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LATE-PCR for LoC Molecular Diagnostics Devices and Its Application to the Sensitive Detection of SARS-CoV-2 †

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Abstract: The emergence of the novel coronavirus, SARS-CoV-2, has highlighted the need for rapid, accurate, and point-of-care diagnostic testing. Lab-on-a-Chip (LoC) devices offer the possibility to run such tests at a low cost, while at the same time permitting the multiplexed detection of several viruses when coupled with microarray detection of the amplified products. Herein, we report the development of a protocol for the qualitative detection of SARS-CoV-2, through the design of appropriate primers that target the evolutionary conserved regions of the virus. The proposed protocol relies on an improved version of asymmetric RT-PCR, the linear-after-the-exponential (LATE)-PCR that uses primers that are deliberately designed for use at unequal concentrations. As a result, LATE-PCR exhibits similar efficiency to symmetric PCR, while promoting accumulation of single-stranded products that can subsequently hybridize to a single-strand DNA probe-spotted microarray. The performance of the developed LATE-PCR protocol was compared to that of symmetric RT-PCR, and validated with the use of artificial viral RNA and nasopharyngeal swab samples from real patients. Furthermore, and in order to illustrate its potential for integration into a biosensor platform, the amplicons were allowed to hybridize with probes that were covalently immobilized onto commercially available functionalized glass, without the need for heat denaturation.

Keywords: SARS-CoV-2; asymmetric PCR; LATE PCR; microarray detection; molecular diagnostics

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1. Introduction

The current pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has stressed the need for testing on a massive scale. Towards this goal, numerous efforts have been made to fabricate reliable and cost-efficient screening devices that can be deployed at the point-of-need [1], such as immunoassays (rapid-tests, ELISA, etc.), but also molecular-based methods that are primarily based on isothermal amplification as a means to substitute the "golden standard" diagnostic procedure, qPCR, since the latter are much more easily incorporated into portable devices. Nevertheless, all of these approaches suffer from low sensitivity, repeatability, and high operation cost, due to the use of highly specialized reagents (LAMP, RPA). Especially in the case of molecular diagnostics, primers for loop-mediated isothermal amplification (LAMP) are hard to be designed [2], while multiplexed analysis [3] in a single chamber is impossible, thus increasing the footprint of the portable device. Recombinant polymerase amplification, on the other hand, is very susceptible to sequence mismatches [4], making this approach inappropriate for viral genetic material detection.

In an attempt, therefore, to develop an amplification protocol for SARS-CoV-2 that is widely applicable, can be easily multiplexed, and whose integration into a portable LoC is straight-forward, we focused on an improved version of asymmetric RT-PCR called linear-after-the-exponential (LATE)-PCR. LATE-PCR overcomes the limitations of traditional asymmetric PCR that uses conventional PCR primers at unequal concentrations to generate ssDNA, but is inefficient, and tends to promote nonspecific amplification [5]. Herein, we

demonstrate that rational primer design for the LATE-PCR amplification of the IP2 gene of SARS-CoV-2 maximized the yield of specific single-stranded DNA products, which was validated by means of the hybridization of the latter with immobilized complementary probes at room temperature, without any further dsDNA denaturation or degradation steps required.

2. Materials and Methods

Samples were collected from patients in primary health care centers in Athens. In addition, artificial RNA control for SARS-CoV-2 was purchased from AMPLIRUN®. The RNA extraction procedure was carried out by automated extractors provided by TANbead (Smart LabAssist 32 (SLA-32)). The extracted RNA was diluted in Tris-HCl pH = 8 and stored at −80 °C until use. The concentration of extracted RNAs was measured at 14.2 ng/μL.

Primer design for the LATE protocol was based on those suggested by the Pasteur Institute (Paris, France) while base additions were made in order to account for the different ratios of the excess and limiting primers (1 up to 4 bases added). The amplification run consisted of 50 min at 55 °C in order to complete the reverse transcription, followed by 3 min at 95 °C as a denaturation step and 45 cycles at 95 °C for 12 s followed by amplification at 58 °C for 30 s. For the standard symmetrical PCR protocol, 0.4 μM of each primer was used. For the LATE protocol, the forward primer (limiting) was used in a concentration of 0.5 μM, whereas the reverse primer (excess) at 1 μM. In both protocols an FAM-labeled molecular probe (0.2 μM in concentration) was used.

For the array-based detection of the amplified product, amino-modified probes complementary to the generated ssDNA fragment were designed and immobilized onto commercially available 2D carboxy-terminated glass slides (PolyAn) following activation EDC-NHS (100 mM/25 mM NHS in MES 0.1 M, pH 5.5). Fluorescent detection of the hybridized product was achieved through tagging of the reverse primer with Cy5 at the 5′ end. The amplified and fluorescently tagged product was allowed to hybridize with the probe-modified surfaces without any prior treatment or dilution for 30 min and was subsequently washed with 1TBST.

3. Results

The results obtained for the amplification of both artificial viral RNA as well as RNA extracted from positive human samples, clearly demonstrate the increased efficiency achieved with the modified primers, as indicated by the significantly lower Cp values (Tables 1 and 2). Furthermore, the addition of two bases to the limiting primer resulted in the lowest Cp value for both types of samples. Most interestingly, the Cp value achieved with the primer that was modified with an extra two bases was only marginally higher than the one achieved with the standard symmetric protocol in the case of artificial RNA, and even lower than that for the RNA extracted from a human sample. What is more, the amplification curves obtained (Figures 1 and 2) illustrate the differences in fluorescence intensity between the symmetric protocol and the asymmetric ones, as for the former they exponentially increase over time, indicating the generation of dsDNA, whereas for the latter they level off when amplification enters the linear phase, which illustrates the accumulation of ssDNA.

The amplified products were subsequently allowed to hybridize at room temperature, with probes that were complementary to the generated ssDNA that were immobilized onto commercially available functionalized glass slides. The results in Figure 3 further validate the efficiency of LATE PCR in accumulating ssDNA versus the symmetric PCR, where no fluorescence signal was recorded due to the presence of dsDNA only.

Table 1. Detection performance comparison of different protocols for RdRp-IP2 gene with 1000 copies SARS-CoV-2 artificial RNA as template.

Protocol	Cp	Standard Deviation
Standard	33.80	0.13
Asymmetric	37.02	0.02
LATE + 1 base added	34.58	0.26
LATE + 2 bases added	32.46	0.12
LATE + 3 bases added	33.16	0.07
LATE + 4 bases added	32.79	0.09

Table 2. Detection performance comparison of different protocols for RdRp-IP2 gene with SARS-CoV-2-positive human sample.

Protocol	Cp	Standard Deviation
Standard	23.00	0.17
Asymmetric	24.23	0.09
LATE + 1 base added	22.88	0.20
LATE + 2 bases added	20.96	0.15
LATE + 3 bases added	22.09	0.16
LATE + 4 bases added	21.55	0.11

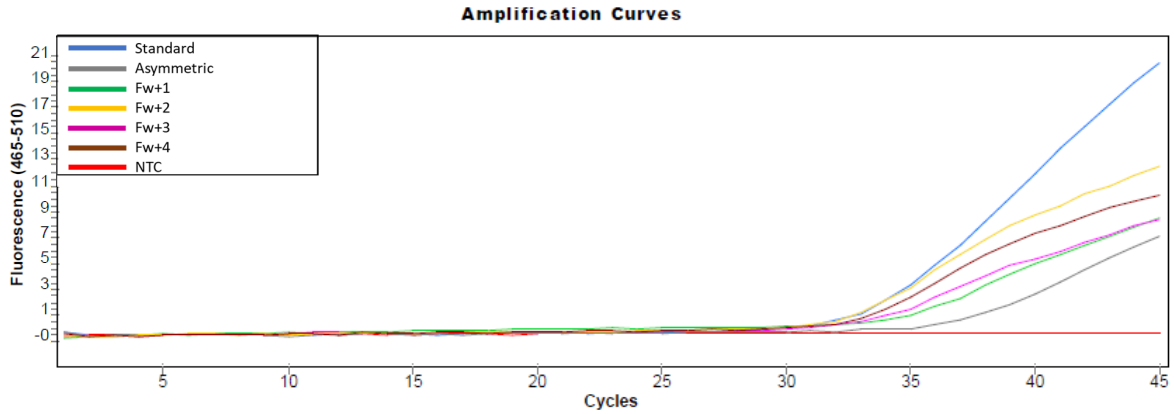


Figure 1. Real-time PCR amplification curves using 1000 copies of artificial RNA as a template for RdRp-IP2 gene.

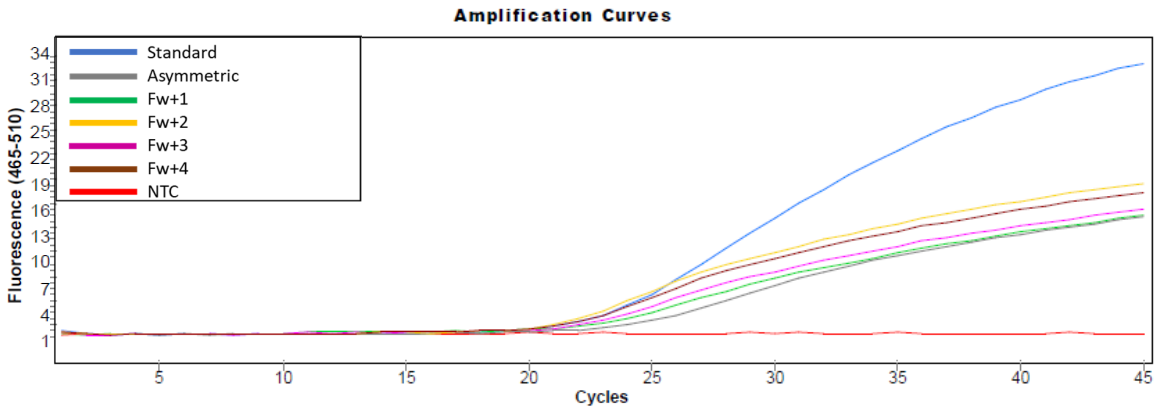
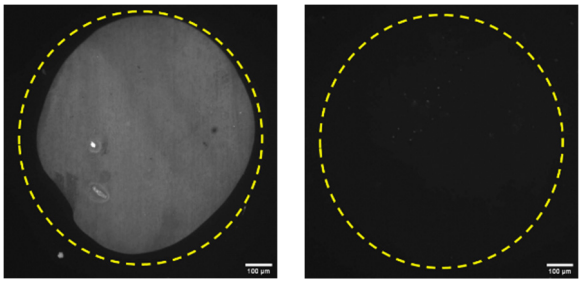


Figure 2. Real-time PCR amplification curves using human positive sample as a template for RdRp-IP2 gene.



(**a**) (**b**)

Figure 3. Fluorescence intensity recorded from carboxy-modified glass slides where probes complementary to the amplified product have been immobilized upon their incubation with (**a**) Cy5-labeled LATE-PCR product and (**b**) Cy5-labeled product generated following conventional symmetric PCR.

4. Conclusions

LATE-PCR effectively eliminates the need for multiple rounds of amplification or laborious steps for single-stranded DNA generation, by making the need for mechanical or enzymatic separation and purification of single-stranded DNA obsolete, or the use of a separate linear amplification step. LATE-PCR is, therefore, ideally suited as an amplification technique for point-of-need LoC devices, especially when coupled with DNA microarray detection. In this way, not only do the individual components that are required for LoC fabrication get simplified, but also multiplexing at a very small footprint can be achieved.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Biomedical Research Foundation of the Academy of Athens (in accordance to the National Public Investment Program of the Ministry of Development and Investment/General Secretariat for Research and Technology, in the framework of the Flagship Initiative to address SARS-CoV-2 (2020ΣΕ01300001)).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflict of interest.

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