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ORIGINAL PAPER

Thermal gradient for fluorometric optimization of droplet PCR in virtual reaction chambers

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Abstract An open system with a thermal gradient is described for the optimization of polymerase chain reactions (PCR). Two thermal electric coolers were used as the heat source. The gradient is measured through encapsulated water-based beads of a temperature-dependent dye inside mineral oil, thereby forming virtual reaction chambers. Nine droplets (with typical volume of 0.7 μL) were used. Using the intrinsic fluorescence of a temperature-sensitive inert dye (sulforhodamine B), the process involves measurement of the fluorescence intensity at a known, uniform temperature together with the instrument-specific calibration constant to calculate an unknown, possibly non-uniform temperature. The results show that a nearly linear thermal gradient is obtained. This gradient function is a useful feature that can be used for optimization of a commonly used enzyme-activated reaction, viz. PCR. The emission spectra of fluorescent droplets during two-step PCR were monitored and the changes in fluorescence between 50 °*C* and 100 °*C* quantified. As the gradient feature allows for testing a range of annealing temperatures simultaneously, the optimal annealing temperature can be easily determined in a single experiment.

Keywords Microfluidics · Droplet PCR · Thermal gradient · Fluorometric sensing · Temperature-dependent dye · Sulforhodamine B

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Introduction

Microfluidic systems, or "labs-on-a-chip", have revolutionized many aspects of quantitative biochemistry and analytical chemistry [1]. The potential advantages, including portability, speed, high efficiency, and reduced reagent consumption [1–4] have been explored by the miniaturization and integration of the various chemical operations. As in digital microfluidics where droplets are manipulated on an open, hydrophobic surface, the virtual reaction chamber (VRC) offers a simple way of exploiting the advantages of microfluidics and droplets while circumventing many of the practical problems. It is formed by encapsulating single aqueous sample droplets with volumes in the low microliter range within slight larger oil droplets [5–7].

Temperature is the most fundamental element in biochemical reactions [8], either in micro or macro scale. Therefore, to obtain robust, unique and clean products, optimization needs to be performed. This optimal temperature is often reaction dependent, or relies on other factors such as the physical characteristics of the molecules in a particular solvent or equipment characteristics. On the other hand, temperature may affect the rate or efficiency of the reaction. Accurate control of sample temperatures in microfluidic systems is often very important, particularly during the reaction and separation. The importance of temperature control in lab-on-a-chip devices has been demonstrated for enzyme-activated reactions [1, 5, 9–11].

One of these enzyme-activated reactions, polymerase chain reaction (PCR) [12], conducted by a deoxyribonucleic acid (DNA) polymerase, is introduced to illustrate the point. PCR utilizes biological and chemical components to orchestrate enzymatic amplification. It gives access to a method of amplifying DNA molecules across several orders of magnitude, which has substantially accelerated the pace of research in many fields of biology.

The sequence and length of PCR primers generally determine the annealing temperature of the thermal cycling reaction for a specific assay. Using too low an annealing temperature can produce non-specific priming of templated DNA or form primer-dimers, whereas if the temperature is too high, little or no product may be produced. Therefore, PCR yield is reduced. These problems can often be avoided by an annealing temperature optimization step [13–15].

Most groups have reported using a temperature sensor to measure the temperature of the substrate of a microfluidic system [6, 7, 9, 16] or on the outside of capillaries [17, 18]. This is perhaps the simplest and easiest way to measure temperature. However, it is not accurate considering the temperature discrepancy between the temperature on the outside of the system and the fluid inside the system. Besides, concerns caused by direct sensor contact within the solution, such as product contamination or inhibition, the added thermal mass of the sensor, and the obstruction of optical measurements become more acute as the sample volume decreases, forcing measurements external to the sample and compromising accuracy during rapid temperature transitions.

A simple solution for non-contact temperature measurement is to use a passive reference dye whose fluorescence varies with temperature but does not inhibit the reaction. The technique takes advantage of the temperature dependence of the fluorescence intensity of a dilute fluorophore added to the fluid [2]. Since the fluorescence of many dyes is temperature-dependent [19, 20], a suitable dye has to be chosen for each specific application. Considering the repeated heating and cooling during thermal cycling, sulforhodamine B has been used for measuring temperature because of its reliable fluorescence over time [19–21]. Moreover, sulforhodamine B exhibits excellent temperature sensitivity.

4 parallel PCR reaction-stations were presented in [15] with a purpose of optimizing annealing temperature in the range of 50–68 °C. Our work, based on VRC, is capable of affording 9 thermal gradients, aiming to optimize PCR reaction in a single run and in more precise temperature scale. More thermal gradients can be obtained by smaller droplet size and tighter posited droplets. Commercially available gradient thermocyclers, such as the 96 Universal Gradient, PeQSTAR (http://www.isogen-lifescience.com), Mastercycler epgradient (http://www.labx.com), and Chromo 4 usually require more than two temperature controlling modules to achieve the same temperature gradient. Most of them either have no real-time detection [22], or require large volumes of the PCR cocktail for the reaction [23]. Multi-zone temperature control may ensure accuracy. Nevertheless, more energy is consumed by multi-heater units. Meanwhile, the footprint is much bigger because of multi-heater units and corresponding control parts. A two-step thermal gradient for fluorometric optimization of droplet PCR in virtual reaction chambers is present here. Sulforhodamine B was used for real-time thermal gradient control and monitoring. The method incorporates a two-step protocol combining the annealing and elongation steps, which leads to significant time-savings and a reduction in reagent use during optimization and standard PCR experiments.

Materials and methods

Surface preparation

As described earlier, the glass surface for the VRC has to be hydrophobic as well as oleo phobic. Chemical vapor deposition method is applied to silanize glass coverslips. A self-assembly monolayer of a fluorosilane with a reproducible contact angle (Drop shape analysis system DAS 10 MK 2, https://www.kruss.de) around 109 ° was achieved. Coating stability was assessed by the INM institute (http://www.leibniz-inm.de). Detailed description on surface coating can be accessed in supplementary material.

Temperature calibration

Theoretically, given a small piece of highly thermally conductive material, a uniform thermal distribution can be reached in seconds or milliseconds. By applying two different temperatures to this material, points along the temperature difference direction should have temperatures in between.

To demonstrate the point, sulforhodamine B, a passive dye which exhibits excellent temperature sensitivity, was chosen for monitoring the temperature. For absolute intensity of the fluorescence to serve as a temperature monitor, the instrument and dye must be stable over time. Temperature calibration was performed at equilibrium temperatures, not while the temperature was changing.

Temperature can be related to fluorescence through a calibration constant:

$$C = ln\left(I/I_{ref}\right)/\left(1/T - 1/T_{ref}\right) \quad (1)$$

Fluorescence intensities I were measured at temperatures T (in *Kelvin*) and related to reference fluorescence intensity I_{ref} at a reference temperature T_{ref}. Instrument-specific calibration constants are used to convert fluorescence to solution temperatures. Afterwards, the solution temperatures were converted into *Celsius* using the following formula:

$$t(°C) = T(K) - 273.15 \quad (2)$$

Where t and T represent temperature in *Celsius* and *Kelvin*, respectively. Detailed temperature calibration description can be found in supplementary material.

Reagents

All buffers were made using deionized water from a Milli-Q PrograD T3 column (http://www.merckmillipore.com/DE/de). The fluorescence of sulforhodamine B (monosodium salt, http://www.sigmaaldrich.com/germany.html) was measured in a "mock" PCR solution (without polymerase) at a final concentration of 0.1 mM; see protocol below. The polymerase was replaced with deionized water.

The performance of the system was verified by performing real-time PCR to detect a DNA segment of an avian virus. The PCR primers for the chosen avian virus segment (detailed sequence can be found in supporting material) were designed by Primer Express 3.0. The sequence of the forward primer:

5-TGTACTCCCCAGTGTCATGATTG-3;

Reverse primer:

5-AAGGGAATAAGCGGCCATATC-3.

The melting temperature for the primer (Eurofins, Germany) is 60.6 °C.

The master mix was prepared by adding 3 μL of 25 mM $MgCl_2$, 9 μL 50 pM of each forward and reverse primer, and 4 μL of the LightCycler FastStart DNA Master SYBR Green I (http://www.roche.de/). 2 μL DNA templates were added to the reaction mixture to the total volume of 27 μL immediately before the onset of the reaction. The template concentration was around 10^5 copies $\cdot \mu L^{-1}$.

On-chip PCR thermocycling

The instrument setup is shown in Fig. 1. Thermocycling of the microfluidic device was achieved using two thermoelectric coolers (1TML10-21 × 21–10, http://www.thermion-company.com/) and a manufactured controller TEC-1122-SV (http://www.meerstetter.ch/). Temperature feedback was accomplished by inserting two 1 mm-thick 22 × 22 mm copper plates on top of each TEC unit with an embedded pt100 (http://de.farnell.com/) temperature sensor. Temperature control was performed by proportional integrated derivative (PID) feedback control. Optimized PID constants were used to achieve a fast yet stable control system. Then, another piece of 1.2 mm thick 20 × 65 mm copper plate was placed between the microfluidic device and two small copper plates to facilitate efficient heat transfer to achieve a uniform heat distribution. A custom-fabricated copper block was placed beneath the TEC device to dissipate waste heat. Finally, a 3-mm silicon wafer was placed between the copper plate and the microfluidic device to help equalize heat distribution and provide a better optical surface for imaging. The VRC used in this work was formed by a 0.7 μL sample, covered with 3 μL of M5904 mineral oil (Sigma-Aldrich, http://www.sigmaaldrich.com/germany.html) and placed on a 170 μm thick hydrophobic/oleo phobic microscope coverslip. Two-step PCR thermocycling was initiated with a 10 min "hot start" at 95 °C to activate the Taq polymerase followed by 40 cycles of ramping between 50 °C and 95 °C using 10 s hold times and a thermal ramp rate of 5 $^{\circ}C \cdot s^{-1}$ .The total PCR thermocycling reaction time required ~45 min. The ability to perform on-chip thermal cycling of droplets is necessary to be able to perform real-time observation of the entire droplet reactor array during PCR amplification.

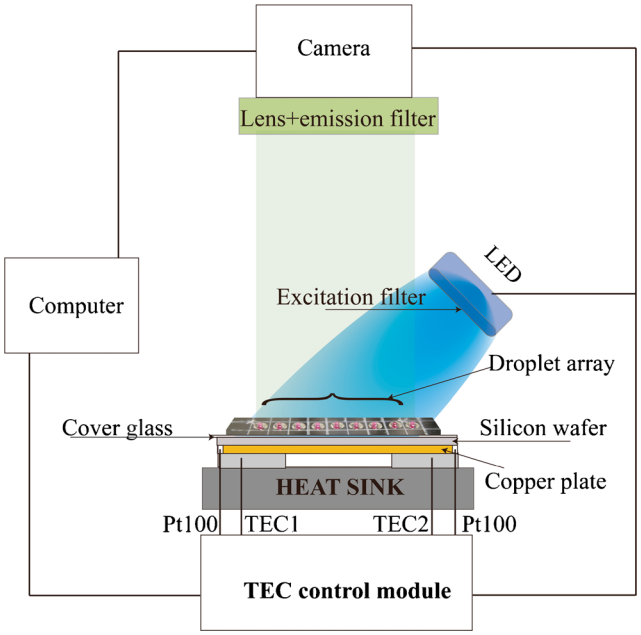

Fig. 1 Instrument setup. The TEC control module sends out a signal to LED and camera at the same time at the end of the annealing step, making sure that the LED light is on while capturing the image

To find the optimal annealing temperature for the reaction, a recommended temperature range ±10 °C above and below the calculated melting temperature of the primers was used. Since the melting temperature for primer was 60.6 °C, the temperature gradient was set to 52 °C ~ 72 °C for optimization.

Image acquisition and processing

Fluorescence imaging of the sulforhodamine B dye was performed using a ProgRes MF Cool CCD camera (https://www.jenoptik.de/). The camera gain was set manually to 1 and kept constant throughout the whole experiment. An appropriate filter set (ET546/22×, ET605/70 m, www.ahf.de) was applied in front of the C-mount fixed focal lens HF 16HA-1B/1.4 (http://fujifilm.jp). Fluorescence imaging of the PCR reaction was the same as that for sulforhodamine B, except the filter set was different (MF469/35 https://www.thorlabs.de/, ET525/50 https://www.chroma.com/).

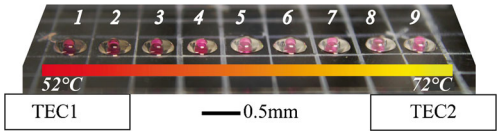

Fig. 2 Droplet array with thermal gradient. From left to right, the temperature increases and the droplets are numbered as droplet 1 to 9

ImageJ software and custom Matlab code were used to systematically detect and quantify fluorescent droplets and analyze the size and the fluorescence intensity.

Melting analysis

Because SYBR Green I bind to all double-stranded DNA, it is necessary to check the specificity of the PCR assay by analyzing the amplified products. After each reaction, a melting curve analysis was run. A BioAnalyzer 2100 (http://www.agilent.com/) and gel electrophoresis were also used to check the specificity of the amplicon. Additional information on amplicon concentration can be accessed through the analysis from the BioAnalyzer. An optimized SYBR Green I PCR reaction should have a single peak in the melt curve, corresponding to a single band on the gel image. By comparing the gel image with the melt curve, one can identify peaks in the melt curve that correspond to specific products, additional non-specific bands and primer dimers.

Commercial instrument

Another group of experiments were performed on a commercial gradient machine, i.e. 96 Universal Gradient, PeQSTAR in house. Unfortunately, the device does not have a real-time function. Because of its large reaction volume, gel electrophoresis was carried out after the reaction. 5 μL of each reaction product from the PeQSTAR commercial gradient machine was resolved on a 2% agarose gel for a period of 30 min at 100 V. 6X DNA Gel loading buffer was added at a ratio of 5:1. Gel images were taken by Bio-Rad (www.bio-rad.com). Gel lanes were processed using ImageJ.

Results

Temperature gradient

When two different temperatures were applied at the two ends of the chip, a group of nearly linear different temperatures was obtained, forming a thermal gradient. Figure 2 illustrates the different temperatures in color. Meanwhile, the thermal gradient is represented by color depth. From left to right, the droplets are numbered droplet 1 to 9. The fluorescence of sulforhodamine B decreased as the temperature increased.

After temperature calibration, a two-step thermal cycling was run with sulforhodamine B monitoring the temperature in real time. Figure 3(a) shows the instrument equilibration of sulforhodamine B assessed at 55 °C while 3(b) shows the temperature profile of each droplet during the PCR reaction, positioned exactly as shown in Fig. 2. In order to demonstrate more clearly, the combined annealing and extension steps were set to 30 s. Each video frame denotes 2 s. The thermal gradient is clearly observable.

Gradient PCR

Nine droplets were prepared in each temperature zone. An additional droplet without template (NTC) was positioned in parallel with the fifth droplet, or can be placed anywhere on the chip except for spaces already taken up by the nine droplets. With a thermal gradient, PCR experiments can be optimized in a single run. The amplification curves of the reaction are shown in Fig. 4(a). The intensity plots reveal that droplet 4 has the highest fluorescence intensity. The best yield of the product was acquired at 61.04 °C.

Melt curve analysis was run to testify the specificity of the product, as shown in Fig. 4(b). Robust, unique, and clean products were obtained during the amplification, without any secondary products such as primer-dimers.

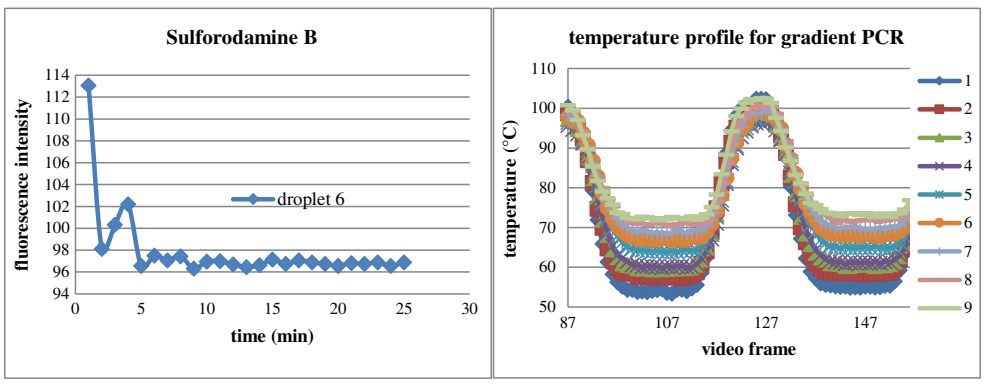

Fig. 3 **a** Instrument equilibration of sulforhodamine B assessed at 55 °C. After 20 min, no evident change in fluorescence as well as evidence of photo bleaching was observed. **b** Temperature profile for gradient PCR. Each line denotes one sample as illustrated in Fig. 2

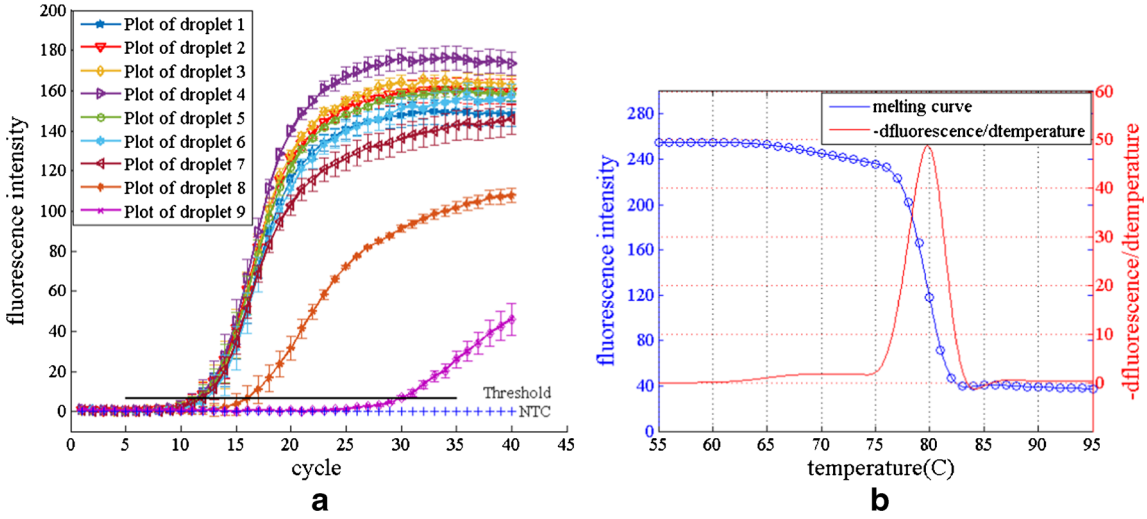

Fig. 4 **a** Amplification curves of gradient PCR. NTC denotes no template control. **b** Melt curve analysis of droplet 4 with the first derivative of the change in fluorescence intensity as a function of the temperature; meanwhile, only a single peak corresponding to the PCR product is observed. The amplicon is clean and specific

Another group of experiments was performed on 96 Universal Gradient, PeQSTAR. Since it is not a real-time machine, gel image of the results was taken by a Bio-Rad imager. The gel lanes of the gel image processed using ImageJ as shown in Fig. 5 show that 61.6 °C was the optimal temperature. The result is in accordance with the result from our device.

Figure 6(a) shows the temperature calculated from the calibration constant (the blue curve). The red curve represents the temperature measured by direct contact of the temperature sensor of the chip without thermal loads. No temporal delay was taken into consideration. Both methods showed an almost linear thermal gradient. Since the droplet volume is small, and so is the temperature sensor size, the discrepancies between the two methods can be neglected. However, care must be taken when using a large volume (thermal loads) for the reaction (most commercial devices use large volumes).

In order to determine the relationship between fluorescence intensity and the final concentration, the results were transferred to a BioAnalyzer 2100 for further analysis. After running the analysis, a correlation analysis between the temperature gradient and the product concentration was performed in Fig. 6(b). The y-axis is the BioAnalyzer analysis from the annealing temperature optimization experiment. The optimal temperature was 60.62 °C.

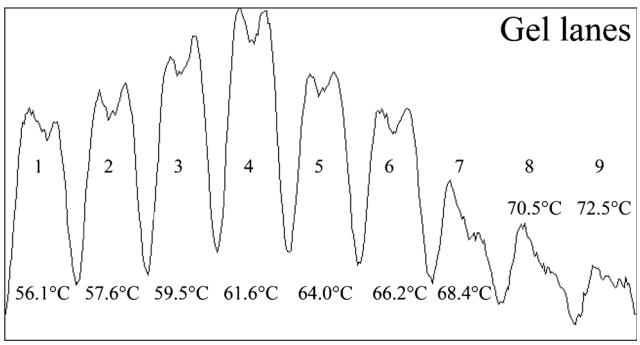

Fig. 5 Agarose gel result of PCR run on a commercial 96 Universal Gradient, PeQSTAR. The columns are gel lanes of the target amplicons. Lane 4 has the best result

Discussion

Different dyes react differently to temperature changes. The fluorescence of most dyes decreases as the temperature is increased. The exact temperature-time course of the sample can be monitored through the use of a temperature-sensitive passive reference dye, which can provide solution temperatures in real time throughout the thermal cycling. Therefore potentially controls the solution temperature. Sulforhodamine B was chosen because of its high temperature sensitivity and stability over repeated heating and cooling cycles. In addition to evaporation and/or condensation, other potential artifacts include instrument drift, thermal degradation of the dye, and fluorescence quenching, all of which might affect fluorescence signal as well. To use fluorescence to monitor temperature, fluorescence variations must be attributable to temperature. After 20 min, no evident change in fluorescence was observed. In addition, no evidence of photo bleaching of sulforhodamine B was observed.

The calibration constant is dependent on the physical characteristics of the fluorescent molecules in a particular solvent. It provides a quantitative way of judging the overall temperature sensitivity of the dye and optics. Different calibration constants range from 1314 ~ 1487 *K* for sulforhodamine B,

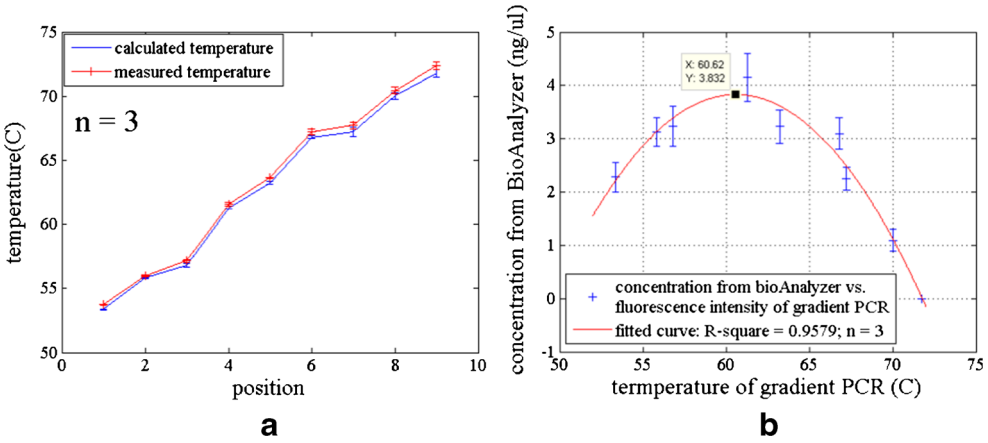

Fig. 6 **a** Thermal gradient formed from droplet 1 to 9. **b** Correlation between the thermal gradient and the BioAnalyzer analysis of amplicon concentrations

with an accuracy of ±0.8 ~ 8%. Continuous acquisition throughout temperature cycling and melting was possible. A higher value of the calibration constant correlates to greater temperature sensitivity and system precision. Solution temperatures were determined using the calibration constant, I_{ref} and T_{ref} as shown in formula (1).

The reaction chamber was made by encapsulation of a water-based sample in mineral oil. As no solid cover or micro channels were required, device fabrications consisted only of deposition and patterning the substrate using chemical vapor deposition. The use of disposable glass slides prevents cross-contamination. The small droplet shape minimized the temperature gradient throughout the droplet. Furthermore, the disposable coverslip was not subject to any processing. The glass thermal conductivity coefficient is 1.1 $Wm^{-1}K^{-1}$, while the surrounding air has a thermal conductivity coefficient of only 0.025 $Wm^{-1}K^{-1}$. Therefore, the temperature of the glass will be determined only by the temperature of the silicon wafer attached to the thermoelectric coolers.

The outcome of optimizing the annealing temperature under a single gradient experiment with the primer set (melting temperature 60.6 °C) was successful under a gradient range of 52 °C to 72 °C. The primer set displayed a range of annealing temperatures that can successfully amplify the specific amplicon. The experiment demonstrates the possibility of optimizing a primer set using a single PCR protocol with a selected range of temperatures. This was also confirmed by running an experiment on a commercial gradient device in house. The gel electrophoresis of the products from the commercial device verified that our device works. PCR was optimized in a single run thanks to the thermal gradient generated based on a temperature-dependent dye. Furthermore, the optimal temperature was related to the relative fluorescence intensity of the gradient PCR, since the fluorescence intensity was proportional to the concentration. Hence, no further post-analysis using a gel or BioAnalyzer is required, saving a lot of time and effort. Moreover, because only a very small volume of the reagent mixture is needed for optimization, reagent costs and sample consumption can be highly reduced. Finally, the device is easy to operate. However, this system is not perfect, such as the droplet preparation and alignment have to be done manually.

Conclusion

A small and simple device with a thermal gradient to optimize PCR was designed, with real-time monitoring of the gradient based on a temperature-dependent dye. This was achieved with no direct contact of the temperature sensor, no time delay and no discrepancies between the device and the droplet inside the oil. The gradient feature greatly reduced the time devoted to determining the optimal annealing temperature. The device is cheap, easy to operate and time-saving. Moreover, more gradients can be obtained using smaller and more tightly arranged droplets. The gradient feature is not limited to the annealing step but also allows for the optimization of the denaturation or extension temperature in one experiment as well. We expect that this temperature gradient feature will be used to optimize many reactions in the future.

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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