NextGenPCR™ evaluated with a fluorescence microplate reader accelerates testing for SARS-CoV-2 viral RNA

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- **NextGenPCR™ significantly accelerates testing procedure to detect SARS-CoV-2 positive samples**
- **Fluorescence-based readout on the FLUOstar Omega enables quick and easy detection of the results**
- **MARS analysis software enables effortless evaluation and interpretation of data**

Introduction

The rapid spread of infectious diseases raises more than ever the need for quick, sensitive and high-throughput diagnostic tools to monitor and interrupt infection chains. Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the current diagnostic gold standard to detect viral RNA from patients[1]. However, RT-PCR is limited due to its run time of approx. 4-6 h which considerably restricts its use for high-throughput test campaigns.

In 2017 Molecular Biology Systems B.V. (MBS) introduced its NextGenPCR™ which allows significantly faster cycling speeds compared to regular PCR and enables a throughput increase. With the NextGenPCR, 30 cycles, switching among 3 temperatures, can be completed in less than 2 min. This is achieved using innovative heating technology that accelerates the temperature ramp rates to 1000°C/s. Still, the general advantages of the PCR method, such as high sensitivity, remain. This is also shown in this recent publication using a 27 min NextGenPCR approach[2]. It also circumvents the need of a preceding nucleic acid extraction. While nasopharyngeal swabs are routinely used for fast screening, analysis based on saliva samples would be preferrable as it is less invasive.

This application note reports on the successful amplification of SARS-CoV-2 RNA in realistic saliva solutions with a NextGenPCR approach and the subsequent detection with the FLUOstar® Omega microplate reader.

Assay Principle

NextGenPCR is based on the same principle as regular RT-PCR. Short sequences called primers are used to select the portion of the genome to be amplified. The assay runs through several cycles switching between 98°C, 60°C and 72°C, to denature, anneal and extend DNA strands with enzymes and nucleotides, respectively (fig. 1 B). The substantial decrease in the overall run time with NextGenPCR is achieved through the compression of wells between two temperature blocks (fig. 1 A). The direct contact of the wells and the temperature block with a very large contact surface, leads to the instant cooling and heating of the samples. For this purpose, wells are made of very thin polypropylene foil. The NextGenPCR reaction mix contains primers and probes targeting the ORF1ab and N gene sequences of SARS-CoV-2 (labelled with a FAM dye) and human ribonuclease P/MRP subunit P30 (*RPP30*, labelled with a Cy5 dye). The latter serves as internal control, to check if the sampling from patients and PCR cycling itself worked efficiently. The included quenched fluorescent probes bind specifically to respective genomic sequences if they are present and amplified (fig. 1C). The fluorescence intensity of these probes increases substantially upon binding since they are liberated from the quencher during elongation of a strand. The fluorescence increase compared to a fluorescent baseline derived from a non-template control (NTC) can be detected with a fluorescence plate reader.

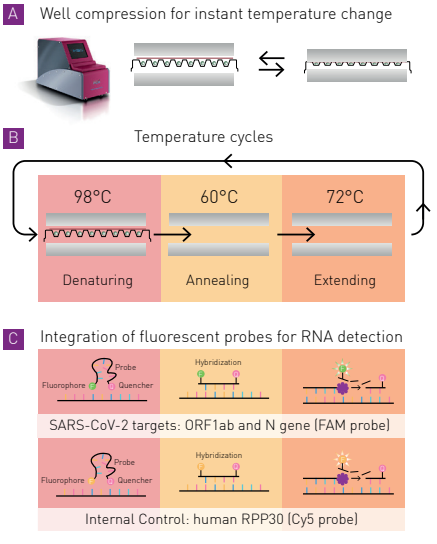

Fig. 1: Assay principle of the NextGenPCR with fluorometric readout.

Materials & Methods

- NextGenPCR SARS-CoV-2 RT-PCR Reagent Kit (#50007, MBS)
- Liquid Amies Transport Medium (#52000, MBS)
- SARS-CoV-2 synthetic RNA Control #2 (#102024, Twist BioScience)
- NextGenPCR White Barcoded Microplates 96 wells, 20µL (#32613, MBS)
- NextGenPCR Semiautomatic Heat Sealer 220v (#10102, MBS)
- NextGenPCR Machine (#10001, MBS)
- FLUOstar Omega (BMG LABTECH)

Experimental procedure

Saliva samples of 4 individuals were pooled and mixed 2:1 or 1:1 with Liquid Amies Transport Medium. Samples were heat-inactivated for 10 min at 98°C. A dilution of synthetic RNA was added to these saliva samples to obtain final concentrations ranging from 1-100,000 copies/NextGenPCR reaction. In parallel, the same dilution was prepared in nuclease-free water instead of saliva.

NextGenPCR reactions (20 µL) contained RT-PCR chemistry, primers and probes, nuclease-free water, and the respective RNA dilution standard. Reactions were loaded in microplates, sealed, and transferred to the

Rev. 04/2022
Keywords: nucleic acid, PCR, polymerase, RNA, viruses

NextGenPCR machine for the PCR protocol (1 cycle for 300 sec at 55°C followed by 60 sec at 98°C followed by 5 cycles at 98°C for 10 sec, 60°C for 20 sec, 72°C for 3 sec, followed by a final 45 cycles at 98°C for 5 sec, 60°C for 12 sec, 72°C for 3 sec). Following the amplification in the NextGenPCR, microplates were read on a FLUOstar Omega plate reader.

Instrument settings

Optic settings	Fluorescence, endpoint, multichromatic	
	Filter settings GFP	Ex 485-12 Em 520-10
	Filter settings Cy5	Ex 640-10 Em 680-10
Well scan	Orbital averaging	Diameter 4 mm
General settings	Number of flashes	10
	Settling time	0 sec

Results & Discussion

Detection of SARS-CoV-2 RNA

After the NextGenPCR, the fluorescence intensities of the RNA dilution series in the different diluents (saliva vs. nuclease-free water) were detected on the FLUOstar Omega and compared to negative controls without synthetic RNA (fig. 2). Except for the lowest standard (1 copy/reaction) all standards of the dilution series were clearly distinguishable from the negative control (purple line). The introduction of human saliva only led to a minor reduction of 7.1-12.5% in fluorescence intensity compared to the samples based on nuclease-free water for higher concentrations of 100-100,000 copies per reaction. Moreover, the NextGenPCR showed a comparable lower limit of detection starting at 10 copies/reaction, independent of the presence of saliva. The successful detection of RNA in clinically relevant conditions including human saliva, confirms the suitability of the amplification of SARS-CoV-2 samples using NextGenPCR, followed by the detection in the FLUOstar Omega.

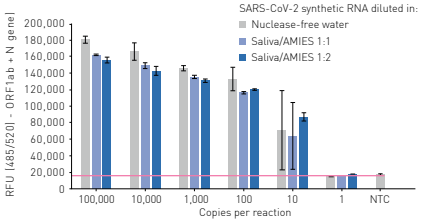

Fig. 2: Fluorescence-based detection of SARS-CoV-2 gene (ORF1ab and N gene) amplification products after NextGenPCR using samples with decreasing concentrations of synthetic SARS-CoV-2 RNA, NTC = non-template control.

Detection of human RPP30 as internal control

Next to the successful detection of reactions containing SARS-CoV-2 RNA with NextGenPCR, human RPP30 was detected in all samples containing human saliva but not those based on nuclease-free water. This additionally confirms the suitability of the attempt using NextGenPCR and a fluorescence-based readout on the FLUOstar Omega to evaluate sampling procedure. In routine use, the monitoring of the internal control and the NTC also provides the opportunity to interpret and reduce false positive or false negative test results.

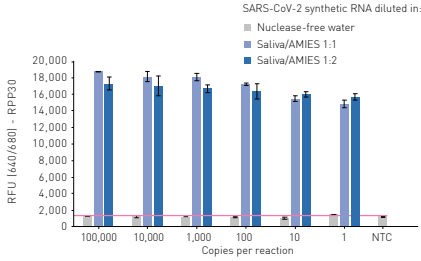

Fig. 3: Fluorescence-based detection of human RPP30 amplification products after NextGenPCR using samples with decreasing concentrations of synthetic SARS-CoV-2 RNA, NTC = non-template control.

With BMG LABTECH's MARS analysis software, validation templates can be set up which allow to obtain an interpretation of the NextGenPCR results with one click.

Conclusion

NextGenPCR combined with a fluorescent readout on the FLUOstar Omega was successfully employed for the detection of SARS-CoV-2 RNA, even in clinically relevant conditions based on saliva samples. The extreme acceleration of the PCR procedure achieved by using NextGenPCR, without the need for preceding extraction steps, enables a significant throughput increase in SARS-CoV-2 testing procedures. The user-friendly options in the MARS analysis software allow easy and fast data evaluation even by untrained personnel.

References

1. Buller, R.S. Molecular detection of respiratory viruses. Clin Lab Med 33, 439-60, doi:10.1016/j.cll.2013.03.007 (2013).
2. Struijk, R.B. et al. Ultrafast RNA extraction-free SARS-CoV-2 detection by direct RT-PCR using a rapid thermal cycling approach. medRxiv preprint doi:10.1101/2021.11.09.21265517 (2021).