RT-qPCR DETECTION OF SARS-CoV-2 RNA FROM PATIENT NASOPHARYNGEAL SWAB USING QIAGEN RNEASY KITS OR DIRECTLY VIA OMISSION OF AN RNA EXTRACTION STEP

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ABSTRACT
The ongoing SARS-CoV-2 pandemic has caused an unprecedented need for rapid diagnostic screening [1]. The primary assay employed is a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay that requires the use of an RNA extraction kit [2,3]. In the United States, the Centers for Disease Control and Prevention (CDC) protocol requires the use of the Qiagen QIAamp DSP Viral RNA Mini kit. The current shortage of this RNA extraction kit during this pandemic has resulted in a severe bottleneck in testing capacity. To address this problem, we tested two alternative strategies: the use of alternative RNA extraction kits or a direct RT-qPCR assay that omits an RNA extraction step altogether. We found that the Qiagen RNeasy Mini kit and the Qiagen RNeasy Micro kit could be substituted for the QIAamp Viral RNA Mini kit. Importantly, we report here that the RT-qPCR assay can be performed directly on patient sample material from a nasal swab, without the need for an RNA extraction step of any kind. Collectively, our findings provide viable options to circumvent supply chain issues in COVID-19 testing. Further, the ability to omit the RNA extraction step from RT-qPCR screening protocols would drastically ease supply chokepoints of COVID-19 screening and should be applicable throughout the world. We would note that our findings are preliminary and based on a single pooled nasopharyngeal swab sample from two previously confirmed positive COVID-19 patients. But due to the urgent need for high volume COVID-19 screening, we wanted to make these findings available immediately while we conduct replicate studies using additional patient samples.

RESULTS/DISCUSSION
There is a current shortage of the recommended viral RNA extraction kit (QIAamp DSP Viral RNA Mini) (Qiagen, Cat. No. 52904/52906) needed for the Centers for Disease Control and Prevention (CDC) RT-qPCR assay to diagnose SARS-CoV-2 infection [4]. To address this problem, we tested i) whether alternative Qiagen RNA extraction kits (RNeasy Mini, Cat. No. 74104/74106 or RNeasy Micro, Cat. No. 74004) could be substituted for the Viral RNA Mini kit and ii) whether the need for RNA extraction could be eliminated altogether by simply adding nasopharyngeal patient sample directly into the RT-qPCR assay.

Qiagen RNeasy Mini and RNeasy Micro kits successfully isolate SARS-CoV-2 for subsequent detection by RT-qPCR. To validate and compare the performance of each viral RNA extraction kit, we first conducted a spiking experiment. A standard quantity of $5x10^4$ copies of purified SARS-CoV-2 RNA (nCoVPC #RV202005) was added into the RNA lysis buffer provided in each kit and the full isolation procedure was performed. Purified RNA samples were then screened by RT-qPCR using the CDC Research Use Only 2019 nCoV_N3 primer/probe set. From each RNA extraction, 5 µl of the eluted RNA was used as input. This equated to inputs of $4x10^3$ copies for the QIAamp Viral RNA Mini and RNeasy Mini kits, and $6x10^3$ copies for the RNeasy Micro kit (due to its lower elution volume [**Table 1**]). As shown in **Figure 1**, each kit isolated sufficient SARS-CoV-2 RNA for detection by RT-qPCR. However, there was a range in the cycle threshold (CT) at which each sample was detected (average CT [of duplicates] of 27.8 for the QIAamp Viral Mini kit, 30.9 for the RNeasy Mini kit, and 33.7 for the RNeasy Micro kit). Thus, these results indicate that all kits are effective for isolation of detectable viral RNA, but that the purification performance varied by kit (QIAamp Viral RNA Mini > RNeasy Mini > RNeasy Micro).

Next, to assess the efficiency of RNA extraction from a nasopharyngeal swab (NP) sample by each type of extraction kit, we obtained NP swabs from two COVID-19 patients who had been previously verified for SARS-CoV-2 infection by the Vermont Department of Health using the

CDC RT-qPCR test (data not shown). Both patient samples, which were originally collected as nasopharyngeal swabs in a volume of 2 mL of M6 viral transport media (termed diluent hereafter), were pooled (equi-volume) due to the small volumes remaining after clinical testing. Following the standard CDC protocol, 140 µL of the pooled patient sample was used for RNA extraction with the Viral RNA Mini kit and was detected as positive via RT-qPCR with a CT of 18.7 (**Figure 2**). The patient sample inputs for both RNeasy kits was 100 µL and RNA recovered by the RNeasy Mini and RNeasy Micro were detected at CT values of 21.0 and 20.1, respectively. For comparison, we also ran duplicate samples of lower quantities of the CDC positive control synthetic RNA extracted with the Viral RNA Mini kit. For 5×10^4 copies, positivity was determined at an average CT of 28; for 5×10^3 copies positivity was achieved at an average CT of 31.9, and for 5×10^2 copies positivity was found at an average CT of 35.0 (**Figure 2**). All extraction methods (Viral RNA Mini, RNeasy Micro, and RNeasy Mini kits) yielded successful detection of SARS-CoV-2 RNA in the control patient sample that was well above the CT limit of detection required by the CDC test. Thus, this demonstrates that alternative viral RNA extraction kits can be used for detection of SARS-CoV-2 RNA by RT-qPCR.

Detection of COVID-19-positive patient RNA by RT-qPCR in the absence of an RNA extraction step. We next tested whether the RNA extraction step could be omitted by adding the pooled COVID-19 patient nasopharyngeal swab diluent directly to the RT-qPCR reaction. To determine whether heating the nasopharyngeal swab diluent before RT-qPCR might improve accessibility of viral RNA to the RT enzyme, we used swab diluent that had either been heated or not for five minutes at 70°C prior the RT-qPCR reaction. As shown in **Figure 3,** SARS-CoV-2 RNA was successfully detected in the absence of an RNA extraction step and preheating the nasopharyngeal swab diluent had no impact on viral RNA detection (average CT positivity in both cases was 23). Compared to the same pooled nasopharyngeal swab diluent extracted with the QIAamp Viral RNA Mini kit, adding the nasopharyngeal swab diluent directly into the RT-qPCR reaction resulted in an ~30-fold drop in sensitivity (**Figure 3**). However the equivalent amount of the original sample analyzed was 60% that of the extracted RNA. Thus, the drop in sensitivity was actually ~18 fold. As controls, **Figure 3** also shows the CTs where known quantities of the CDC positive control synthetic RNA extracted with the QIAamp Viral RNA Mini kit were detected. The pooled nasopharyngeal patient sample was detected much earlier (CT of 23) compared to the top spiked control of $4x10^3$ copies of purified SARS-CoV-2 RNA (CT of 28). These results demonstrate that successful detection of SARS-CoV-2 RNA from patient NP sample by RT-qPCR can be done in the absence of an RNA extraction step.

CONCLUSIONS

All three Qiagen RNA extraction kits yield sufficient SARS-CoV-2 RNA from a patient NP sample for successful RT-qPCR detection. The Viral RNA Mini kit performs slightly better than the other two kits (~four-fold better RT-qPCR signal when used on the pooled patient sample), while the RNeasy Mini and RNeasy Micro kits perform similarly to each other. Importantly, our results suggest that it is possible to skip the RNA extraction step entirely and still detect SARS-CoV-2 RNA from a patient nasopharyngeal swab sample. Each of the methods tested here (the three RNA extraction kits or omission of the RT step) allowed detection of viral RNA in patient samples at levels significantly higher than 4×10^3 copies of the CDC positive control RNA and well before the 40 cycle CT cutoff the CDC is recommending for positive sample identification. Further, the samples we obtained were swabs collected into 2 mL of transport medium. This

volume could be reduced to i) increase the resulting viral RNA copies present per µL of the sample and enhance detection and ii) to spare the transport medium (another potential bottleneck). Sensitivity could also be increased by raising the volume of the RT-qPCR reaction to accommodate more patient input material. It should be noted that these results are preliminary and represent data from only one pooled patient sample due to extremely limited reagents (patient sample volume, primers, one-step master mix). They should thus be interpreted with caution, as we currently have no data on whether the viral RNA quantity present in the pooled NP sample used here is broadly representative of levels seen across the spectrum of COVID-19 patients. Replicates of these experiments in a greater number of patient samples will be ongoing in our laboratories, and results will be published as soon as they are available.

Methods:

1. The three Qiagen kits (QIAamp Viral RNA Mini (Qiagen, Cat. No. 52904/52906); RNeasy Mini kit (Cat. No. 74104/74106); or RNeasy Micro (Cat. No. 74004)) were used according to the manufacturer's instructions with the following modifications.
    a. As the RNeasy Mini kit does not include carrier RNA, 120 ng/reaction of the carrier RNA (Qiagen Cat. No. 1017647 if purchased separately) from the RNeasy micro kit was added to the RNeasy Mini kit in order to increase yield (i.e. help precipitate/isolate the targeted viral RNA). This amount of carrier RNA is calculated based on the amount used in the RNeasy Micro kit and adjusted for the increased column surface area.
    b. The RNeasy Mini and RNeasy Micro kits are designed for use on cell pellets and purification of RNA. As a liquid sample (nasopharyngeal swab diluent) was being lysed (rather than a cell pellet) no QIAshredder (cell homogenization) step was used. 100 µL of liquid sample was instead added directly to the RLT lysis buffer containing 2-Mercaptoethanol (10uL/1000uL of RLT).
    c. Sample input, elution volumes, and concentrations of carrier RNA per reaction are provided (**Table 1)**. Additionally, for synthetic SARS-CoV-2 RNA spiking experiments, the quantities of RNA used are described in text.

2. Nasopharyngeal swabs collected from 2 COVID-19 confirmed patients were collected in a 2 mL volume of M6 transport media. For this study, limited quantities of this material were available so we pooled equal amounts of each sample for our assays. Sample manipulations (mixing with viral RNA extraction lysis buffer or direct addition to RT-qPCR plates) were done in a Class II Biosafety Cabinet using BSL-2 precautions. For the viral RNA extraction kits, nasopharyngeal swab diluent was added directly to the lysis buffer for each kit, vortexed, and incubated for 10 minutes to allow for complete viral inactivation. For direct (no viral RNA extraction) RT-qPCR, the master mix was prepared and pipetted into the RT-qPCR plate followed by 7 µl of the pooled patient sample (or as a negative control the M6 viral transport media). The plate was sealed inside the biosafety cabinet and then removed for RT-qPCR.

3. 5 µL of extracted RNA (from the final eluted material at the end of the viral RNA extraction for each kit) or 7 µl of nasopharyngeal swab diluent was used as input material for the New England Biolabs Luna Universal Probe One Step RT qPCR kit (cat #E3006S, lot #10066679) according to the IDT recommendation for primers/probe (1.5 µL primer/probe

per reaction) using primer set N3- lot #0000499772 for IDT's Research Use Only kit for the 2019-nCoV CDC qPCR Probe Assay. The primer-probe sequences and One Step RT-qPCR conditions are as follows:

Name	Description	Oligonucleotide Sequence (5'>3')
2019-nCoV_N3-F	2019-nCoV_N3 Forward Primer	5'-GGG AGC CTT GAA TAC ACC AAA A-3'
2019-nCoV_N3-R	2019-nCoV_N3 Reverse Primer	5'-TGT AGC ACG ATT GCA GCA TTG-3'
2019-nCoV_N3-P	2019-nCoV_N3 Probe	5'-FAM-AYC ACA TTG GCA CCC GCA ATC CTG-BHQ1-3'

Assay	Temperature	Time	Cycles
RT reaction	55ºC	10 minutes	1
	95ºC	1 minute	
qPCR reaction	95ºC	15 seconds*	45
	60ºC	1 minute	

*CDC uses 3 seconds, we increased to 15 seconds to ensure complete denaturing of dsDNA.

4. RT-qPCR was performed on a ABI QuantStudio Flex6 according to the CDC guidelines for Research Use Only Real-Time RT-PCR Protocol for Identification of 2019-nCoV [5].
5. Data was analyzed on QuantStudio Real Time PCR software.

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ETHICAL STATEMENT
Patient samples were de-identified and were not considered Human Subjects Research due to the quality improvement and public health intent of the work.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Table 1: Comparative nasopharyngeal swab diluent inputs and elution volumes used in the three Qiagen RNA extraction kits

	QIAamp Viral RNA Mini	RNeasy Mini	RNeasy Micro
Input Volume	140 µL	100 µL	100 µL
Elution Volume	60 µL*	60 µL	40 µL
carrier RNA	5.6 µg	120 ng	20 ng
Media spike-in synthetic RNA (CDC positive control RNA provided by VDH)	5 µL	5 µL	5 µL

The CDC positive control RNA (as provided by the Vermont Department of Health) was used to make serial dilutions in M6 transport media.

*Vermont Department of Health uses 100 µl

FIGURES

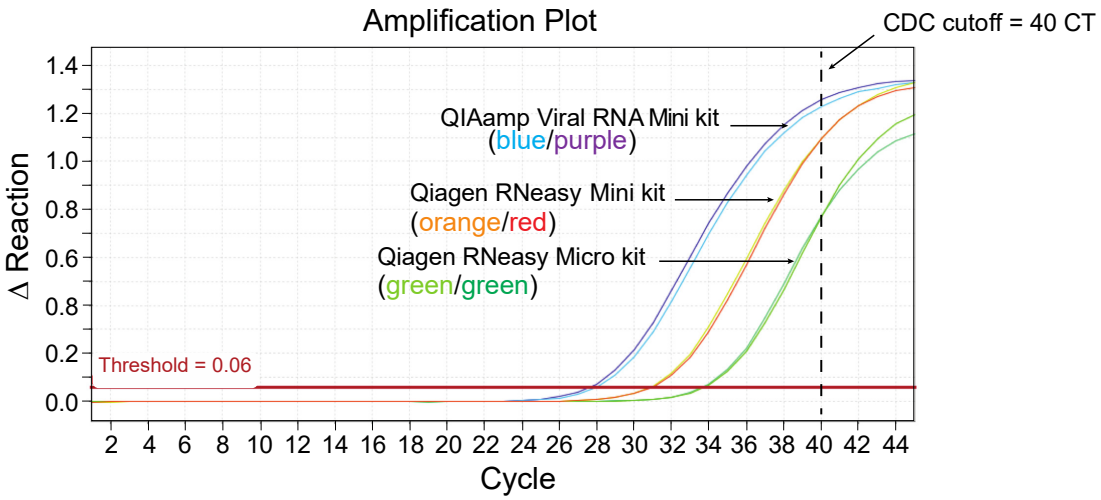

Figure 1 | The Qiagen QIAamp Viral RNA Mini, RNeasy Mini, and RNeasy Micro kits can purify "spiked in" quantities of purified SARS-CoV-2 RNA for subsequent detection by RT-qPCR. A standard quantity ($5x10^4$ copies) of the CDC SARS-CoV-2 synthetic RNA was mixed with M6 transport media. Aliquots of this mixture were subjected to RNA isolation using the QIAamp Viral RNA Mini, RNeasey Mini, or RNeasy Micro kits and then screened by RT-qPCR using the N3 SARS-CoV-2 primer/probe set. Of the initial $5x10^4$ copies of SARS-CoV-2 synthetic RNA that was spiked into the M6 transport media, approximately $4x10^3$ copies (for QIAamp Viral RNA mini and RNeasy Mini) and $6x10^3$ copies (for RNeasy micro) were loaded onto the RT-qPCR plate (after accounting for dilution of the input RNA during the RNA extraction final elution step). The resulting RT-qPCR amplification curves are shown and each sample was run in duplicate.

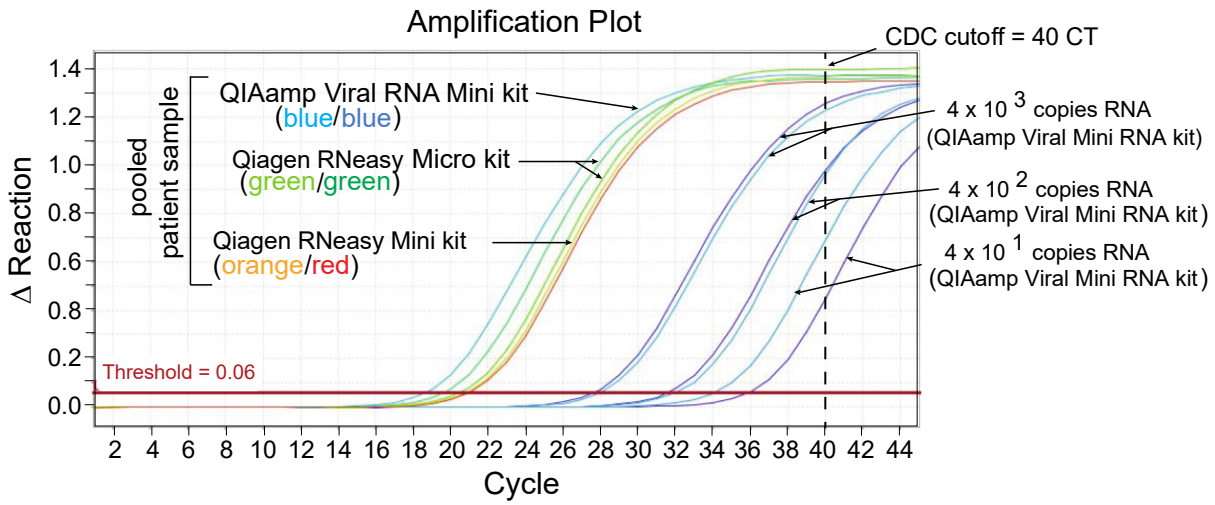

Figure 2 | Qiagen RNeasy Mini and RNeasy Micro kits can be used to successfully isolate SARS-CoV-2 RNA for subsequent detection by RT-qPCR. The nasopharyngeal swab diluents from two confirmed COVID-19 patients were pooled and then subjected to RNA extraction using the Qiagen QIAamp Viral RNA Mini, RNeasy Mini, and RNeasy Micro kits. Note that the original volume of M6 transport media added to each swab was 2 mL per patient. For our screen, following viral RNA extraction, approximately 11.7 µL (QIAamp Viral RNA Mini), 8.3 µL (RNeasy Mini), or 12.5 µL (RNeasy Micro) of the pooled nasopharyngeal swab diluent was used in the RT-qPCR assay and amplification curves are shown (each sample was run in duplicate). As a control, the indicated quantities of SARS-CoV-2 synthetic RNA were spiked into M6 transport media, purified using the QIAamp Viral RNA Mini kit, and screened by RT-qPCR. The resulting amplification curves are shown.

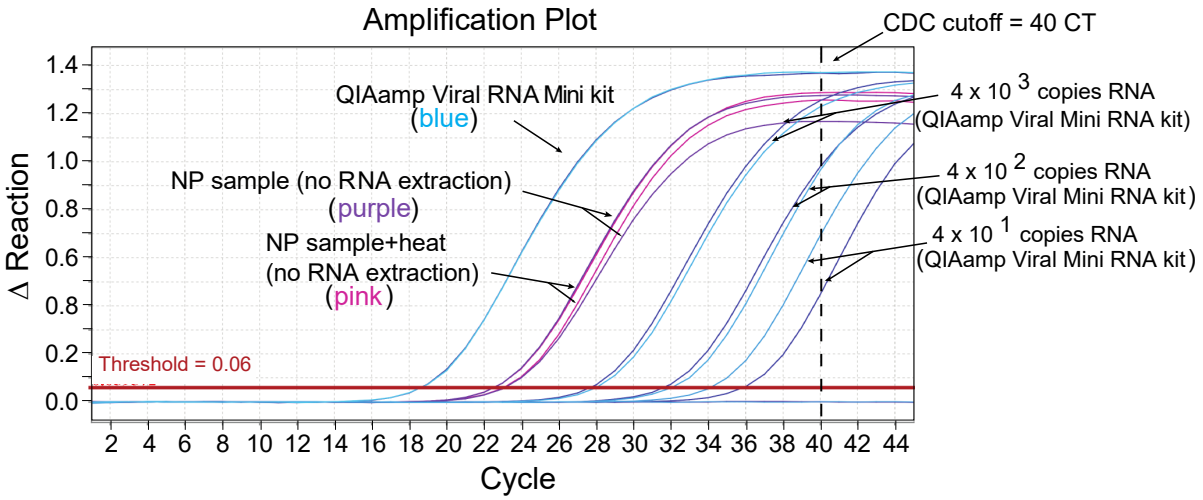

Figure 3 | Direct detection of COVID-19 RNA from a pooled patient nasopharyngeal swab by RT-qPCR in the absence of an RNA extraction step. The nasopharyngeal swab diluents from two confirmed COVID-19 patients were pooled and this mixture was then i) subjected to RNA extraction using the Qiagen QIAamp Viral RNA Mini kit followed by subsequent screening by RT-qPCR or ii) directly added to the RT-qPCR reaction (to omit an RNA extraction step). Note that the original volume of M6 transport media added to each swab was 2 mL per patient. For the RT-qPCR screen, 7 µL of nasopharyngeal swab diluent was added for the direct RT-qPCR (ie. no RNA extraction) approach while an equivalent of ~11.3 µL of diluent was loaded following RNA extraction via the Qiagen QIAamp Viral RNA Mini kit. For the direct, no viral RNA extraction method, we tested whether a preheating step (five minutes at 70°C) might improve availability of the viral RNA for RT (this curve is designated "NP sample + heat"). As a control, the indicated quantities of SARS-CoV-2 synthetic RNA were spiked into M6 transport media, purified using the QIAamp Viral RNA Mini kit, and screened by RT-qPCR. The amplification curves of all samples are shown (each was done in duplicate).