UltraPlex® 1-Step ToughMix® for Extraction-free, High Capacity COVID-19 Testing

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

ABSTRACT

Nucleic acid amplification testing (NAAT) is becoming a preferred methodology for disease monitoring and more so during the current COVID-19 pandemic caused by SARS-CoV-2. This pandemic has infected >150 million people worldwide with more infectious strains evolving and being transmitted daily.

NAAT for COVID-19 testing has encountered two key obstacles affecting both accurate virus detection and test result turnaround times. First, use of RNA extraction based protocols (either manual or robotic extraction as is used by most COVID-19 testing facilities) is time consuming, expensive and resource limiting. Second, there is great variability in sensitivity and specificity of primers and reaction master mix combinations, resulting in varying proportions of percent positive and false negative detection contributing to increased public risk. In this application note, we describe the use of UltraPlex 1-Step ToughMix (Quantabio) in an extraction-free methodology as a practical solution to COVID-19 high capacity testing. We demonstrate robust, reproducible and inexpensive testing with no loss of sensitivity or specificity when compared to standard extraction based NAAT. The use of an extraction-free approach with UltraPlex 1-Step ToughMix circumvents the need for expensive extraction devices, allowing for greater than 3-4 fold increase in testing capacity while providing the sensitivity needed for detection of weak COVID-19 positives, significantly reducing the turnaround time of test results.

INTRODUCTION

The COVID-19 pandemic has massively affected the health, well-being and day-to-day lives of people. Initially thought to only affect the elderly or immunocompromised individuals, it is now known this is not the case as new mutations have arisen giving highly transmissible variants affecting all populations and ages.[1,2] Many have predicted that COVID-19 testing will still be required into the fall/winter of 2021/2022 (even in the presence of vaccine rollouts) that will certainly engage and burden many health care systems.[3–5] Thus, there is a need to develop methodologies that accelerate testing and achieve faster reporting turnaround times, while maintaining high detection rates of both strong and weak positives, with low false negative rates. Extraction-free COVID-19 detection offers an accelerated testing solution achievable with the use of UltraPlex 1-Step ToughMix. Several groups have now employed UltraPlex 1-Step ToughMix to achieve a limit of detection (LOD) of less than one genome copy per reaction for detection of COVID-19 genes in a multiplex assay with a sensitivity of 97% and specificity approaching 99% (in original or pooled samples). In this application note, we describe a robust, reproducible and inexpensive methodology for high capacity COVID-19 testing that can achieve the specificity and sensitivity needed to aid in meeting the increased need for testing.

Figure 1 Overview of Extraction-free protocol using heat-treatment methodology.

METHODS

qPCR primers/probes

NAAT for COVID-19 was carried out utilizing primers/probes to E, N1 and RNase P gene targets obtained from Integrated DNA Technologies (IDT) in a multiplex assay. Heat inactivated virus (ATCC VR-1986HK™) was used to determine the LOD of the COVID-19 assay using UltraPlex 1-Step ToughMix (#95166-01K, Quantabio).

A single-tube mix of primers and probes for COVID-19 targets was prepared according to Table 1.

Sample preparations for direct RT-qPCR

Swab specimens were gently vortexed, 100 µl of the swab solution was transferred to a 96-well qPCR plate and incubated at 65°C for 15 min for virus inactivation. Alternatively, swab tubes (as collected) were placed in a water bath at 65°C for 30 min with equivalent results. After heating, the samples were cooled down for 5-10 min and 5 µl used directly in RT-qPCR, or were stored at -20°C for longer term storage. The heat inactivated virus (ATCC VR-1986HK™) did not require any further thermal inactivation and was used for the LOD by dilutions in COPAN UTM media (#3C047N, COPAN).

RT-PCR detection of SARS-CoV-2

5 µl of the heated sample or viral diluted samples were used for NAAT for COVID-19. Thermal cycling was carried out with UltraPlex 1-Step ToughMix (5 µl) with E.N1.RNaseP primers/probe mix (1 µl) in a total reaction volume of 20 µl. Reverse transcription was carried out at 50˚C for 10 min, denaturation at 95˚C for 3 min (1 cycle) and amplification for E.N1.RNaseP multiplex for 50 cycles (of 95˚C for 5 s then 60˚C for 30 s) with total qPCR time of ~70 min. In the data presented, both COVID-19 E and N1 genes have FAM-based probes, resulting in additive fluorescence signal which aids in detection of SARS-CoV-2. RNase P is an Atto647 probe detected in the Cy5 channel.

Contrived Samples

For the purpose of this application note, COVID-19 positive samples were contrived samples obtained by dilutions from the SARS-CoV-2 heat inactivated virus, ATCC VR-1986HK. 'Strong', 'medium' and 'weak' contrived positives were prepared using heat inactivated SARS-CoV-2 at 50000, 10000 and 100 copies per reaction (2500, 500 and 5 copies/µl), respectively. For contrived samples and dilution of contrived samples, the diluent was a COPAN UTM swab solution from a control (COVID-19 negative) throat swab as described for each figure.

Interpretation of qPCR results

The determination of a COVID-19 positive and negative sample is evaluated based on the Cq, fluorescence and sigmoidal nature of the qPCR curve produced. Each instrument and assay should be validated using 20–50 positive and 20–50 negative samples to determine cut-offs for utilizing Cq, fluorescence and sigmoidal nature to call COVID-19 positive or negative.

A positive result is typically based on Cq <40, fluorescence levels >7 units and a sigmoidal nature of the curve to clearly show amplification of the viral nucleic acid. An analysis of Cq and fluorescence values of 20–50 negatives will allow one to determine cut offs for calling COVID-19 negative samples and for calling invalid samples. The inclusion of human RNase P

Primer/probe	Sequence of primer/probe (5' to 3')	Final concentration in primer/probe mix (µM)	Final concentration in RT-qPCR reaction (nM)
E-gene FP	ACAGGTACGTTAATAGTTAATAGCGT	4	200
E-gene RP	ATATTGCAGCAGTACGCACACA	4	200
E-gene Probe	FAM-ACACTAGCA/ZEN/TCCTTACTGCGCTTCG-IABkFQ	2	100
N1-gene FP	GACCCCAAAATCAGCGAAAT	13.4	670
N1-gene RP	TCTGGTTATGCCAGTTGAATCTG	13.4	670
N1-gene Probe	FAM-ACCCCGCAT/ZEN/TACGTTTGGTGGACC-IABkFQ	3.4	170
RNase P FP	AGATTTGGACCTGCGAGCG	13.4	670
RNase P RP	GAGCGGCTGTCTCCACAAGT	13.4	670
RNase P Probe	Atto647NN-TTCTGACCT/TAO/GAA GGCTCTGCGCG-IABRQSp	3.4	170

Table 1 Primers and probes used for COVID-19 detection. FP, forward primer; RP, reverse primer.

into the assay will determine sample quality, with fluorescence value of the RNase P signal of <1 generally indicating poor sample quality/swabbing and re-collection should be triggered. For samples with Cq <40 and fluorescence levels > 7 with poor sigmoidal curves, a second platform or second gene confirmation is advised to confirm COVID-19 positivity. Some labs recommend utilizing an E.N1 Cq <33/35 as the cut off for a robust positive with samples with Cq >33/35 but <40 needing second gene confirmation. Second gene confirmation has been carried out by certain researchers as an Orf1ab/N2/RNase P multiplex gene assay, reporting detection rates of >98% with <1% false negatives with the use of UltraPlex 1-Step ToughMix. For strong positives with Cq <33/35 and high fluorescence values, RNase P fluorescence tends to be very low or even absent due to the efficient qPCR reaction for the COVID-19 genes but a positive can still be called. LED based qPCR machines (such as the Roche LightCycler® 480 II, qTOWER systems and Quantabio Q instrument) produce higher fluorescence signals with increasing ease of detection of weak positives.

RESULTS

We first explored the use of UltraPlex 1-Step ToughMix to detect SARS-CoV-2 virus by a LOD assay. The LOD obtained was between 0.5 and 1 copy per reaction or 100 and 200 copies/ml with an E.N1 gene multiplex readout (Figure 2, E and N1 are COVID-19 specific genes). This was in contrast to results obtained for other master mixes (most have an LOD >1000 copies/ml), other primer/probe assays (such as the use of Rdrp with an LOD >5000 copies/ml) and other non-LED qPCR machines (such as the detection of E.N1 on the Roche LightCycler 480 with an LOD >1000 copies/ml).[6,7]

Next, extraction-free detection was investigated for pooled samples. When testing large numbers of samples for COVID-19, pooling samples can offer reductions in materials, time and labor required. Three contrived positive samples (strong, medium and weak) were prepared in a swab solution from a control (COVID-19 negative) throat swab to provide a source of RNase P (the internal control). They were then used as neat samples (no dilution) or diluted as indicated and RT-qPCR was carried out with UltraPlex 1-Step ToughMix in an E.N1.RNaseP multiplex assay. All positive samples were successfully detected using the multiplex readout for the COVID-19 genes using UltraPlex 1-Step ToughMix (Figure 3A). In addition, diluting the sample does not significantly affect the detection of the internal control, RNase P, suggesting that even poorly collected swabs can still

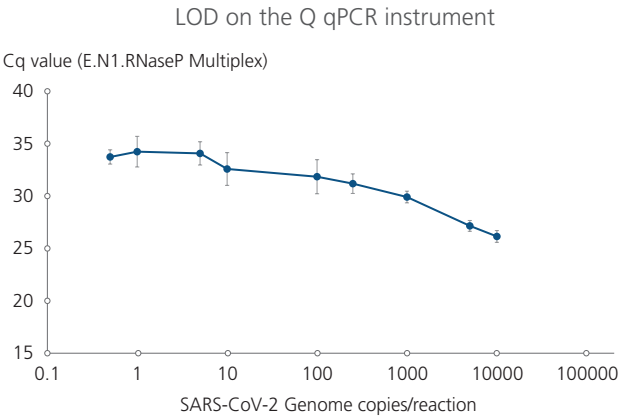

Figure 2 **Limit of detection of the E.N1.RNase P multiplex assay.** Viral dilutions from 0.5–10000 copies per reaction were utilized to determine the limit of detection using the E.N1.RNase P COVID-19 multiplex assay and UltraPlex 1-Step ToughMix. qPCR was performed on the Quantabio Q instrument. The LOD was found to be 0.5 copies per reaction equivalent to 100 copies/ml.

be easily evaluated for RNase P (Figure 3B). Furthermore, strong positives (≥ 2500 copies/µl) can be detected at high dilution up to 1:50 or 1:100 (data not shown), demonstrating the high sensitivity of the assay.

A concern with an extraction-free methodology is of possible matrix effects (from the swab solution) that may inhibit the qPCR reaction. UltraPlex 1-Step ToughMix comprises a 'ToughMix' formula to overcome PCR inhibitors and allow DNA and RNA amplification from crude samples. To test matrix effects on SARS-CoV-2 detection using UltraPlex 1-Step ToughMix, contrived positive samples were prepared at 10000 copies/µl in three common viral transport media: eSWAB™ (#480C, COPAN), UTM® (#3C047N, COPAN) or VTM (#26490, Liofilchem) and assayed using the E.N1.RNaseP multiplex assay. There was no significant difference in the Cq value with any of the viral transport media demonstrating media tolerance of UltraPlex 1-Step ToughMix (Figure 3C).

Caution is advised with some viral transport media, such as with GDLKorea VTM tubes (#GDLS1011) or the clear Biologix saline swab tubes, where clients have reported lack of compatibility with UltraPlex 1-Step ToughMix. It is thus recommended that users carry out 1:4 to 1:10 dilutions of positives in the transport media of choice to determine matrix effects that may inhibit the RT-qPCR reaction.

As demonstrated above, the use of a single fluorophore for both COVID-19 genes (E and N1) in the multiplex assay can result in higher signal for detection of weak positive samples. However, separate detection of each gene may be necessary, for example, if mutation screening is needed for one of the

COVID genes, such as an S gene mutation screen. The use of UltraPlex 1-Step ToughMix enables efficient multiplexing of genes with probes in different channels to allow for robust detection of all targets (Figure 4).

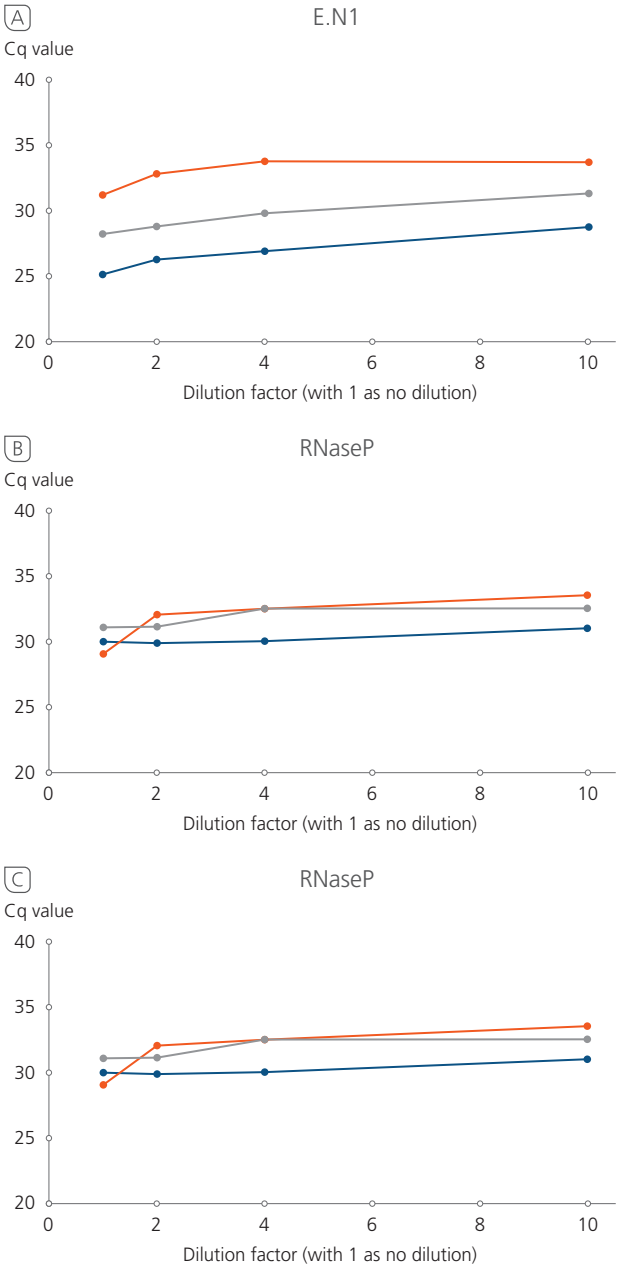

Figure 3 **Robust detection of SARS-CoV-2 contrived samples using UltraPlex 1-Step ToughMix.** A Dilutions of contrived positive samples were prepared at neat (dilution of 1), 2 (1:2), 4 (1:4) or 10 (1:10). Dilutions were prepared in a UTM (COPAN) swab solution from a control (COVID-19 negative) throat swab. RT-qPCR was carried out with E.N1.RNaseP multiplex assay on examples of strong (10000 copies/µl, orange), medium (2000 copies/µl, gray) or weak (20 copies/µl, blue) positives. B Detection of RNase P in the strong, medium or weak contrived positive samples of (A). C Effect of matrix on SARS-CoV-2 detection using E.N1.RNaseP multiplex assay. Contrived samples (2000 copies/µl) were diluted in eSWAB (orange), UTM (blue) or VTM (gray) and RT-qPCR carried out with E.N1.RNaseP multiplex assay. Again, solutions were diluted in the respective swab solution from a control (COVID-19 negative) throat swab.

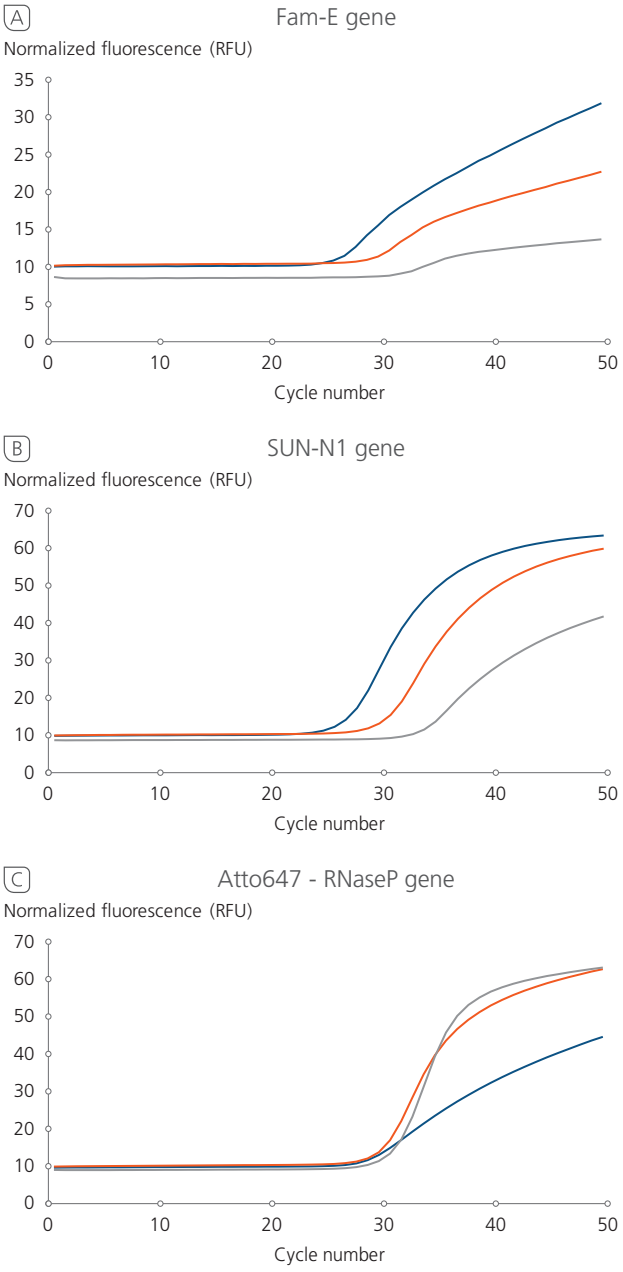

Figure 4 **Analysis of a three-gene multiplex in separate channels using UltraPlex 1-Step ToughMix.** Three contrived strong (10000 copies/µl, blue), medium (2000 copies/µl, orange) or weak (20 copies/µl, gray) positive samples were prepared in a negative throat swab sample as a source of human RNase P. These were then amplified using a multiplex qPCR reaction containing FAM-E-gene primers/probe (A), SUN-N1 primers/probes (B) and Atto647-RNaseP primers/probe (C) that allowed detection in three separate channels. UltraPlex 1-Step ToughMix is robust enough to amplify both E and N1 COVID-19 genes in separate channels and RNase P in a third channel as before.

CONCLUSION

In this application note, a robust extraction-free methodology for SARS-CoV-2 detection using a E.N1.RNaseP multiplex assay and UltraPlex 1-Step ToughMix has been demonstrated. The assay was shown to be reliable for detection from samples of various concentrations ('strong', 'medium' and 'weak' positives), suitable for use with pooling strategies, and compatible with various commonly used swab collection tubes and viral transport media.

Several researchers have used this extraction-free thermal protocol to prepare nucleic acid for genetic testing from patient derived throat, nasopharyngeal or nasal swabs. Compared to standard NAAT, this extraction-free protocol saves > 2.5 h in preparation time compared to automated robotic extraction systems. In our hands, the use of the UltraPlex 1-Step ToughMix allows for a detection rate of > 98% with < 1% false negatives using either E.N1 or Orf1ab/N2 COVID-19 gene multiplex assays. Rapid qPCR times of between 60 and 70 minutes are obtained depending on the qPCR machine used.

The key feature in the success of this extraction-free NAAT protocol is the use of UltraPlex 1-Step ToughMix which provides exceptional inhibitor tolerance and a rapid, simple workflow. The UltraPlex 1-Step ToughMix has now been utilized by several researchers to amplify targets from crude extraction-free human, viral and bacterial genomic RNA and DNA sample preparations with robust detection.

Advantages of UltraPlex 1-Step ToughMix for extraction-free COVID-19 testing:

- Robust compatibility with viral swab solutions to amplify viral and human nucleic acid
- Robust compatibility with bacterial swab solutions to amplify bacterial nucleic acid
- Little to no background fluorescence when performing COVID-19 qPCR on negative swab samples
- Can be utilized for qPCR detection of 2-4+ multiplexed targets with no loss of Cq values and nominal loss of fluorescence units
- Affordable for high capacity testing

References

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