

# Determination of Advantages and Limitations of qPCR Duplexing in a Single Fluorescent Channel

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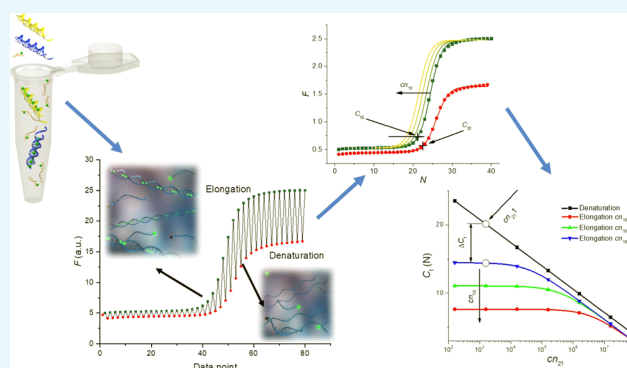


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Supporting Information

**ABSTRACT:** Real-time (quantitative) polymerase chain reaction (qPCR) has been widely applied in molecular diagnostics due to its immense sensitivity and specificity. qPCR multiplexing, based either on fluorescent probes or intercalating dyes, greatly expanded PCR capability due to the concurrent amplification of several deoxyribonucleic acid sequences. However, probe-based multiplexing requires multiple fluorescent channels, while intercalating dye-based multiplexing needs primers to be designed for amplicons having different melting temperatures. Here, we report a single fluorescent channel-based qPCR duplexing method on a model containing the sequence of chromosomes 21 (Chr21) and 18 (Chr18). We combined nonspecific intercalating dye EvaGreen with a 6-carboxyfluorescein (FAM) probe specific to either Chr21 or Chr18. The copy number ( $cn$ ) of the target linked to the FAM probe could be determined in the entire tested range from the denaturation curve, while the  $cn$  of the other one was determined from the difference between the denaturation and elongation curves. We recorded the amplitude of fluorescence at the end of denaturation and elongation steps, thus getting statistical data set to determine the limit of the proposed method in detail in terms of detectable concentration ratios of both targets. The proposed method eliminated the fluorescence overspilling that happened in probe-based qPCR multiplexing and determined the specificity of the PCR product via melting curve analysis. Additionally, we performed and verified our method using a commercial thermal cycler instead of a self-developed system, making it more generally applicable for researchers. This quantitative single-channel duplexing method is an economical substitute for a conventional rather expensive probe-based qPCR requiring different color probes and hardware capable of processing these fluorescent signals.



## 1. INTRODUCTION

Polymerase chain reaction (PCR) is a powerful technique to amplify a small number of specific sequences of deoxyribonucleic acid (DNA) molecules up to a detectable level.<sup>1</sup> Since its invention in 1986, variants of PCR-based methods have been developed,<sup>2</sup> such as standard PCR (end-point PCR),<sup>3</sup> real-time/quantitative PCR (qPCR),<sup>4</sup> and digital PCR (dPCR).<sup>5</sup> qPCR is capable of monitoring the PCR progress in real-time and determining the initial concentration of the target genes. It is currently a gold standard for nucleic acid (NA) detection, especially during the coronavirus disease 2019 (COVID-19) pandemic.<sup>6</sup> It is performed using either nonspecific intercalating fluorescent dyes, such as SYBR Green I or EvaGreen,<sup>7</sup> or specific oligonucleotides (probes) with fluorophores,<sup>8</sup> such as 6-carboxyfluorescein (FAM)<sup>9</sup> and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC).<sup>10</sup>

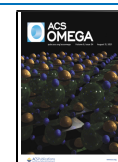
Multiplexing of any PCR expands its detection capability as more than one target gene is simultaneously amplified and detected.<sup>11–14</sup> qPCR multiplexing is often used for the detection of a DNA component with minor content with a

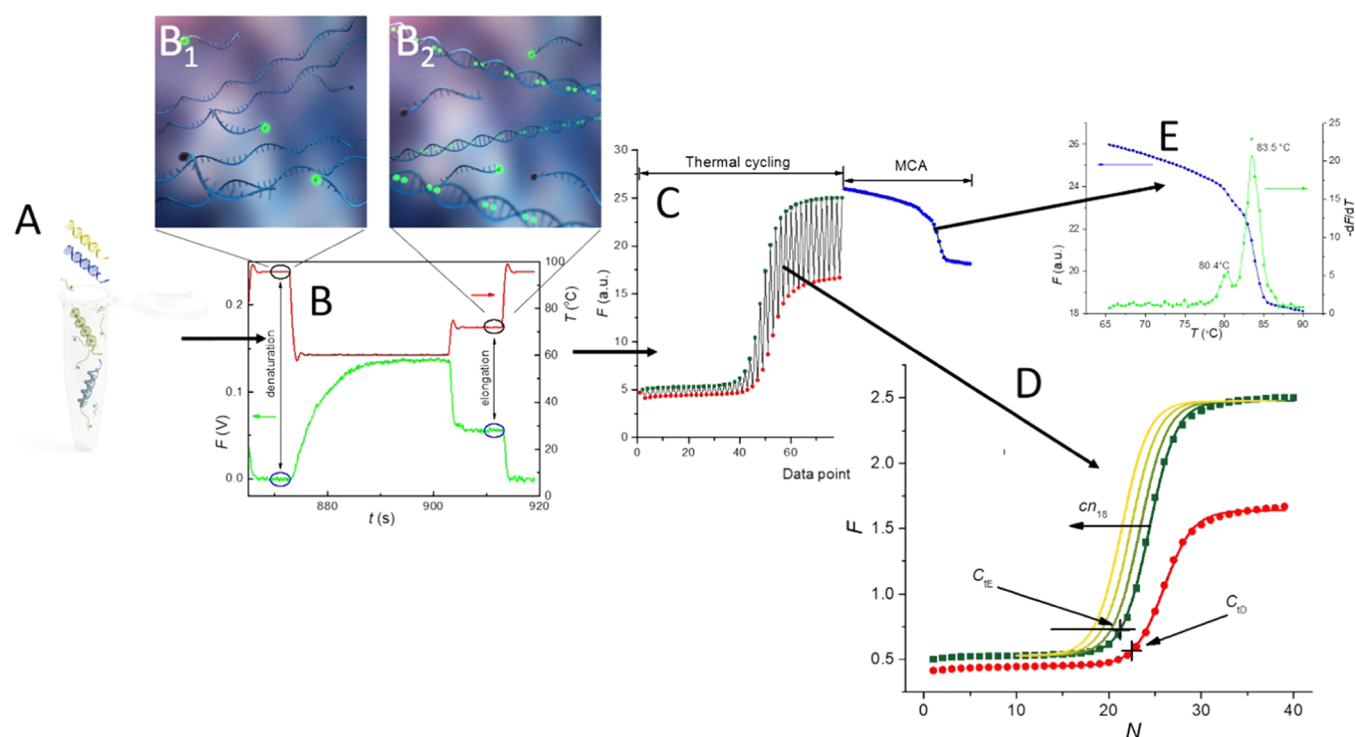
background of another more abundant DNA, such as gene mutations with wild types in oncological research and diagnostics.<sup>15,16</sup> The infectious agents in the background of human sequences are often determined in microbiology and virology.<sup>17</sup> The genes of genetically modified organisms are searched in food control.<sup>18</sup> Paternally inherited disease-associated sequences of DNA are investigated in the background of cell-free DNA in the plasma of pregnant females, and they are used in noninvasive prenatal diagnostics.<sup>19,20</sup> The correct quantification of both mixture components by qPCR is complicated once there are large differences in their representation in the mixture.

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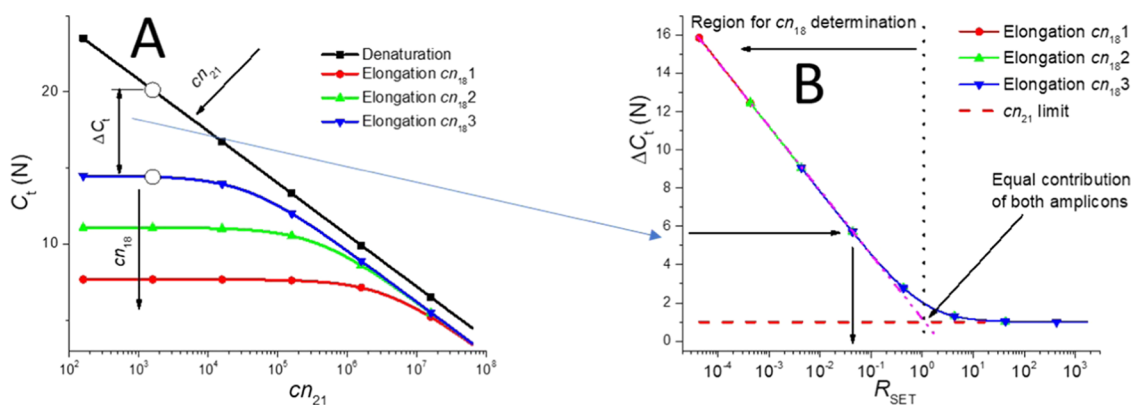
**Figure 1.** Principle of the proposed qPCR multiplexing method. (A) We prepared the PCR master mix containing two DNA templates, two sets of relevant forward and reverse primers, a FAM probe specific to one DNA template, and EvaGreen intercalating dye. (B) We performed the PCR and captured the  $F$  (green curve) during each cycle at the end of the denaturation and elongation steps, while the red curve represents the heater temperature. Contributions to the  $F$  at the denaturation step are only from the FAM probe (inset B1).  $F$  at the elongation step consists of both the FAM probe linked to Chr21 as well as the EvaGreen intercalating dye with both targets (inset B2). (C) All of the collected data during the PCR followed by the MCA were split (D) into two sets and plotted separately, which represents the denaturation amplification curve (red) and the elongation amplification curves in gradient colors from dark to light green for different  $C_{n_{18}}$ . (E) We also plotted an MCA as well as the negative derivation of  $F$  showing the presence of two different amplicons in the PCR master mix.

Probe-based qPCR multiplexing utilizes probes attached to fluorophores with different emission wavelengths. The fluorescent signals are then split and detected using different optical channels. The number of target genes for the concurrent detection is theoretically unlimited as long as there is no emission wavelength overlap between the different probes. However, it is limited by the optical detection system as the increasing number of detectable colors makes the system complicated and costly. Additionally, the design of the probes and primers for each target gene should also be done carefully otherwise the interaction between them inhibits the PCR multiplexing efficiency. Expression analysis using qPCR highly depends on the quantification of all of the simultaneously analyzed targets. It is assumed that qPCR multiplexed amplification does not alter each other's efficiency.<sup>21,22</sup> The prerequisites for the successful interpretation of duplexed qPCR experiments were extensively studied, which resulted in a generally accepted methodology of  $2^{-\Delta\Delta C_t}$ , which is considered as the gold standard for the relative quantification of mixtures.<sup>23</sup>

An intercalating dye-based qPCR multiplexing method simplifies the optical detection system as only one fluorescent channel is required, and there is no need to design different probes. The genes are typically distinguished based on different melting temperatures ( $T_M$ ) during the melting curve analysis (MCA) after temperature cycling.<sup>24,25</sup> However, it is an endpoint detection, and primers specific to each target gene must be designed to have a different  $T_M$ . Alternatively, it can be performed using the continuous fluorescence monitoring

(CFM) method allowing monitoring the PCR progress and optimizing it.<sup>26</sup> It also enables to perform dynamic MCA during the transition from the elongation to denaturation step and to construct the PCR amplification curves of two<sup>27</sup> or three genes,<sup>28</sup> or perhaps more. Another CFM-based method was proposed combining the FAM probe with the EvaGreen dye for simultaneous detection as both FAM and EvaGreen share the same emission wavelength,<sup>29</sup> which effectively doubles the PCR throughput. Unfortunately, both of these CFM-based methods are restricted from being further applied as they only allow one experiment at a time and require a customized setup. Recently, a novel single fluorescent channel-based multiplexing method was proposed using intercalating dyes, and the result was processed based on multidimensional standard curves.<sup>30–32</sup> Moreover, the data set was achieved by commercial qPCR instruments, and the absolute quantification was determined, thus extending the use of these devices.

In this work, we performed a comprehensive study of single-channel multiplexing based on the combination of EvaGreen and a FAM probe. We used a commercial qPCR instrument with 96 wells, which allowed us to study multiplexing using robust, statistically significant data sets. We showed the strengths and weaknesses of the proposed method and also compared it with the dual-channel multiplexing method, thereby making this PCR duplexing universal. Although the proposed work is based on the same principle as our previous one,<sup>29</sup> we performed a quantitative study of the method, having statistically significant data set using a commercial thermocycler instead of a home-made system to determine the



**Figure 2.** Determination of the copy numbers of both targets based on calculation. (A) Values of  $C_{tD}$  and  $C_{tE}$  as a function of  $cn_{21}$  with  $cn_{18}$  as the parameter. The black lines with squares are three denaturation curves overlapping each other that have been calculated using eq 1. The blue curve with downward triangles, the green with upward triangles, and the red with circles are elongation curves calculated by eq 2. Each of them represents different values of  $cn_{18}$ . (B)  $\Delta C_t$  values as a function of  $R_{SET}$  with  $cn_{18}$  as the parameter that has been extracted from Figure 2A. All three curves overlap, which shows that it is the  $R_{SET}$  value determining the  $\Delta C_t$  instead of an absolute value of  $cn_{18}$ .

limit of this method in detail in terms of detectable concentration ratios of both targets. The proposed methodology was more generally applicable for system optimization compared with the previous one, as researchers could perform it using any commercial qPCR instruments. We show fully quantitative duplexing capable to be performed by a conventional thermocycler and thus available to anyone.

## 2. PRINCIPLE OF THE PROPOSED MULTIPLEXING METHOD

We assumed that we prepared PCR master mix using a fragment of chromosome 21 (Chr21) specific to a FAM probe with a fixed number of copies ( $cn_{21}$ ) and a fragment of chromosome 18 (Chr18) with a fixed number of copies ( $cn_{18}$ ) as the parameter (Figure 1A). We then performed the PCR in the presence of EvaGreen while collecting the fluorescent amplitude ( $F$ ) at the end of the denaturation as well as the elongation in each PCR cycle (Figure 1B), followed by an MCA (Figure 1C). We split the collected data and formed two amplification curves, the denaturation and elongation curves (Figure 1D). The denaturation curve only consists of the FAM probe as all of the double-stranded DNA molecules melted into single-stranded DNAs, and EvaGreen's contribution to  $F$  is negligible. Changing  $cn_{18}$  has no influence on the denaturation curve. The elongation curves represent EvaGreen intercalated with both Chr21 and Chr18 amplicons in combination with the FAM probe linked to Chr21. Changing  $cn_{18}$  causes the PCR curve shift. The MCA extracted at the end of the PCR proved the presence of both amplicons in the PCR product (Figure 1E).

The threshold of the denaturation curve ( $C_{tD}$ ) of the mixture is only determined by the value of  $cn_{21}$  as the only FAM probe presence, and its concentration contributes to the  $F$  value at  $T_D$  (Figure 2A, black line with squares)

$$C_{tD} = A_D - B_D \times \log(cn_{21}) \quad (1)$$

where  $A_D$  is an offset and  $B_D$  is a PCR curve slope determined by the reaction efficiency of Chr21.

The threshold of the elongation curve ( $C_{tE}$ ) is affected by both  $cn_{18}$  and  $cn_{21}$ , and we can create a plot of  $C_{tE}$  as a function of  $cn_{21}$  with  $cn_{18}$  as the parameter

$$C_{tE} = A_E - B_E \times \log(cn_{18} + cn_{21}) \quad (2)$$

where  $A_E$  is an offset and  $B_E$  is the overall PCR efficiency (Figure 2A, blue, red, and green lines). Equation 2 can be split into two limiting situations,  $cn_{18} \gg cn_{21}$

$$C_{tE18} = A_1 + B_1 \times \log(cn_{18}) \quad (3)$$

and  $cn_{18} \ll cn_{21}$

$$C_{tE21} = A_2 + B_2 \times \log(cn_{21}) \quad (4)$$

where  $A_1$  and  $A_2$  are the offsets and  $B_1$  and  $B_2$  are the individual PCR efficiencies of Chr18 and Chr21 amplifications, respectively.

We then extracted the differences from the corresponding  $C_t$  values between the elongation and denaturation curves ( $\Delta C_t$ ) and plotted them as a function of the ratio between the  $cn$  of the amplicon with and without a link to FAM ( $R_{SET}$ ) with  $\log cn_{18}$  as the parameter. Here, it would be  $R_{SET} = cn_{18}/cn_{21}$ . It shows that the  $\Delta C_t$  value is a function of  $R_{SET}$  independent of the  $cn$  absolute values (Figure 2B). The  $\Delta C_t$  can be expressed as

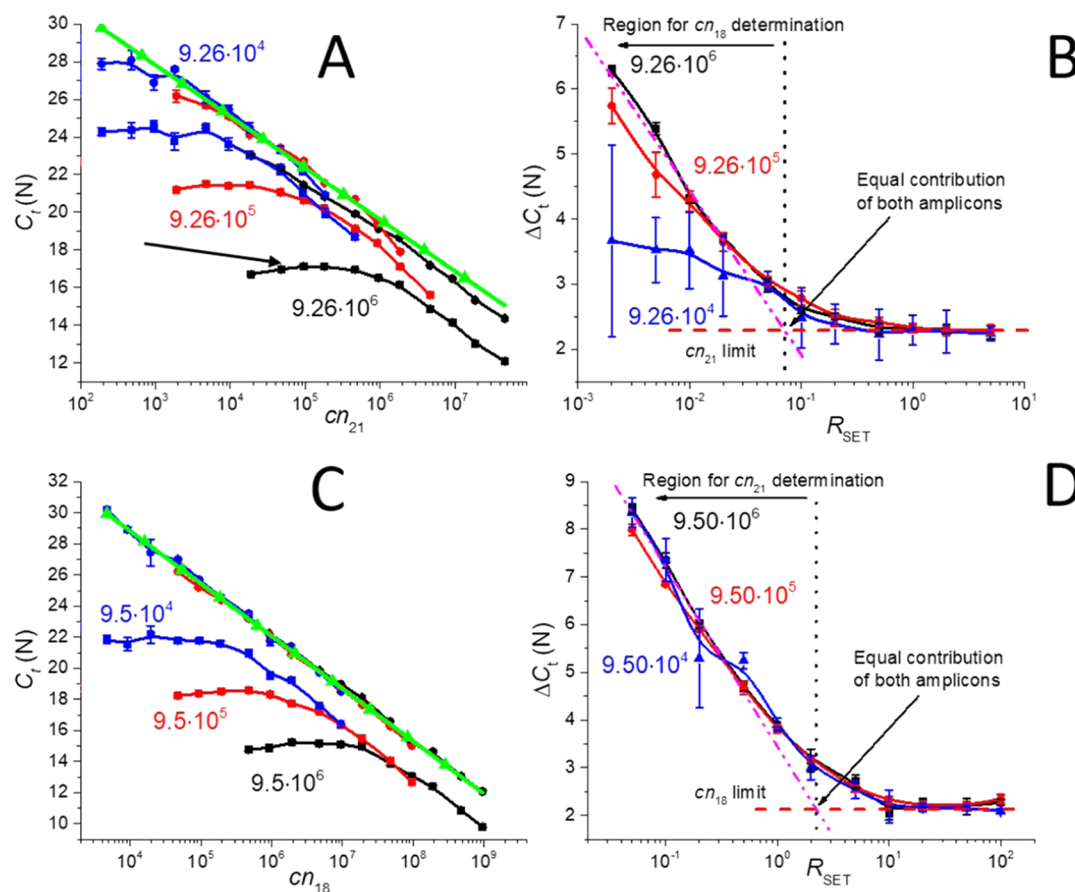
$$\begin{aligned} \Delta C_t &= A_D - A_E + B \times [\log(cn_{21}) - \log(cn_{18} + cn_{21})] \\ &= A + B \times \log\left[\frac{cn_{21}}{cn_{18} + cn_{21}}\right] \end{aligned} \quad (5)$$

where  $A$  is intercept with the Y-axis and  $B$  is a slope, respectively. We can then, with eq 4, simplify the relationship as

$$\begin{aligned} \Delta C_t &= A + B \times \log\left[\frac{cn_{21}}{cn_{21}R_{SET} + cn_{21}}\right] \\ &= A + B \times \log\left[\frac{1}{R_{SET} + 1}\right] \end{aligned} \quad (6)$$

to show its independence on an absolute value of  $cn_{21}$  and  $cn_{18}$  (Figure 2B). Parameter  $B$  defines the precision of  $R_{SET}$  and thus  $cn_{18}$  determination.

Thus, once we form the PCR standard curve, as shown in Figure 2A, we can determine the  $cn_{21}$  of an unknown sample from the denaturation curve (marked by a black arrow). Then, we calculate the  $\Delta C_t$  value and determine the  $cn_{18}$  based on the extracted  $R_{SET}$  values from Figure 2B. Note that the  $cn_{18}$  can only be determined from the left part of the graph starting at



**Figure 3.**  $C_t$  values extracted from the PCR with EvaGreen intercalating dye and the FAM probe. (A) PCR standard curves of the variable  $cn_{21}$  values with three different  $cn_{18}$  values as the parameter with the denaturation standard curves marked with circles and the elongation with squares. The  $cn_{21}$  values could be determined in an entire range from the denaturation standard curves. The arrow pointed to the curved region where minor Chr21 clearly influences Chr18 amplification. The green line marked with triangles was formed by the curve fitting of an average value from all three denaturation curves. (B) Values of  $\Delta C_t$  plotted as a function of  $R_{SET}$  showing the importance of the  $R_{SET}$  value and not the individual  $cn_{21}$  or  $cn_{18}$ . (C) Equivalent of (A) with  $cn_{18}$  as the variable and  $cn_{21}$  as the parameter with elongation curves marked by squares and the denaturation by circles. (D) Equivalent of (B) with  $cn_{18}$  as the variable and  $cn_{21}$  as a parameter.

the point where both amplicons' contribution is equal; otherwise,  $cn_{21}$  dominates the change of  $C_{tE}$ .

### 3. RESULTS AND DISCUSSION

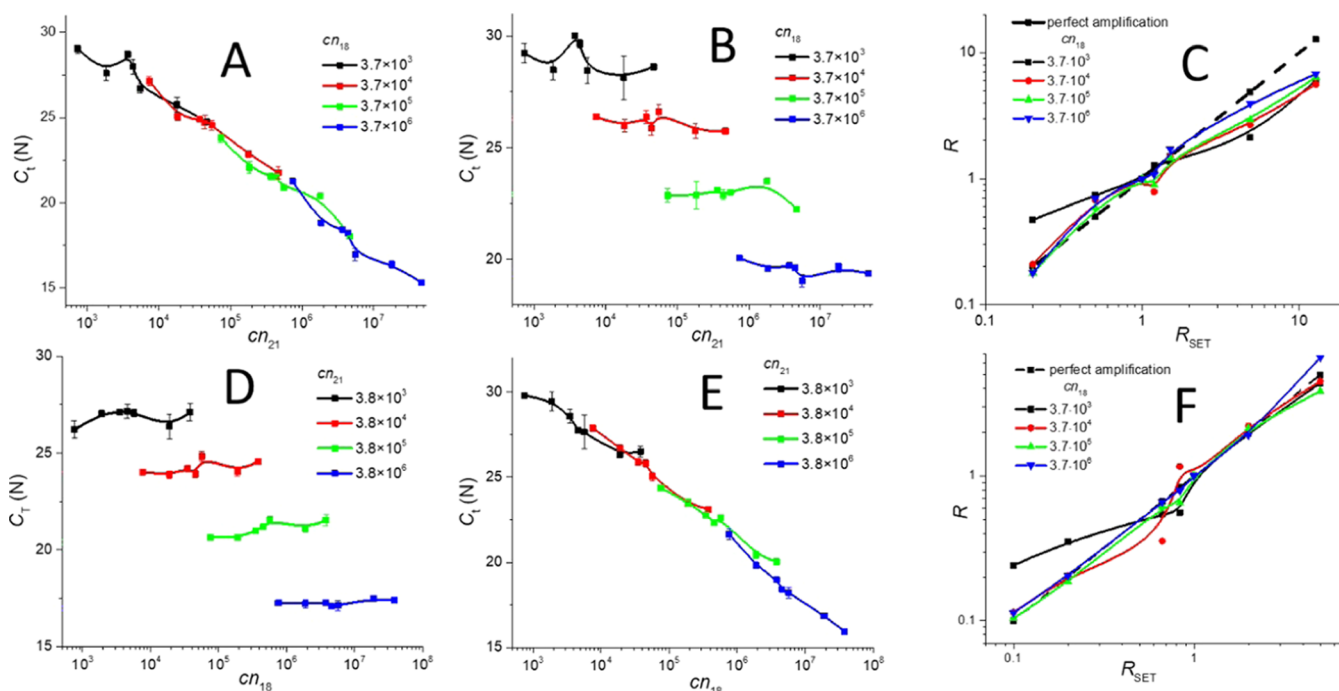
**3.1. Preliminary Experiments.** We first performed the PCR and extracted elongation and denaturation standard curves of Chr21 with  $cn_{21}$  from  $9.5 \times 10^1$  to  $9.5 \times 10^6$  with and without a FAM probe to determine the  $C_t$  shift from the elongation to the denaturation as well as the PCR efficiency for both curves. Each experiment was repeated four times to suppress any random error. Then, the same experiment was conducted using Chr18 from  $cn_{18}$  between  $9.26 \times 10^2$  and  $9.26 \times 10^6$  with a VIC probe (Supporting Information, Section S1). We used a VIC specific to Chr18 as a reference to the single-channel multiplexing method. We found that the PCR efficiency was very similar for all combinations, and its value is  $(-3.46 \pm 0.03) N \times (\log cn)^{-1}$  (mean  $\pm$  fitting error from four measurements), which demonstrated the correct experiment setting as well as the minimal pipetting error (Supporting Information, Section S1).

**3.2. Multiplexing Using the Target  $cn_{18}$  Constant and the  $cn_{21}$  Variable with FAM Specific to Chr21.** We then mixed Chr21 with a FAM probe and Chr18 with the ratio  $R_{SET}$  with three different  $cn_{18}$  values of  $9.26 \times 10^4$ ,  $9.26 \times 10^5$ , and

$9.26 \times 10^6$  and a  $cn_{21}$  variable (Supporting Information, Section S2). We performed the PCR and extracted the denaturation and elongation standard curves (Figure 3A). The denaturation curves (black, red, and blue line with the squares) were solely determined by  $cn_{21}$ , but they were nevertheless influenced by the abundance of Chr18, as exhibited by the curve deviation from the straight line.

The elongation curves (black, blue, and red with circles) show two distinguished regions, one determined by  $cn_{18}$  and the other by  $cn_{21}$ , as predicted by eqs 2–6, and their graphical representation is shown in Figure 2A. We performed the curve fitting (green line in Figure 3A) of the average of all three denaturation curves using eq 1 and obtained  $A_D$  and  $B_D$  as  $(36.03 \pm 0.56) N$  and  $(-2.74 \pm 0.11) N \times (\log cn_{21})^{-1}$  (both mean  $\pm$  fitting error), respectively.

The saturation at the elongation curve determined by the  $cn_{18}$  value exhibited a curved shape instead of a flat line parallel to the X-axis (pointed by an arrow in Figure 3A), a clear sign of  $cn_{21}$  influence. With a lower value of  $cn_{21}$ , the  $C_{tE}$  was also lowered, demonstrating that the influence of Chr21 was diminishing. All of the PCR experiments were accompanied with MCAs, and there was a trend showing the transition from the region dominated by Chr21 to a region dominated by Chr18 with an increased number of amplicons of Chr18, while



**Figure 4.**  $C_t$  values extracted from the PCR with FAM and VIC probes. (A) FAM channel with a  $cn_{21}$  variable with  $cn_{18}$  as the parameter showing the problems with the dual-channel amplification as the standard curves with different values of  $cn_{18}$  should be collinear and form a single line regardless of the  $cn_{18}$  value. (B) Same PCR mixtures using a VIC channel also showed the influence of  $cn_{21}$  on  $cn_{18}$  as the results should be independent of  $cn_{21}$  since it was Chr18 with the VIC probe. (C) Implementation of the  $2^{-\Delta\Delta C_t}$  method showing its shortcoming with the noncompensated data for fluorescence spillover. (D–F) Similar results for a mixture with the  $cn_{18}$  value variable and the  $cn_{21}$  as the parameter.

the product of Chr21 dominated regardless of  $cn_{21}$  (Supporting Information, Section S3).

We then calculated the values of  $\Delta C_t$  and plotted them as a function of  $R_{SET}$  (Figure 3B). We found that each amplicon had different contributions to the  $C_{tE}$  value as the point of the equal contribution of both the amplicons was not for  $R_{SET} = 1$ . We then modified the original elongation curves per eq 2 as

$$C_{tE} = A_E - B_E \times \log(cn_{18} + R_C \times cn_{21}) \quad (7)$$

where the critical ratio ( $R_C$ ) was a coefficient reflecting the different contributions of each amplicon to the  $C_{tE}$  value. Then, eq 6 would convert to

$$\begin{aligned} \Delta C_t &= A + B \times \log\left[\frac{cn_{21}}{cn_{18} + R_C \times cn_{21}}\right] \\ &= A + B \times \log\left[\frac{R_C}{R_{SET} + R_C}\right] \end{aligned} \quad (8)$$

We extracted the slope  $B$  from the curves as  $(-2.40 \pm 0.03) N \times (\log R_{SET})^{-1}$  (mean  $\pm$  fitting error), thereby defining the sensitivity of  $R_{SET}$  determination as well as the  $R_C$  value of  $\approx 0.07 R_{SET}$ . The low  $R_C$  value in comparison with  $R_{SET}$  showed strong dominance of Chr21 contribution to the  $C_{tE}$  values. As predicted by eq 7, the  $\Delta C_t$  was a function of  $R_{SET}$  instead of an absolute number of either  $cn_{21}$  or  $cn_{18}$ .

We determined the absolute value of  $cn_{21}$  in the entire range from the denaturation curve (curves in Figure 3A marked with circles) with a precision of  $(-2.74 \pm 0.11) N \times (\log cn_{21})^{-1}$  (mean  $\pm$  fitting error). The  $cn_{18}$  value was identified by extracting the  $R_{SET}$  value from Figure 3B and multiplying it with  $cn_{21}$ .

We found that the precision of the  $cn_{18}$  determination is  $(-2.40 \pm 0.03) N \times (\log R_{SET})^{-1}$  (mean  $\pm$  fitting error) for  $R_{SET}$  values smaller than  $R_C \approx 0.07$ . The lower values of  $cn_{18}$  deviated from the described behavior because the presence of Chr21 probably inhibited the PCR of Chr18, as shown by the MCAs (Supporting Information, Section S3).

**3.3. Multiplexing Using a Target  $cn_{21}$  Constant and a  $cn_{18}$  Variable with FAM Specific to Chr18.** We repeated the same measurement with a FAM probe specific to Chr18 and Chr21 with  $cn_{21}$  fixed at the three different values with the  $cn_{18}$  variable and plotted the results into Figure 3C (Supporting Information, Section S4). Here, the  $R_{SET}$  was  $cn_{21}/cn_{18}$ . We performed the curve fitting as it was done in the previous section and found that the transition  $R_C$  changed from 0.07 to 2.26, which showed the dominance of the Chr21 regardless of the FAM probe specificity. We also calculated the  $\Delta C_t$  and plotted it as a function of  $R_{SET}$  (Figure 3D). The results verified our original hypothesis that  $\Delta C_t$  was determined by the value of  $R_{SET}$  instead of the absolute values of  $cn_{18}$  and  $cn_{21}$ .

The absolute value of  $cn_{18}$  could be determined in an entire range from the denaturation curve (curves in Figure 3C marked with squares) with a precision of  $(-3.38 \pm 0.03) N \times (\log cn_{21})^{-1}$  (mean  $\pm$  fitting error). The  $cn_{21}$  value could be extracted from Figure 3D using the  $R_{SET}$  values and a value of  $cn_{18}$  with a precision of  $(-3.48 \pm 0.26) N \times (\log R_{SET})^{-1}$  (mean  $\pm$  fitting error) for the  $R_{SET}$  values smaller than  $R_C \approx 2.26$ .

**3.4. Multiplexing with Target  $cn_{21}$  Having a Chr21-Specific FAM Probe and  $cn_{18}$  with a Chr18-Specific VIC Probe.** We performed a PCR using Chr21 and Chr18 specific to FAM and VIC probes, respectively, with either a  $cn_{18}$

constant and a  $cn_{21}$  variable or vice versa (Supporting Information, Section S5).

Each experiment was conducted five times. We then extracted the  $C_t$  values using eq 11 and plotted  $C_t$  as a function of  $cn_{21}$  from the FAM and VIC channels, as shown in Figure 4A,B, respectively.

We calculated the average errors in  $C_t$  determination (Table 1), and they were rather small—between 2.4 and 1.5% for the

**Table 1. Results from Two Fluorescence Channels Showing  $C_t$  Determination Errors**

	$cn$	intercept ( $N$ )	slope ( $\log cn$ )	mean value ( $N$ )
$cn_{21}$ variable				
FAM channel		$38.60 \pm 0.43$	$-3.05 \pm 0.08$	
VIC channel	$3.7 \times 10^3$			$29.03 \pm 0.70$
	$3.7 \times 10^4$			$26.13 \pm 0.33$
	$3.7 \times 10^5$			$22.92 \pm 0.35$
	$3.7 \times 10^6$			$19.55 \pm 0.30$
$cn_{18}$ variable				
FAM channel	$3.8 \times 10^3$			$26.89 \pm 0.37$
	$3.8 \times 10^4$			$24.16 \pm 0.35$
	$3.8 \times 10^5$			$20.99 \pm 0.42$
	$3.8 \times 10^6$			$17.22 \pm 0.16$
VIC channel		$39.59 \pm 0.39$	$-3.07 \pm 0.07$	

lowest and highest  $cn_{18}$ . The determination of the individual  $C_t$  values varied much more, especially for the lowest value of  $cn_{18}$  (black line in Figure 4B). We used the  $2^{-\Delta\Delta C_t}$  method<sup>23</sup> with the mixture having  $R_{SET} = 1$  as the calibrator. We plotted the extracted  $2^{-\Delta\Delta C_t}$  values as a function of  $R_{SET}$  (Figure 4C). The results showed that even the widely accepted method of  $2^{-\Delta\Delta C_t}$  had a problem with determining  $R$  precisely when applied to the data without any compensation for fluorescence spillover.<sup>33</sup>

We repeated the same experiment with the  $cn_{18}$  variable and  $cn_{21}$  as the parameter and extracted the results in the same fashion as for the first experiment (Figure 4D–F), and the extracted data and errors were shown in Table 1.

**3.5. Comparison of the Single-Channel Duplexing Method with the Method Employing Two Probes with Different Fluorophores.** We performed the single-channel duplexing qPCR method using Chr21 with a FAM-specific probe and Chr18 both in the presence of EvaGreen intercalating dye. We also alternated the mixture composition having a FAM-specific probe to the Chr18 sequence instead of Chr21. We found that we could determine the value of the  $cn$  of the amplicon with the FAM probe in the entire range of the

values. The second amplicon,  $cn$ , could only be determined once the  $R_{SET}$  value was smaller than the  $R_C$ , which could vary for each individual pair of targets. In comparison, we performed standard multiplexing using FAM and VIC probes. The multichannel technique exhibited large error especially for the lower values of  $cn$ s, where the  $C_t$  value varied in a range between 27 and 30 (black line in Figure 4B). We then processed the extracted data using the  $2^{-\Delta\Delta C_t}$  method<sup>23</sup> without performing channel overspilling compensation. It showed that even these widely accepted methods did not produce precise data (Figure 4C,F). Single-channel multiplexing produced more precise data, but it could determine both amplicons only for  $R_{SET} < R_C$  in half of the total range of  $cn$ .

## 4. CONCLUSIONS

We proposed and verified a method of quantitative polymerase chain reaction (qPCR) duplexing using a single fluorescein isothiocyanate (FITC) fluorescent channel with a commercially available qPCR system with a 96-well platform. We amplified the following two sequences: one from chromosome 21 (Chr21) and one from chromosome 18 (Chr18) in the presence of the EvaGreen intercalating dye. We added a 6-carboxyfluorescein (FAM) probe specific to either Chr21 or to Chr18 and extracted the PCR curves after the denaturation and elongation steps, and then, we plotted the PCR standard curves, one for denaturation and the second one for elongation. We were able to determine the copy number ( $cn$ ) of an amplicon with a FAM-specific probe in the entire range of the  $cn$  values from the denaturation standard curve with precision values of  $(-2.74 \pm 0.11) N \times (\log cn_{21})^{-1}$  and  $(-3.38 \pm 0.03) N \times (\log cn_{18})^{-1}$  (both mean  $\pm$  fitting error) for Chr21 and Chr18, respectively.

The  $cn$  value of the other amplicon was extracted from the difference of the critical thresholds between the denaturation and elongation curves with precision values of  $(-2.40 \pm 0.03) N \times (\log R_{SET})^{-1}$  and  $(-3.48 \pm 0.26) N \times (\log R_{SET})^{-1}$  (both mean  $\pm$  fitting error) for Chr21 and Chr18, respectively.

This proposed method has better precision than the standard technique of working with differently labeled probes, which eliminates the problem of overspilling between fluorescent channels. However, this method is not capable of operating in the entire range of possible ratios of mixture components as the  $R_{SET}$  value has to be greater than the  $R_C$  to determine the  $cn$  of the target without the FAM probe. Nevertheless, if the determination of the value with  $R_{SET} < R_C$  is required, it is possible to change the system and use a FAM probe that is specific to the second target. The combination of the probe-based and intercalating dye-based methods brings the potential drawbacks of both such as the high cost for

**Table 2. Information of Target Sequences and the Corresponding Primers and Probes**

target gene	gene location <sup>a</sup>	primer type	primer sequence (5'–3')	amplicon size (bp)	amplicon $T_M$ (°C)
Chr21	14099141-14099218	forward	ctaggagactgtccctgagctt	78	83.5
		reverse	agggggaacatagaggcttg		
		LNA probe	FAM-ccctgcctct-BHQ1		
Chr18	1248496-1248575	forward	ccatctccatacccaataacc	80	80.4
		reverse	ccttgcacaacctcatgttga		
		LNA probe	VIC-cccacctcca-BHQ1 FAM-cccacctcca-BHQ1		

<sup>a</sup>Gene location according to the human genome assembly version hg19/Genome Reference Consortium Human Build 37.

probe-based qPCR and the low specificity for intercalating dye-based qPCR. However, it is not necessary to have two expensive probes and a complex fluorescence system; thus, the proposed method can lower the cost as only one probe is applied. Additionally, it eliminates the fluorescence over-spilling, which exists in probe-based qPCR multiplexing. Moreover, the melting curve analysis conducted immediately once the thermal cycling is completed will determine the amplicon specificity.

This proposed duplexing method using a single FITC fluorescent channel can be implemented for a wide variety of examinations where mixture analysis is desired once either multifuorescence channel is not a viable option or to lower the test cost. This qPCR duplexing thus opens new horizons for practical applications, providing an original, easily applicable technical solution.

## 5. MATERIAL AND METHODS

**5.1. DNA Sequences and PCR Master Mix.** We used the synthesized target fragments of Chr21 and Chr18 as well as primers and probes for both, as shown in Table 2. The chosen amplicons, designed primers, and the lock nucleic acids (LNA) probes were used beforehand for the noninvasive prenatal diagnostics using dPCR, and they were optimized to perform in multiplex reactions.<sup>34</sup> We then prepared different types of PCR master mixes based on the following designed experiments by adjusting the volume of probes for both targets (Supporting Information, Section S6). The final volume of the PCR master mix was  $\approx 10 \mu\text{L}$  by adding sterilized deionized  $\text{H}_2\text{O}$ .

**5.2. qPCR Protocol.** We performed the qPCR with the protocol set in the following way: a hot start at  $95^\circ\text{C}$  for 30 s to activate the polymerase followed by 40 cycles of denaturation at  $95^\circ\text{C}$  for 10 s, annealing at  $52^\circ\text{C}$  for 30 s, and elongation at  $72^\circ\text{C}$  for 10 s using a commercial 96-well real-time PCR system collecting two  $F$  values during each PCR cycle at the end of the elongation and denaturation steps. We then conducted an MCA from  $65$  to  $95^\circ\text{C}$  with a ramping rate set to  $0.5 \text{ K}\cdot\text{s}^{-1}$ .

**5.3. Data Processing.** The collected data were processed using a MATLAB script by first splitting them into two blocks. The first 80 points contained the PCR amplification curves, and the rest represented the MCA. During each cycle, we collected two data points corresponding to the two adjacent sample points,  $F$  values were collected at the end of the denaturation and elongation steps. Then, we split the amplification curve data according to their sequence. The data whose serial number was odd (even) were selected to form the denaturation (elongation) amplification curve. The elongation curve was the same as the conventional PCR amplification curve. The denaturation curve was different since the PCR cycle started with denaturation and ends with elongation. We skipped the first point of the denaturation curve as it had no meaning since no PCR amplification had occurred by that point in time. As a result, the denaturation curve had only 39 points, while the elongation had typical 40 points resulting from 40 cycles. After that, the amplification curves were processed independently.

**5.4. Data Normalization.** We normalized the amplification curves according to the following formula

$$F_N = \frac{F - F_{\min}}{F_{\max} - F_{\min}} \quad (9)$$

where  $F_N$  is the normalized  $F$  amplitude value and  $F_{\max}$  is the maximal amplitude value of the  $F$  data.  $F_{\min}$  is manually selected for each curve due to the unpredictable fluctuation in the  $F$  value of the first few data. If the variation range ( $F_{\max} - F_{\min}$ ) of a curve is judged to be lower than 50 000 arbitrary units (a.u.), this curve is regarded as a nonamplification curve, and the  $F_N$  value is set to 0 a.u.

**5.5.  $C_t$  Value Extraction.** We performed the curve fitting using a Boltzmann function similar to the methods published earlier<sup>35,36</sup>

$$F_N(i) = \frac{1}{1 + \exp\left(\frac{i - i_0}{dF}\right)} + F_{\min}/F_{\max} \quad (10)$$

where  $F_N(i)$  is the normalized amplitude of the  $F$  value of cycle number  $i$ ,  $i_0$  is an inflexion point of the Boltzmann curve, and  $dF$  determines the Boltzmann curve slope at  $F(i_0)$  by  $\frac{dF_N(i)}{di} = -\frac{1}{4} \times \frac{F_{\max}}{dF}$ . In previous work,<sup>36</sup> the author defined  $C_t$  as a value of  $N$  at a fluorescence inflection point. Here, we calculated the  $C_t$  value as an  $N$  value for a 10% increase of the  $F$  value above its baseline

$$C_t = F_{\min} + 0.1(F_{\max} - F_{\min}) \quad (11)$$

This method is similar to the one conventionally used for calculating the  $C_t$  defining the  $C_t$  value as a cycle number for 10% of  $F$  increased above the baseline. The Boltzmann function-based  $C_t$  value determination was then implemented using the MATLAB script.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c02971>.

Standard curves of single genes, amplicon ratios of Chr21 and Chr18 with Chr21 variable, MCAs between Chr21 with the FAM probe and Chr18 having  $cn_{18}$  with three different values and  $cn_{21}$  variable, amplicon ratios of Chr21 and Chr18 with Chr18 variable and MCA after PCR, amplicon ratios of Chr21 and Chr18, and PCR master mix (PDF)

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## Notes

The authors declare no competing financial interest.

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