NextGenPCR Application Brief:

Accelerating direct detection of SARS-CoV-2 RNA from Amies Transport Medium using ultra-fast endpoint PCR

Introduction

Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the gold standard testing method for detecting viral RNA. However, most thermocyclers take an hour to complete a reaction, so RT-PCR has been characterized as not a rapid test. With Molecular Biology Systems'(MBS) introduction of NextGenPCR in 2017, laboratories received an ultra-fast PCR (30 cycles in less than 10m)[1] thermocycler that processes up to 384 samples on SBS-formatted microplates. In June of 2020, researchers demonstrated direct quantification of fluorescent endpoint PCR for the detection of SARS-CoV-2 using NextGenPCR.[2] This ultra-fast RT-PCR method highlighted a new bottleneck, RNA isolation, which decreases laboratory testing capacity. In this study, we present the successful combination of heat-enabled RNA extraction together with ultra-fast RT-PCR.

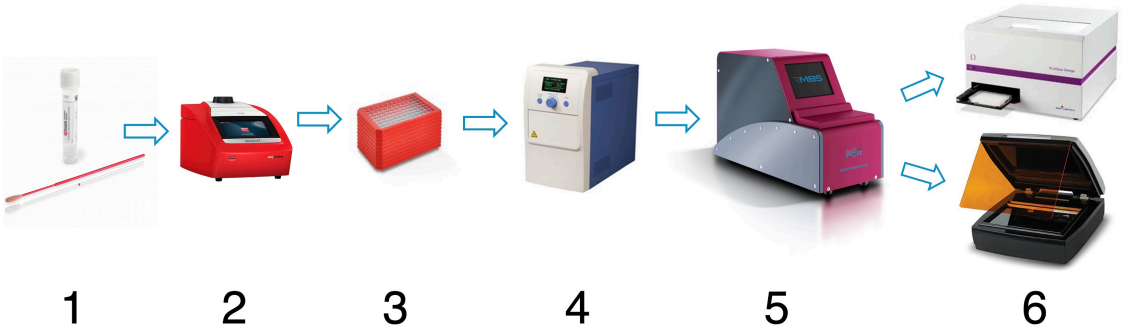


Fig. 1: Overview of NextGenPCR workflow for fluorescent endpoint detection. Swabs deposited into Amies medium and heat-treated (1), re-heated (2), and then aliquoted into ultrafast RT-PCR reactions (3) are sealed (4). The PCR plates were then cycled in the NextGenPCR machine (5) before being read on a multicolor plate reader (6-top) or a single color flatbed scanner (6-bottom).

NextGenPCR Direct RT-PCR protocol

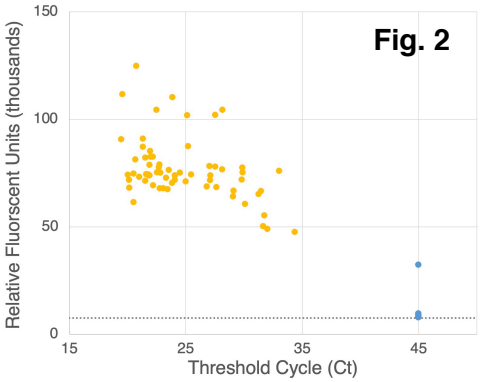
For direct SARS-CoV-2 testing, nasopharyngeal swabs were deposited (Copan Diagnostics, Italy) in 1.0mL of Liquid Amies Transport Medium (bioTRADING, Netherlands). Subsequently, samples were heat treated for 10m at 98°C for viral inactivation. Samples were refrigerated until processing. Shortly before assembling the RT-PCR reactions, 200uL of each Amies media sample was transferred to a PCR tube and heated to 98°C for 5m in a Biometra thermocycler (Analytik Jena, Germany).

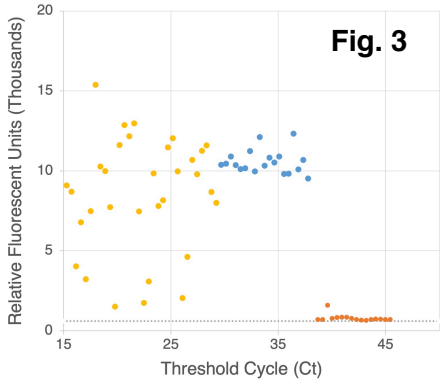
PCR reactions (20uL) contained 10 uL RT-PCR Chemistry-2× (MBS), 1.6 uL SARS-CoV-2 and Human RNase P Primers and Probes (MBS), 4.4 ul of nuclease-free water (MBS), and 4 uL of heat-inactivated Amies media sample. Reactions were loaded in 96-well×20uL microplates (MBS) and sealed with clear heat seals (MBS) in a heat sealer (MBS) for 1.5s. at 165°C. Reactions were performed using a NextGenPCR machine (MBS). The RT-PCR protocol: 1 cycle for 300s at 55°C followed by 60s at 98°C followed by 5 cycles at 98°C for 10s, 60°C for 20s, 72°C for 3s, followed by a final 45 cycles at 98°C for 5s, 60°C for 12s, 72°C for 3s.

The microplates were removed from the NextGenPCR machine, snapped into an imaging anvil (MBS), and scanned with an Omega Fluostar (BMG Labtech) to detect both the SARS-CoV-2 ORF1ab and N gene RNA (FAM) and human RNase P (Cy5). The fluorescent signal was then analyzed using the Omega Fluostar software. After scanning, the sealed plates were inverted and placed directly on a flatbed Bio1000F Imager (MBS) and the FAM signal was captured using the 4× setting. Fluorescent signal captured by the Bio1000F Imager were then quantified using the QuickDetect software (MBS).

Results

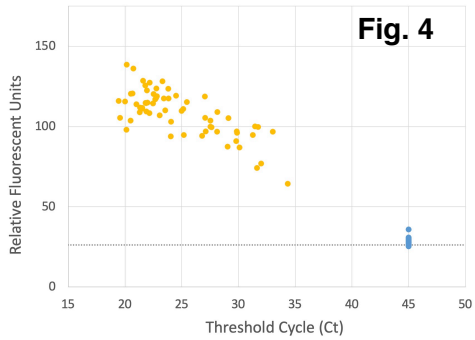
To establish SARS-CoV-2 status, 100 samples were tested concurrently using the Roche Lightcycler, yielding 64 SARS-CoV-2 positive, 20 SARS-CoV-2 negative, and 16 media only samples. Initially, samples processed with the ultrafast direct RT-PCR protocol were measured with a multicolor fluorescent plate reader that detects both FAM signal (viral RNA targets ORF1AB and N genes) and Cy5 signal (human RNase P). In Figure 2, the fluorescent values greater than 47,000 units cluster together with samples that were determined to be SARS-CoV-2 positive (yellow) with C_T values below 35. The negative SARS-CoV-2 samples (blue) has fluorescent values near the fluorescence detected in the no template control (gray line). In Figure 3, the fluorescent values in the Cy5 channel (human RNase P) for both the SARS-CoV-2 positive (yellow) and negative (blue) samples were plotted against their respective C_T values. All of the samples run on NextGenPCR showed detectable amplification of human RNase P. This result confirms that the samples with low fluorescent values in Figure 2, had been successfully extracted and amplified.





The wide variation of Cy5 fluorescent values for SARS-CoV-2 positive samples likely stems from the RNaseP reaction being outcompeted by the two viral RNA amplifications.

As an alternative, a single color fluorescent detection method was tested and compared to the corresponding C_T values. This was performed after completing the multi-color scan, the microplate/imaging anvil was inverted and placed on the Bio1000F imager. An .jpeg image was captured and analyzed using the Quick Detect software. In Figure 4, the fluorescent values greater than 60 units cluster together with samples that were determined to be SARS-CoV-2 positive (yellow) with C_T values below 35. Fluorescent values below 40 units clustered near the average fluorescence seen in the no template control (grey line). All of these samples were SARS-CoV-2 negative (blue) as determined by qPCR. Taken together, this data suggests that the fluorescent signal generated using direct extraction and ultrafast RT-PCR correlates well with slower qRT-PCR methods.



Summary

Ultrafast RT-PCR with NextGenPCR is compatible with direct extraction of viral RNA from liquid Amies Transport Medium that results in a ~**26 minute sample-to-result** workflow. This workflow would translate to amplifying **4,320 samples per day** with a single NextGenPCR machine.

References

1) "The kinetic requirements of extreme PCR" Biomolecular Detection and Quantification. Millington, AL, et. al. Mar. 13, 2019.
2) "Ultra-fast one-step RT-PCR protocol for the detection of SARS-CoV-2" medRxiv. Asghari, E, et. al. Jun. 26, 2020. (Preprint)

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