilizing the Quantabio mastermix, then 15 weak positives may be missed in every 100 identified or 1 missed in every 7. What we have observed based on our population testing, the incidence of weak positives is actually about 1 in 250 samples analyzed. Therefore, we would need to analyze 1750 samples before we have one false negative. So our estimated accuracy approaches 1749/1750 or 99.9%. No one molecular assay is 100% sensitive. We are confident that the heat extraction protocol we have developed can attain a 98% – 99% detection rate of positives overall. What all of these observations now suggest is that the selection of specific mastermixes and, more importantly, of multiplex gene assays, contributed significantly to the accuracy and sensitivity of our COVID-19 molecular assay.

Overall, the analysis of several cohorts of patient positives with the multiplexes and mastermixes suggests that the mastermixes from Roche and Quantabio appear to be the best choices for detecting positives with the latter producing a percent detection rate ≥ 98% when used in combination with the E/N1 or Orf1ab/N1 multiplex (Fig. 2d and Supplementary Fig. S3).

When fined tuned, detection and interpretation of patient positives is quite straightforward. However, we observed some issues with the interpretation of patient negatives utilizing the extractionless heated approach. In our study, use of E/N1 multiplex/Roche mastermix combination generated a small number of false positive results in some patient negative samples displaying Cq values with higher fluorescence signal (>2). Utilizing the Orf1ab/N multiplex/Roche mastermix or the E/N1 multiplex/Quantabio mastermix combinations would circumvent this problem as they both generate on average significantly lower fluorescence in patient negative samples.

Varying degrees of "false positive" calls can be obtained depending on the specific gene assay used. Several conclusions/solutions can be derived from all of our observations with gene assays: (1) background fluorescence in patient negative samples is usually high when utilizing the Roche Diagnostic's primer/probe multiplex containing E-gene/EAV primers but not observed when utilizing the E-gene without EAV primers/probes (E-gene WHO from IDT, Supplementary Fig. S5a); (2) the fluorescence in negative samples is more abundant if a sample is heated at 95°C (translating into the occurrence of possibly more false positives); (3) a fluorescence threshold set between 1 and 2 may need to be assigned to negatives of a heated sample in order to distinguish from positives that may have fluorescence >2 but < 10 and Cq < 33. 4) Confirmation of uncertainties can be carried out on another platform such as Hologic Panther or Seegene (as we have done) or with alternative gene assays (Supplementary Fig. S7). In summary, in order to alleviate the above issues, we would recommend mitigating false positive results by (1) changing mastermix; (2) changing gene targets for the rRT-PCR assay; (3) establishing a slightly higher fluorescence cut off threshold for interpreting positives based on your multiplex assay and detection output. By utilizing some of the above mitigation strategies at *DynaLIFE* Medical Labs, we have eliminated all false positive results and were able to attain a specificity of 100% relative to the traditional extraction method with the Roche system.

4.3. Performance of the extractionless protocol on pooled clinical samples

None of the pooled clinical samples at either 1:2 or 1:4 pooling ratio escaped detection by our extractionless protocol. On average, for every doubling in sample pooling, Cq values appeared to increase by a multiple of 1.00 while fluorescence values dropped by a multiple of 0.77. We also noted that Cq values shared a linear relationship with the viral load in a sample. This means that Cq values can be directly used to extrapolate the actual viral load in any given clinical sample. From our observations, we conclude that the extractionless protocol can reliably detect SARS-CoV-2 RNA in up to 1:4 pooling ratio even when the viral load in the sample approaches the lowest limit of detection of our assay.

4.4. The stability of SARS-CoV-2 RNA in transport media

Based on the analysis of 255 positive samples in this study, patient samples with Cq value of ≤ 33 are easily detected with a fluorescence > 10 arbitrary units. For those samples with Cq > 33, we generally observe a sigmoidal curve and a fluorescence > 10 with fresh samples and > 5 with those that have been frozen and thawed several times. The latter scenario will generally not occur in a high capacity COVID-19 diagnostic laboratory as testing would only be conducted on fresh samples or on samples kept at 4°C in the fridge for 1–2 days. We noted that, under such circumstances, there is no significant loss in Cq value (Supplementary Figure S1b).

4.5. Effect of various transport media

The composition of swab solutions has been previously reported to alter Cq values of positives, and is especially the case with the use of saline swabs (Smyrlaki et al., 2020). Of the ones tested, interference was noted with swab solutions from GDL Korea when utilizing Roche Diagnostics mastermix. As we could not explore all the swabs or swab solutions available for COVID testing, we highly recommend a quick verification to ensure any viral transport media used is compatible with the heated approach.

4.6. Establishment of an internal control

To date, we have not seen a study that introduced an internal control to a heat extraction protocol so that quality control can be duly established. As with most assays, an internal control should be included to ensure that (1) the rRT-PCR amplification worked, and (2) there is adequate (good quality) sample collected to produce a result.

Most automated extraction protocols include an extraction control such as EAV (employed in the Roche COBAS 4800/6800/8800 extraction protocol) or Escherichia virus MS2 (MS2, employed in the Seegene Health Canada approved kit) that are utilized to control for extraction and rRT-PCR runs. However, both EAV and MS2 do not monitor the quality and validity of the submitted material. Both EAV and MS2 are usually spiked into swab samples before an extraction is carried out such that all samples will give an EAV or MS2 reading upon rRT-PCR run.

From our evaluation, EAV appears to be a viable candidate for use as an internal control for the rRT-PCR for heated samples. A potential issue, however, is the increased background fluorescence introduced by the E-gene/EAV multiplex when combined with N1 (Supplementary Figure S1b). This was more problematic with negative patient specimens.

Like with EAV, Human ribonuclease P (RNaseP) can also be utilized to control for the rRT-PCR amplification, and the extraction process (if needed). Unlike that seen with EAV, however, we observed minimal to no background fluorescence when using RNaseP as internal assay control. Since RNaseP is present in every human cell, another major advantage with its usage as internal control is that you can also control for the quality of the submitted specimen, and can thereby ensure that there is an adequate starting cellular material in the specimen to produce a reliable result. Given the clean and encouraging results we have seen with the use of RNase P as internal control, we ended up adapting it to our routine clinical testing.

4.7. Additional multiplex rRT-PCR combinations for COVID-19 nuclei acid testing

We have noted that the specific primer/probe targeting Rdrp used in this study is not as robust as those for E and N1 and can only achieve a ~70% detection rate of positive samples. On the other hand, we have noted robust signals and >98% analytical sensitivity with the use of Orf1ab/N1/RNaseP or Orf1ab/N2 multiplex combinations.

In summary, we have explored whether our optimized heat extraction protocol was compatible with varying transport media, rRT-PCR mastermixes and whether it was amenable to sample pooling (see Table 2). We observed that mastermix choice and gene assays for rRT-PCR were key elements in determining percent detection and accuracy of a heat extraction protocol. We demonstrated that the Roche LightMix, Quantabio Toughmix and Fast Track Diagnostics mastermixes were compatible with the heated protocol at >95 % detection.

An extractionless system, consisting of a heat extraction step and a preparation time of 45 minutes, offers a process that is

Table 2
Summary of the optimal parameters of the heated protocol.

Parameter	Recommended choice
Viral Transport Media	Compatible with COPAN UTM, Yokon VTM and Saline
Temperature Treatment	1) 65°C for 15 min in rRT-PCR plate; OR 65 °C for 30 min in a water bath; 2) 5–10 min cool down at 4°C
Optimal Mastermix	Quantabio UltraPlex 1-Step ToughMix > Roche LightMix > FTD > Promega/Agilent
Gene Usage for rRT-PCR assays	Orf1ab.N1 > E (WHO).N1 > N1 > E (Roche or WHO)
Internal Control	RNaseP (Atto647 Probe, IDT) > EAV (Cy5 Probe, Roche Diagnostics)
rRT-PCR Program	
Reverse transcription	50°C (Quantabio[a])/55°C (Roche[b])/45° C (Promega[c]) for 10 minutes
Taq-polymerase activation	95°C for 3 min
Each amplification cycle (total 50 cycles)	95°C for 3 s/60°C for 30 s
Cooling	Cooling at 37°C for 1 s
rRT-PCR COVID-19 Standard	EURM-019 (Sigma) at 1:4000 – 1:8000 dilution (5 μL per 20 μL reaction)
Volume of heated sample for rRT-PCR	5 μL
Time to complete analysis of 93 samples	110 –120 min

[a] (Quantabio Inc., Beverly, Massachusetts, USA).
[b] (Roche Diagnostics, Mississauga, Ontario, Canada).
[c] (Madison, Wisconsin, USA).

>2.5 hours faster than automated robotic extraction systems needing to extract nucleic acid for molecular testing. Our methodology is capable of attaining a detection rate of > 98% and a throughput of 93 samples (+ 3 quality controls) every 80 minutes. We also provided additional proposed multiplex combination alternatives that can be used in the detection of COVID-19. The use of our methodology translates into ~1,128 tests per day (non-pooled capacity) and a theoretical ~4,500 tests per day (with pooling and not factoring in delays introduced by the pooling process) utilizing one single 96 well rRT-PCR machine and no additional extraction devices. If a 384 well rRT-PCR machine is utilized, then ~4,600 samples can be analyzed (non-pooled) per day. As we implemented this extractionless molecular assay in our routine clinical testing of patient samples, we saw a doubling of our throughput compared to that which we are getting using automated robotic extraction systems. And even though we have not yet implemented additional pooling strategy with the extractionless protocol, we have done so with our automated robotic extraction systems. With a 1:4 sample pooling strategy of community (non-hospitalized) patient samples, we witnessed a three-fold increase in our throughput capacity. The additional pre- and post-analytic steps required with the sample pooling strategy invariably introduced delays to the whole testing process. Overall, the heated approach: (Tosi and Campi, 2020) can be safely carried out in a level 2 clinical laboratory with respect to both specimen preparation and specimen processing, (Li et al., 2020) is affordable, (Wiersinga et al., 2020) is flexible with the use of multiplex and mastermix from several sources, (Fang and Meng, 2020) adaptable to high-throughput workflows, (Vogels et al., 2020) amenable to quick turn-around times and (6) can be utilized in remote areas in the world that may not have access to expensive COVID-19 testing instrumentations. All of the aforementioned features highlight the clinical usefulness of our proposed heated protocol that will aid in meeting the ever-increasing demands in clinical testing.

Data availability

Dataset analysis, clinical validation of reporting algorithm and information about protocols and gene assays in this study can be obtained by contacting the corresponding author.

Declaration of competing interest

The authors report no conflicts of interest relevant to this article

Acknowledgments

We would like to thank Mr. Harold Jahn of Healthy Life Innovations for his support in utilizing his heat inactivated virus during the quality improvement and development phase of establishing the heated protocol for COVID-19 testing. We would also like to thank Mark Malick and his entire COVID-19 testing team for their support and help during the quality improvement and development phase of establishing the heated protocol for COVID-19 testing. Furthermore, we would also like to thank LeeAnn Turnball at Alberta Precision Labs for her help in procuring patients samples and Bruno Johnson at Roche Diagnostics for his help in utilizing the Roche COBAS 4800 system and software.

Financial support

No funding was associated with this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2021.115458.

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