Extraction-free COVID-19 detection with Quantabio's RT-qPCR ToughMixes

Keywords: COVID-19, SARS-CoV-2, RT-qPCR, one-step, virus detection, extraction-free

ABSTRACT

RT-qPCR is considered the "gold-standard" for COVID-19 nucleic acid detection. Detection from purified RNA provides the most sensitivity and reliability, but in the wake of increased demand for testing with faster turn-around-times, extraction-free detection methodologies have also been developed. In this application note we demonstrate the use of Quantabio RT-qPCR reagents for the detection of SARS-CoV-2 from purified RNA, heat-treated viral transport medium samples and saliva samples. We compare these results to published examples and conclude that Quantabio RT-qPCR reagents provide robust, broadly applicable solutions for RNA virus detection.

INTRODUCTION

In response to the global pandemic, routine widespread testing for the SARS-CoV-2 virus was quickly identified as key to monitor and control its spread. Most COVID-19 assays are based on viral nucleic acid detection via amplification of a small number of specific viral target loci by RT-qPCR. By combining first strand cDNA synthesis and subsequent DNA amplification, one-step RT-qPCR offers an elegant, fast and reliable method for quantitative SARS-CoV-2 detection. The highly reproducible performance of one-step RT-qPCR in kitted formats offers protocols that can be easily implemented to allow rapid, high-throughput validation of samples.

Quantabio's portfolio of one-step RT-qPCR master mixes offer key advantages for COVID-19 testing. Each master mix product comes preformulated containing engineered reverse transcriptase, DNA polymerase and an optimized buffer formulation for easy reaction set up and high performance. The buffer incorporates Quantabio's proprietary ToughMix® technology, a blend of Tough additives that optimize enzyme performance and confer resistance to a broad spectrum of PCR inhibitors (e.g. heparin, salt, hemoglobin and others). Over the past seven years, Quantabio has actively supported development of testing protocols for influenza, polio and other infectious diseases in partnership with public health agencies.[1–3]

In relation to the COVID-19 pandemic, Quantabio's RT-qPCR reagents have provided highly sensitive, reliable solutions as a key component for testing via RT-qPCR.[4–11]

In this application note, we provide an overview of the performance of the following Quantabio RT-qPCR ToughMixes: qScript® XLT 1-Step RT-qPCR ToughMix, qScript 1-Step Virus ToughMix and UltraPlex® 1-Step ToughMix, using the EUA-approved SARS-CoV-2 RT-qPCR assay from the Centers for Disease Control and Prevention (CDC).[11] Protocols are described for SARS-CoV-2 detection from purified RNA, heat-treated samples in viral transport media and saliva samples.

	qScript XLT 1-Step RT-qPCR ToughMix	qScript 1-Step Virus ToughMix	UltraPlex 1-Step ToughMix
Formulation	2x master mix	2x master mix	4x master mix
RT Hot-Start	No	Yes	Yes
Multiplex capability	Up to 4 targets	Up to 4 targets	Up to 5 targets
Recommended in CDC protocol	✓	(was not yet launched)	✓
Detection purified RNA	✓	✓	✓
Extraction-free detection	✓	–	✓

Table 1 Overview Comparison of Quantabio's RT-qPCR master mixes.

MATERIALS AND METHODS

Reference materials

The following reference materials were used instead of SARS-CoV-2 live virus or RNA isolated from SARS-CoV-2 virus: Twist Synthetic SARS-CoV-2 RNA Controls (1–7) (Twist Biosciences, #103086), Armored RNA Quant™ SARS-CoV-2 (Asuragen #52030) and AccuPlex™ SARS-CoV-2 Verification Panel - Full Genome (Seracare, #0505-0168).

Name	Description	Oligonucleotide Sequence (5' > 3')	Label 1	Final concentration
2019-nCoV_N1-F	2019-nCoV_N1 Forward Primer	GAC CCC AAA ATC AGC GAA AT	None	500 nM
2019-nCoV_N1-R	2019-nCoV_N1 Reverse Primer	TCT GGT TAC TGC CAG TTG AAT CTG	None	500 nM
2019-nCoV_N1-P	2019-nCoV_N1 Probe	FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1	FAM, BHQ-1	125 nM
RP-F	RNase P Forward Primer	AGA TTT GGA CCT GCG AGC G	None	500 nM
RP-R	RNase P Reverse Primer	GAG CGG CTG TCT CCA CAA GT	None	500 nM
RP-P	RNase P Probe FAM	TTC TGA CCT GAA GGC TCT GCG CG	FAM, BHQ-1	125 nM

Table 2 **CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Panel N1 and RP target assays.**[11]

Extraction-free protocol

Armored RNA Quant SARS-CoV-2 samples were heated at 72°C for 3 min to release the RNA from the virus particles. 5 µl of this crude extract was used as input for the RT-qPCR experiments.

Direct detection from saliva

Samples were prepared by mixing 90 µl negative saliva and 10 µl AccuPlex™ SARS-CoV-2 Verification Panel - Full Genome. 10 µl Proteinase K (Qiagen #19131) was added to the sample and the tubes were placed in a plate mixer and vortexed for 2 min at 2500 rpm. Mixed samples were heated for 5 min at 95°C on a thermocycler, and then 5 µl of processed saliva was used as input for RT-qPCR experiments.

RT-qPCR

RT-qPCR reactions were carried out using Quantabio UltraPlex 1-Step ToughMix (#95166-100), qScript XLT 1-Step RT-qPCR ToughMix (#95132-100) and qScript 1-Step Virus ToughMix (#95131-100). Master mixes were prepared according to table 3 and mixed by gentle vortexing and centrifuged briefly. 15 µl of each master mix was dispensed into the wells of a 96-well plate pre-chilled on an ice block. 5 µl of RNA or water was added to each well, mixed thoroughly using a plate mixer and centrifuged briefly. Each entire 20-µl reaction mix was then transferred to a 'Q' tube. Q tubes were then loaded into the Q cycler and run according to the conditions in table 4.

All RT-qPCR reactions used the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Panel N1 target assay. Data shown in figure 4 was conducted in a duplex assay with human RP gene control.

Reagent	Volume per reaction
UltraPlex 1-Step ToughMix (4x)	
Nuclease-free water	9 µl
20x combined primer-probe mix for N1	1 µl
20x combined primer-probe mix for RP or nuclease-free water	1 µl
UltraPlex 1-Step ToughMix	5 µl
Total volume	15 µl
qScript XLT 1-Step RT-qPCR ToughMix (2x)	
Nuclease-free water	3 µl
20x combined primer-probe mix for N1	1 µl
qScript XLT 1-Step RT-qPCR ToughMix	10 µl
Total volume	15 µl
qScript 1-Step Virus ToughMix (2x)	
Nuclease-free water	3 µl
20x combined primer-probe mix for N1	1 µl
qScript 1-Step Virus ToughMix	10 µl
Total volume	15 µl

Table 3 **1-Step RT-qPCR reaction master mix set up.**

Master Mix	Reverse Transcription	Initial Denaturation	Thermal Cycling (45X)
UltraPlex 1-Step ToughMix	50°C, 10 min	95°C, 3 min	95°C 3 sec 55°C 30 sec
qScript XLT 1-Step RT-qPCR ToughMix	50°C, 10 min	95°C, 3 min	95°C 3 sec 55°C 30 sec
qScript 1-Step Virus ToughMix	50°C, 10 min	95°C, 3 min	95°C 3 sec 55°C 30 sec

Table 4 **One step RT-qPCR protocols for all three Quantabio master mixes used in this study.** All reactions included 45 cycles of denaturation and annealing based on the CDC's recommendation. The protocols were the same for both singleplex and duplex reactions.

PBS tolerance assay

Master mixes were prepared with UltraPlex 1-Step ToughMix as detailed in table 3, replacing up to 12 µl nuclease-free water with PBS (Sigma #806544). The master mixes were gently vortexed, briefly spun down and distributed into the wells of a 96-well plate. 2 µl Twist Synthetic SARS-CoV-2 RNA Controls (500 copies/µl) or water (for NTC) were added to each well of the plate. RT-qPCR experiments were carried out as described in table 4.

RESULTS AND DISCUSSION

Detection of purified SARS-CoV-2 RNA

qScript XLT 1-Step RT-qPCR ToughMix and UltraPlex 1-Step ToughMix were identified in the CDC EUA RT-qPCR protocol for the detection of SARS-CoV-2 RNA purified from the viral transport media (VTM) in which nasopharyngeal swabs were stored.[11,12] In the assay, both mixes detected viral loads of purified RNA as low as 1 copy/µl. The methodology was also validated with clinical samples, where both qScript XLT 1-Step RT-qPCR ToughMix and UltraPlex 1-Step ToughMix demonstrated 100% positive and 100% negative agreement compared to retrospective RNA samples extracted from respiratory specimens.

We validated the detection of SARS-CoV-2 using the above mentioned reagents along with and qScript 1-Step Virus ToughMix for the CDC N1 target assay (Table 2). Twist Synthetic SARS-CoV-2 RNA Controls (1-7) (Twist Biosciences) were used as a representative of RNA extracted from live virus samples. Each RT-qPCR mix yielded consistent and comparable amplification curves with strong signals above baseline (Figure 1A). A dilution series of input RNA copy number was performed from 0.5- 500 copies/µl. All three Quantabio mixes were able to detect purified SARS-CoV-2 RNA with Cq values less than 38 cycles, demonstrating highly sensitive detection over a wide dynamic range (Figure 1B).

These findings are in agreement with a number of studies that have used Quantabio RT-qPCR reagents for COVID-19 detection with RNA extracted from nasopharyngeal swab, combined nasal- and oropharyngeal swab and saliva samples.[10,13–15]

Detection from heat-treated samples in VTM

Currently, the majority of SARS-CoV-2 detection assays require RNA extraction before RT-qPCR.[16] RNA extraction improves sensitivity and reproducibility of detection but extraction kits can be expensive and the need for extraction adds complexity

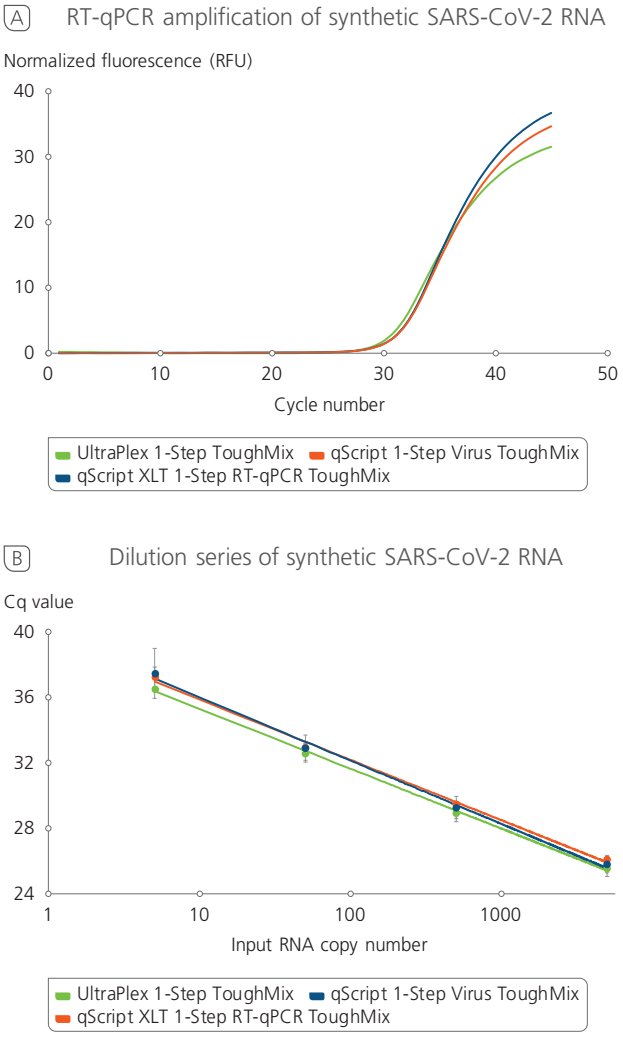

Figure 1 **Detection of purified SARS-CoV-2 RNA by RT-qPCR using CDC N1 target assay and either qScript XLT 1-Step RT-qPCR ToughMix, qScript 1-Step Virus ToughMix or UltraPlex 1-Step ToughMix** **A** Amplification curves from detection of 500 copies/µl SARS-CoV-2 RNA, **B** Cq values for dilution series of SARS-CoV-2 RNA from 0.5–500 copies/µl.

and time to the workflow. In July 2020, the CDC amended the EUA COVID-19 testing protocol to recognize UltraPlex 1-Step ToughMix as an effective heat-treatment alternative for RNA extraction.[11] The EUA recommended that if RNA extraction reagents were in short supply, public health laboratories could instead follow a protocol in which VTM was incubated at 95°C for 1 min then cooled before use directly as input for RT-qPCR.

To investigate the heat-treatment protocol with Quantabio reagents, MS2 Coated Armored SARS-CoV-2 RNA (Armored RNA Quant SARS-CoV-2, Asuragen) was used as a substitute for viral RNA in a protein coat. As with purified RNA, qScript XLT 1-Step RT-qPCR ToughMix, qScript 1-Step Virus ToughMix and UltraPlex 1-Step ToughMix all performed well with heat-treat-

ed samples, giving strong amplification curves (Figure 2A). A dilution series of MS2 Coated Armored SARS-CoV-2-RNA demonstrated highly sensitive detection down to 1 copy/µl with Cq less than 38 cycles (Figure 2B). Cq values with qScript XLT 1-Step RT-qPCR ToughMix and UltraPlex 1-Step ToughMix were 1 cycle lower than with qScript 1-Step Virus ToughMix (37 cycles versus 38 cycles) at 1 copy/µl input RNA, but these results demonstrate that all three mixes can be used for extraction-free SARS-CoV-2 detection.

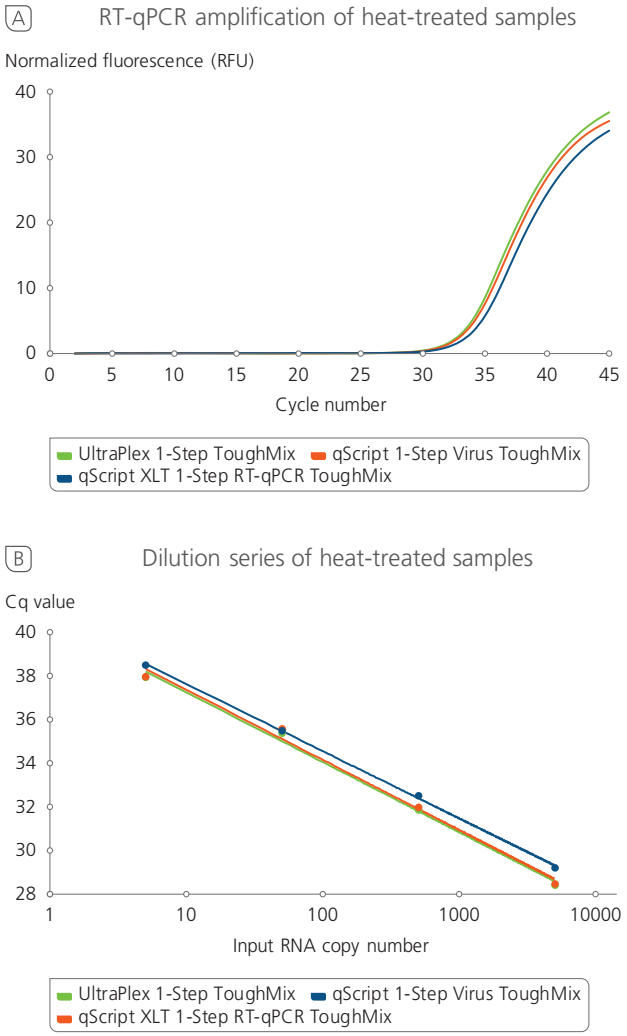

Figure 2 **Direct detection of SARS-CoV-2 RNA in heat-treated samples.** **A** Amplification curves from detection of 500 copies/µl SARS-CoV-2 RNA, **B** Cq values for dilution series of SARS-CoV-2 RNA from 0.5–500 copies/µl.

The CDC validated this assay with heated-treated nasopharyngeal swab samples in VTM with UltraPlex 1-Step ToughMix. In a study of 39 samples, heat-treatment alternative showed similar sensitivity to detection from purified RNA, with 94.7% positive agreement and 100% negative agreement.[11] In another study, this heat-treatment methodology was also demonstrated with qScript XLT 1-Step RT-qPCR ToughMix and CDC N1/N2 assays on nasopharyngeal swab diluent samples (UTM VTM, Copan, heated for 95°C for 10 min). Samples tested using a standard RNA extraction or direct (heat-treated) method showed agreement in 17/20 samples, indicating a sensitivity of 85% for direct detection.[7]

A concern with heat treatment methodology is that the high salt concentration from sample collection media (e.g. VTM) could inhibit the RT-qPCR reaction. To test the effect of high salt concentration on RT-qPCR using Quantabio reagents, reactions with synthetic SARS-CoV-2 RNA were spiked with 0-12 µl PBS to mimic VTM. UltraPlex 1-Step ToughMix demonstrated high salt concentration in the RT-qPCR reaction with little or no change in Cq value with up to 8 µl PBS (61 mM NaCl, 1.2 mM KCl final concentration in reaction) (Figure 3). In contrast, addition of just 5 µl PBS inhibited the alternative master mix from Supplier T (ΔCq = –3.9) and with 8 µl PBS addition the assay was greatly inhibited such that the Cq value was >40.

PBS	UltraPlex 1-Step ToughMix	Supplier T
5 µl	0.3	–3.9
8 µl	0.0	–22.0
12 µl	–21.0	–22.0

Enhancement Neutral Inhibition

Figure 3 **Impact of PBS on CDC N1 assay.** RT-qPCR reactions with synthetic SARS-CoV-2 RNA and either UltraPlex 1-Step ToughMix or an alternative master mix from 'Supplier T' were spiked with 0-12 µl PBS to mimic VTM. Data shown is change in Cq value with addition of PBS compared to the standard N1 assay run with each master mix.

This result was echoed in a published study of multiplexed and extraction-free SARS-CoV-2 detection assays. The study demonstrated that VTM samples amplified with the CDC singleplex assays (N1 and N2) using qScript XLT 1-Step RT-qPCR ToughMix were able to reliably detect as few as 2 copies/µl viral RNA.[6] In a comparison of commercially available RT-qPCR mixes, UltraPlex 1-Step ToughMix and qScript XLT 1-Step RT-qPCR ToughMix were the most tolerant to various VTMs (S2, GG and M4RT VTM) and even demonstrated some amplification enhancement in two of the three VTMs.[6] In an independent study, Thompson et al. demonstrated extraction-free detection

of SARS-CoV-2 RNA from patient nasal or throat swab samples in VTM (Σ-Virocult, Medical Wire).[17] The study compared the effect of VTM on the results from commercially available kits and either qScript XLT 1-Step RT-qPCR ToughMix or UltraPlex 1-Step ToughMix combined with CDC N1/N2 assays following an alternative protocol of heat treatment at 75°C for 10 min. They concluded that, the Quantabio-CDC assays showed superior accuracy and sensitivity for COVID-19 detection.[17]

It should be noted that compositions for VTM can vary and care should be taken to use a VTM that is similar to those in the supporting publications. Sample collection media that is intended to inactivate virus and/or preserve nucleic acids that contain components such as guanidinium/guanidine, sodium dodecyl sulfate (SDS) or high concentrations of ethylenediaminetetraacetic acid (EDTA) are not compatible with direct detection using UltraPlex 1-Step ToughMix (data not shown).

Direct detection from saliva

Collection of saliva offers a non-invasive, alternative sampling method to nasopharyngeal swabs. The combination of saliva sampling and direct detection of viral RNA from saliva, without extraction, could vastly increase the frequency and throughput of testing. Recently, Vogels et al. published a one-step RT-qPCR assay, SalivaDirect assay, for extraction-free detection of SARS-CoV-2 in saliva using the CDC N1 probe set (with human RNase P control assay).[18] The protocol involves pre-treatment of saliva with proteinase K and vortexing, followed by incubation at 95°C for 5 minutes.

We repeated this protocol with UltraPlex 1-Step ToughMix, using AccuPlex SARS-CoV-2 Verification Panel (Seracare) spiked in negative saliva as the input sample. The AccuPlex SARS-CoV-2 Verification Panel is comprised of the full SARS-CoV-2 genome encapsulated in recombinant alphavirus, and so provides a good substitute for live virus. RT-qPCR of treated samples demonstrated detection of 2.5-5 copies/µl viral RNA (Figure 4), which is in line with the published limit of detection of the SalivaDirect assay, 6-12 copies/µl.[18] This demonstrates proof-of-concept that UltraPlex 1-Step ToughMix can be used for extraction-free virus detection from saliva.

Alternative protocols for extraction-free SARS-CoV-2 detection have also been developed. For example, in an open-access protocol, Alarcon et al. detailed a method for direct-lysis saliva SARS-CoV-2 detection using UltraPlex 1-Step ToughMix.[4]

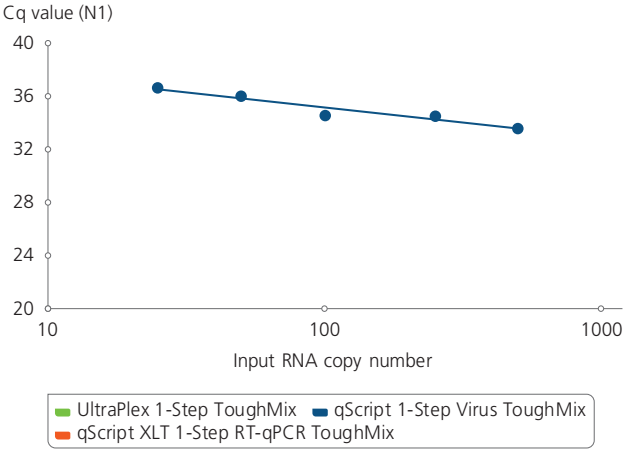

Figure 4 **Extraction-free detection of SARS-CoV-2 RNA in saliva.** A dilution series of AccuPlex SARS-CoV-2 Verification Panel was spiked into negative saliva and treated according to the SalivaDirect protocol. 5 µl treated saliva sample was used as input for RT-qPCR using UltraPlex 1-Step ToughMix.

The protocol involves heat-inactivation of the sample at 65°C for 30 minutes or 95°C for 5 minutes followed by lysis with 30% Triton® X-100 and direct RT-qPCR. Additionally, Chomczynski et al. demonstrate the use of qScript XLT 1-Step RT-qPCR ToughMix for direct RT-qPCR of saliva samples, following a rapid and simple protocol of treatment with alkaline-glycol solution and incubation at room temperature for 5-30 minutes.[5] This once again highlights the broad utility of Quantabio's reagents for SARS-CoV-2 detection in inhibitor rich samples.

CONCLUSION

In this application note we have demonstrated highly sensitive detection of SARS-CoV-2 RNA down to 1 copy/µl using both purified RNA and heat-treated viral sample medium as inputs. Particularly for extraction-free detection, qScript XLT 1-Step RT-qPCR ToughMix and UltraPlex 1-Step ToughMix show high tolerance to PCR inhibitors and robust virus detection. We also present proof-of-concept data demonstrating direct detection of SARS-CoV-2 RNA in saliva with UltraPlex 1-Step ToughMix. In conclusion, Quantabio continues to expand utility for virus detection, supporting the effort to control and overcome the Corona virus pandemic.

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