PCR Multiplexing Based on a Single Fluorescent Channel Using Dynamic Melting Curve Analysis

Haoqing Zhang, Martina Gaňová, ZhiQiang Yan, Honglong Chang, and Pavel Neužil*

ABSTRACT: Since its invention in 1986, the polymerase chain reaction (PCR), has become a well-established method for the detection and amplification of deoxyribonucleic acid (DNA) with a specific sequence. Incorporating fluorescent probes, known as TaqMan probes, or DNA intercalating dyes, such as SYBR Green, into the PCR mixture allows real-time monitoring of the reaction progress and extraction of quantitative information. Previously reported real-time PCR product detection using intercalating dyes required melting curve analysis (MCA) to be performed following thermal cycling. Here, we propose a technique to perform dynamic MCA during each thermal cycle, based on a continuous fluorescence monitoring method, providing qualitative and quantitative sample information. We applied the proposed method in multiplexing detection of hepatitis B virus DNA and complementary DNA of human immunodeficiency virus as well as glyceraldehyde 3-phosphate dehydrogenase in different concentration ratios. We extracted the DNA melting curve and its derivative from each PCR cycle during the transition from the elongation to the denaturation temperature with a set heating rate of 0.8 K·s−1 and then used the data to construct individual PCR amplification curves for each gene to determine the initial concentration of DNA in the sample. Our proposed method allows researchers to look inside the PCR in each thermal cycle, determining the PCR product specificity in real time instead of waiting until the end of the PCR. Additionally, the slow transition rate from elongation to denaturation provides a dynamic multiplexing assay, allowing the detection of at least three genes in real time.

INTRODUCTION

Patients with an acquired immunodeficiency syndrome are infected by human immunodeficiency virus (HIV), thus weakening the human immune system in its fight against other diseases such as hepatitis B and tuberculosis. In HIV-infected patients, HIV exacerbates the symptoms of hepatitis B virus (HBV) infection and accelerates the progression of the liver disease leading to cirrhosis as well as hepatocellular carcinoma. Disease progression to cirrhosis in HIV-positive patients is almost 3 times faster than in HIV-negative patients, and the interaction of HIV and HBV remains the leading cause of death.1−3 Growing globalization and human migration have accelerated the spread of these diseases and no country has been immune from them.4−6 Therefore, to lower death rates, it is essential to have early diagnosis and treatment of these diseases.

The polymerase chain reaction (PCR) was invented in 1986 to detect the presence of specific deoxyribonucleic acid (DNA)7 and, subsequently, by adding a reverse transcription step (RT-PCR) to detect ribonucleic acid (RNA).8 In the next three and a half decades, the PCR was developed9 into a method of choice to detect DNA or RNA, and very recently, the RT-PCR became an accepted method for COVID-19 diagnoses.10 Researchers detected the PCR product (amplicon) using electrophoresis, either with gel11 or a capillary.12 The location of the DNA bands is compared with a standard DNA ladder to identify the number of base pairs (bp) in the sample. A later modification to the method, real-time/quantitative PCR (qPCR), enabled the PCR progress to be monitored and the number of DNA copies in the original sample to be determined, using either nonspecific intercalating dyes such as SYBR Green13 or specific fluorescent probes such as TaqMan.14 Intercalating dyes exhibit fluorescence (F) only in the presence of double-stranded DNA (dsDNA). Once the PCR is completed, the amplicon is gradually heated from a temperature (T) of ≈72 to 95 °C. There is a sudden drop in the F amplitude at the melting temperature (Tm) as each dsDNA molecule melts into two single-stranded DNA (ssDNA) molecules and intercalating dye loses its ability to produce fluorescence called melting curve analysis (MCA).15 This method is widely used to determine the specificity of the
amplicon to perform genotyping and to detect single-nucleotide polymorphism (SNP) by high-resolution MCA.

qPCR multiplexing was subsequently developed to detect two or more specific nucleic acids in a single reaction primarily using TaqMan probes specific to each amplicon and having different color fluorophores; it has been used to detect the presence of viruses and pathogens for species authentication, and for food safety. This method requires hardware with multiple optical fluorescence channels and wide optical spectrum photodetectors using either photodiodes or photomultiplier tubes (PMTs) or a single-channel system utilizing a spectrum analyzer. A single fluorescent channel with a PMT has been used with a combination of a 6-carboxyfluorescein (FAM) probe and an intercalating dye to extract the F amplitude in each PCR cycle, twice, before and after DNA denaturation, resulting in two PCR amplification curves, effectively doubling the PCR throughput.

Intercalating dye-based end-point PCR multiplexing has also been shown to detect specific serotypes of Vibrio cholerae or dengue fever viruses. Alternatively, utilization of the continuous fluorescence monitoring (CFM) method to capture hundreds of data points in each cycle allows the observation of PCR progress, including reaction kinetics, while providing both F and T as functions of time (t). Eliminating t during the transition from elongation to denaturation during PCR gives F as a function of T, i.e., MCA. This method was utilized to multiplex hemagglutinin and neuraminidase genes in avian influenza RNA virus, as well as to determine the original number of copies, using the intercalating EvaGreen dye with an assumption that the T_M difference between two amplicons was at least 5 °C. Unfortunately, the experimental data and the results of the multiplex qPCR processing method could not clearly differentiate peaks due to the high scanning rate of 20 K·s⁻¹ or more. The same technique of PCR multiplexing was used later for digital PCR. Recently, a new method for multiplexing using intercalating dyes was proposed based on multidimensional standard curves. The data obtained by this novel method were achieved by commercial qPCR instruments, thus extending the use of these devices.

In this paper, we propose a PCR multiplexing method based on data extracted from MCA performed during each thermal cycle. The PCR uses the CFM method with one, two, and three genes in different volume ratios, demonstrating that 2 °C difference in T_M of amplicons is sufficient to subsequently demultiplex quantitative data for individual genes. Our proposed method allows researchers to detect multiple genes in real time and to decide whether to stop or optimize the experiment based on the real-time results. As a result, the process can be shortened and become more efficient. Additionally, this method can also be applied to detect SNP.

### RESULTS AND DISCUSSION

#### Principle of the Method.

The PCR master mix of HIV, HBV, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in different volume ratios was prepared and initially verified using a commercial PCR cycler to identify the respective C_T values and T_Mi, T_M for HIV, HBV, and GAPDH was ≈83.0, 87.5, and 79.0 °C (Section S1 in the Supporting Information), respectively. The same PCR protocol was then performed in a droplet on the TEC with a set temperature ramping rate of 0.8 K·s⁻¹ from elongation to denaturation, and the T and F signals were recorded for further processing (Figure 1).
At that point, we chose to perform the PCR experiment on the HIV gene only, as an example of data extraction. We performed the PCR protocol based on a CFM method to monitor \( F \) (Figure 1A) as a function of \( t \), concurrently recording the heater temperature \( (T) \) (Figure 1A). Then, we converted the \( T \) value into sample temperature \( (T_s) \) with a MATLAB script using the differential equation

\[
dT_s = 1/r \cdot (dT - T_s)
\]  

(1)

where \( r \) is an experimentally determined system time constant of \( \approx 1.4 \) s. The fluorescence signal \( F(t) \) and temperature \( T(t) \) were split in successive PCR cycles and plotted as a function of \( t \) with the PCR cycle number \((N)\) as a parameter (Figure 1B). Then, we performed MCA by eliminating \( t \) from \( F(t) \) and \( T_s(t) \), giving \( F \) and its derivative \(-dF/dT_s \) as functions of \( T_s \) (Figure 1C). Finally, we extracted the amplification curve (black squares in Figure 1D) from the PCR (Figure 1A) using the conventional approach of registering the \( F \) amplitude at the end of the extension phase in each PCR amplification cycle. We plotted the peak value of \(-dF/dT_s \) as a function of \( N \) (Figure 1D). The critical threshold \( (C_T) \) was determined by the following way. The amplification curve was first fitted using nonlinear curve fitting and the function

\[
Y = a + b \cdot N^c
\]

(2)

where \( Y \) is the extracted signal data, either \( F \) or \(-dF/dT_s \) and \( a, b, \) and \( c \) are fitting parameters. Then, we determined the \( C_T \) values by solving eq 3 using the formula

\[
C_T = 10^{\frac{\log Y_T - a}{b}}
\]

(3)

where \( Y_T \) is the \( F \) value at the set value of \( C_T \) defined as 10% of the signal increase, either \( F \) or \(-dF/dT_s \). Here, the \( C_T \) values determined from the PCR curve and MCA were \( \approx 16.41 \) and \( 16.29 \) cycles, respectively, differing only by \( \approx 0.12 \) cycle, suggesting that these two methods of \( C_T \) extraction are equivalent.

PCR Multiplexing. We conducted the PCR of HIV, GAPDH, and HBV genes individually and in combination to demonstrate the multiplexing capability of the method. We chose a combination of HIV with GAPDH in three different ratios; for the combination of all three, the GAPDH content was again kept constant while the other two were varied (Table 1). Details of all processed data are in the Supporting Information Section S2–S9.

Here, we only show the results for genes with a volume ratio of 5:5:5 to demonstrate the multiplexing method. First, we performed the PCR with scan rate \( (\nu) \) set to 0.8 K s\(^{-1}\) while recording both \( T \) and \( F \) as a function of \( t \), followed by MCA with \( \nu \) set to 0.1 K s\(^{-1}\) (Figure 2A). The data were recorded by an oscilloscope and then registered and processed with a MATLAB script, as described in the section Principle of the method. The \( F \) and \( T \) data from all cycles were split into individual cycles and plotted as a function of \( t \) (Figure 2B).

Then, \( t \) was eliminated and we plotted \( F \) and \(-dF/dT_s \) as a function of \( T_s \) including the static MCA extracted directly from Figure 2A (Figure 2C). There are three peaks corresponding to HIV, GAPDH, and HBV amplicons with respective \( T_M \) values compared to those extracted from static MCA. The difference between \( T_M \) of GAPDH and HIV is only \( \approx 2.72 ^\circ \)C, and the results are clearly differentiated showing that their \( T_M \) difference could perhaps be significantly smaller. Then, we plotted a composite PCR amplification curve using the \( F \) values at the end of the elongation steps (Figure 2A) and individual PCR amplification curves (Figure 2D) as peak values of respective genes from \(-dF/dT_s \) in Figure 2C. Finally, we calculated the \( C_T \) values by the method described above.

The experiments show that the different DNA molecules during multiplex PCRs influenced each other as they competed for the same limited pool of component supplies, especially enzymes and nucleotides. The outcome was that the more efficiently amplified gene negatively affected the yield of other amplicons, changing their PCR amplification efficiency, but the extracted \( C_T \) values match the gene copy number in the sample (Table 1). The presented method of data processing dynamically provided qualitative and quantitative information about the target genes in the sample and was confirmed for every volume ratio.

### CONCLUSIONS

In this paper, we have proposed a method of quantitative PCR multiplexing based on performing melting curve analysis during each PCR cycle by controlling the ramping rate in the transition phase from elongation to denaturation. We used a single fluorescent channel with an optical wide-band detector, an intercalating dye, and different values of \( T_M \) for individual amplicons to verify the proposed method. We demonstrated the capability of this method by multiplexing up to three genes. Then, we converted the heater temperature to the sample temperature and extracted DNA melting curves from each PCR cycle. We plotted the peak values of \(-dF/dT_s \) for each extracted DNA melting curve, constructing a PCR amplification curve for each gene to determine the original DNA concentration in the sample.

The proposed methodology offers the chance for users to look inside the PCR within each cycle, determining the PCR product specificity in real time instead of waiting until the end of the PCR. Additionally, the lower transition rate set to 0.8 K s\(^{-1}\) from elongation to denaturation provides data used for multiplexing assay, which could also be used in SNP and genotyping.

We envisage that these advantages of simple single-fluorescent channel multiplexing will inspire developers of new qPCR systems to enhance their hardware and software capability to enable it. It would be a rather simple task for newly developed portable devices for rapid detection of infectious diseases outside of laboratories, in the field, and at the point of care. These new multiplexed systems could also be
connected to a global health system as part of the Internet of Things.35

■ MATERIALS AND METHODS

Experimental Setup. PCR was performed in droplets with a volume of \( \approx 0.3 \mu L \) consisting of a master mix with dsDNA templates and relevant primers covered with mineral oil with a volume of \( \approx 2.0 \mu L \), forming a virtual reaction chamber (VRC) (Figure 3A). Both droplets were placed on a microscope coverslip with a size of \( \approx (10 \times 10) \) mm\(^2\) coated with 1H,1H,2H,2H-perfluorodecytriethoxy silane,36 resulting in a surface water contact angle of \( \approx 108^\circ \). This glass and a miniaturized resistance temperature detector of type Pt100 were placed next to each other on a thermoelectric cooler (TEC) with a size of \( \approx (12 \times 12) \) mm\(^2\). The TEC was connected into an H-bridge and its top-plate temperature was controlled by both pulse-width modulation and the direction of flow of an electric current \( (I) \) through the TEC. We employed a closed feedback loop system with a proportional integrative derivative mode of operation controlled from a personal computer via a universal serial bus interface. Glass with the VRC was positioned under an objective lens with a magnification of 5X mounted in a fluorescent optical microscope (Figure 3B). The microscope was equipped with a fluorescein isothiocyanate filter set (Filter) and a light source consisting of a light-emitting diode (LED) with a principal

Figure 2. Dynamic MCA results from amplification of three genes. (A) Continuous fluorescence intensity from melting curve analysis (green) and heater temperature (red) from 40 PCR cycles. (B) Extracted data of sample temperature and fluorescence signal change from each cycle during the transition from the annealing to the denaturation temperature with a scan rate of \( \nu \) set to 0.8 K s\(^{-1}\) as a function of time. (C) Split MCA curves \( F(T_s) \) and its derivative \( -dF/dT_s(T_s) \) of each cycle as a function of sample temperature. The blue curves represent the result of static MCA recorded at the end of PCR amplification, and the static MCA reproduces the shape of the dynamic MCA. (D) Demultiplexed quantitative data represented by three amplification curves extracted from the peak values correspond to the three individual genes (red for GAPDH, green for HIV, and blue for HBV). The fourth amplification curve (black) is extracted from the PCR amplification (from panel A). The blue line is a baseline to extract a threshold cycle for every gene contained in the sample.

Figure 3. (A) Photograph of a VRC on a TEC with a droplet of fluorescein covered with mineral oil. (B) Schematic representation of the LED illuminating through a microscope objective lens the VRC. (C) Diagram of the testing setup.
The fluorescence emitted from the VRC during PCR was captured by a PMT attached to the microscope c-mount, and the signal was then processed by a lock-in amplifier with a set sensitivity of 1 V and a time constant of 50 ms (Figure 3C). Both T and F values were continuously monitored by an oscilloscope with a data sampling frequency of 20 Hz.

**Primers and Genes.** Patients with HIV have a greater risk of being coinfected by other diseases, such as HBV, that share similar transmission modes. GAPDH, a well-known reference gene, is typically expressed stably in cells of interest. As a result, we chose HIV, HBV, and GAPDH as the three genes for the experiments. The primers for their PCR amplification were designed for convered sequences of each gene to have different melting temperatures. The ramping rate in each thermal cycle depending on the system. Of particular note is the temperature transition from elongation to denaturation, which is typically 100 times higher than it is in static MCA, resulting in a discrepancy between the sample and heater temperature; this was confirmed by measuring a quasi-static MCA with different \( \nu \) values. Therefore, we established the optimized \( \nu \) value of 0.8 K s\(^{-1}\) as the ramping rate from elongation to denaturation for our PCR multiplexing to distinguish the three individual genes in real time.

**PCR Master Mix and the Protocol.** The Taq polymerase PCR master mix consisted of \( \approx 0.3 \) \( \mu \)L Taq polymerase with a content of 5 U \( \mu \)L\(^{-1}\), 1 \( \mu \)L of PCR buffer, 0.8 \( \mu \)L of deoxyribonucleoside triphosphate, 0.5 \( \mu \)L of 20X EvaGreen, and 1 \( \mu \)L of 5 mg mL\(^{-1}\) bovine serum albumin. We used synthesized DNA of HIV, HBV, and GAPDH as templates and the corresponding forward and reverse primers at a final concentration of 0.4 \( \mu \)M. The final volume of the PCR master mix was adjusted to 10 \( \mu \)L by adding sterilized H\(_2\)O. The initial contents of HIV, HBV, and GAPDH were \( \approx 2.04 \times 10^7 \) copies \( \mu \)L\(^{-1}\), \( \approx 2.72 \times 10^7 \) copies \( \mu \)L\(^{-1}\), and \( \approx 8.42 \times 10^7 \) copies \( \mu \)L\(^{-1}\), respectively. We prepared the PCR master mix for each gene and then mixed it in different ratios (Table 2).

![Figure 4](https://pubs.acs.org/journal/acsodf)

**Figure 4.** (A) MCA performed at different set values of \( \nu \) from 0.1 up to 60 K s\(^{-1}\) (B) and its negative derivation. With \( \nu \) greater than 2 K s\(^{-1}\), the GAPDH peak is no longer recognizable and with \( \nu \) above 4 K s\(^{-1}\), the HIV peak disappears. With \( \nu \) above 7 K s\(^{-1}\), the MCA is incomplete; thus, we show here only MCAs with a value of \( \nu \) up to 6 K s\(^{-1}\).

| exp order | HIV (copies/\( \mu \)L\(^{-1}\)) | HBV (copies/\( \mu \)L\(^{-1}\)) | GAPDH (copies/\( \mu \)L\(^{-1}\)) | volume ratio |
|-----------|-------------------------------|-------------------------------|-------------------------------|--------------|
| 1         | 2.04 \times 10^7               | 0                             | 0                             | 10:0:0       |
| 2         | 0                             | 2.72 \times 10^7               | 0                             | 0:10:0       |
| 3         | 0                             | 0                             | 8.42 \times 10^3               | 0:0:10       |
| 4         | 1.02 \times 10^7               | 0                             | 4.2 \times 10^3                | 1:0:1        |
| 5         | 6.8 \times 10^6                | 0                             | 5.6 \times 10^3                | 1:0:2        |
| 6         | 1.4 \times 10^7                | 0                             | 2.8 \times 10^3                | 2:0:1        |
| 7         | 6.8 \times 10^6                | 9.1 \times 10^6                | 2.8 \times 10^3                | 5:5:5        |
| 8         | 7.3 \times 10^6                | 7.8 \times 10^6                | 3.0 \times 10^3                | 5:4:5        |
| 9         | 8.1 \times 10^6                | 5.4 \times 10^6                | 3.4 \times 10^3                | 5:2:5:5      |
| 10        | 5.1 \times 10^6                | 6.8 \times 10^6                | 4.2 \times 10^3                | 2.5:2.5:5:5  |

The PCR was performed using a protocol with the following set values: a hot start for 20 s at 95 °C followed by 40 cycles of three-step PCR amplification consisting of denaturation for 4 s at 95 °C, annealing for 30 s at 56 °C, and elongation for 10 s at 72 °C, followed by MCA from 75 to 95 °C with a \( \nu \) value depending on the system.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04766.

Designed primers for each gene with different \( T_M \) the extracted results from PCR curve of GAPDH; the
extracted results from PCR curve of HBV; the extracted results from PCR curve of HIV and GAPDH in a volume ratio of 1:1; the extracted results from PCR curve of HIV and GAPDH in a volume ratio of 1:2; the extracted results from PCR curve of HIV, HBV, and GAPDH in a volume ratio of 5:4:5; the extracted results from PCR curve of HIV, HBV, and GAPDH in a volume ratio of 5:2:5:5; the extracted results from PCR curve of HIV, HBV, and GAPDH in a volume ratio of 2.5:2.5:5; and \( \Sigma M \) of three genes from MCAs with different set \( \nu \) values (PDF).

## AUTHOR INFORMATION

**Corresponding Author**

Pavel Neuzil — Ministry of Education Key Laboratory of Micro/Nano Systems for Aerospace, Department of Microsystem Engineering, School of Mechanical Engineering, Northwestern Polytechnical University, Xi’an, Shaanxi 710072, P. R. China; Central European Institute of Technology and Department of Microelectronics, Faculty of Electrical Engineering and Communication, Brno University of Technology, 612 00 Brno, Czech Republic; orcid.org/0000-0001-9040-281X; Email: pavel.neuzil@nwpu.edu.cn

**Authors**

Haqing Zhang — Ministry of Education Key Laboratory of Micro/Nano Systems for Aerospace, Department of Microsystem Engineering, School of Mechanical Engineering, Northwestern Polytechnical University, Xi’an, Shaanxi 710072, P. R. China

Martina Gaňová — Ministry of Education Key Laboratory of Micro/Nano Systems for Aerospace, Department of Microsystem Engineering, School of Mechanical Engineering, Northwestern Polytechnical University, Xi’an, Shaanxi 710072, P. R. China; Central European Institute of Technology, Brno University of Technology, 612 00 Brno, Czech Republic

ZhiQiang Yan — Ministry of Education Key Laboratory of Micro/Nano Systems for Aerospace, Department of Microsystem Engineering, School of Mechanical Engineering, Northwestern Polytechnical University, Xi’an, Shaanxi 710072, P. R. China

Honglong Chang — Ministry of Education Key Laboratory of Micro/Nano Systems for Aerospace, Department of Microsystem Engineering, School of Mechanical Engineering, Northwestern Polytechnical University, Xi’an, Shaanxi 710072, P. R. China; orcid.org/0000-0003-0400-3658

Complete contact information is available at: https://pubs.acs.org/doi/10.1021/acsomega.0c04766

**Author Contributions**

**H.Z.** and **M.G.** are considered as first authors.

**Notes**

The authors declare no competing financial interest.

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