Published in final edited form as:

Anal Chem. 2012 March 20; 84(6): 2825–2829. doi:10.1021/ac203307h.

Quantitative polymerase chain reaction using infrared heating on a microfluidic chip

Yingjie Yu^{*,1}, Bowei Li^{*,1}, Christopher A. Baker¹, Xinyu Zhang¹, and Michael G. Roper^{†,1,2}

¹Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL, 32311

²Program in Molecular Biophysics, Florida State University, Tallahassee, FL, 32311

Abstract

IR-mediated PCR in microdevices is an established technique for rapid amplification of nucleic acids. In this report, we have expanded the applicability of IR-PCR to quantitative determination of starting copy number by integrating fluorescence detection during the amplification process. Placing the microfluidic device between an IR longpass filter and a hot mirror reduced the background to a level that enabled fluorescence measurements to be made throughout the thermal cycling process. The average fluorescence intensity during the extension step showed the expected trend of an exponential increase followed by a plateau phase in successive cycles. PUC19 template at different starting copy numbers were amplified and the threshold cycle showed an increase for decreasing amounts of starting DNA. Amplification efficiency was 80% and gel separation indicated no detectable non-specific product. A melting curve was generated using IR heating and indicated a melting temperature of 85 °C for the 304 bp amplicon which compared well to the melting temperature obtained using a conventional PCR system. This methodology will be applicable in other types of IR-mediated amplification systems, such as isothermal amplification and in highly integrated systems that combine pre- and post-PCR processes.

Keywords

qPCR; IR; fluorescence; microchip

INTRODUCTION

Amplification of DNA by the polymerase chain reaction (PCR) has revolutionized multiple fields from molecular biology to forensic science. To further the use of PCR in sample-limited settings, microfluidic devices have been utilized.¹⁻³ As these microfluidic systems continue to be developed, a number of advantages have emerged besides simply reducing the volume of sample and reagents used. Numerous upstream and downstream sample-processing steps have been integrated with PCR enabling rapid analyses from complex samples.^{4,5}

Another benefit to microfluidic devices is the ability to perform rapid PCR amplification due to the small thermal mass of the device or the solutions within the device. Rapid amplification of DNA is useful in point-of-care devices and in situations where high throughput screening is needed, such as forensic cases or in large clinical studies.⁶ Contact heating, where thermal elements are in direct contact with the microfluidic device, offer the

[†]Address Correspondence to: Dr. Michael G. Roper, Department of Chemistry and Biochemistry, Florida State University, 95 Chieftain Way, Dittmer Building, Tallahassee, FL 32306, Ph 850-644-1846, Fx 850-644-8281, roper@chem.fsu.edu.

^{*}authors contributed equally

ability to integrate fabrication of heating elements within the microfluidic system and can produce rapid heating rates.^{3,7,8} Non-contact heating methods, such as obtained by heating with IR radiation, have also been used for rapid thermal cycling since nL to pL volumes can be selectively heated, producing rapid heating and cooling rates.⁹⁻¹¹ Another advantage of IR-PCR is that the temperature control system can be built outside the microfluidic device allowing simple and inexpensive devices to be used, which could lead to disposable devices.¹²

IR-PCR has been integrated with multiple sample processing steps, including upstream solid phase extraction of genetic materials from complex samples¹³ and downstream gel separations to determine the size of the amplified fragment.¹⁴ One process that has not received as much attention is integration of fluorescence detection during DNA amplification which allows quantitative determination of the amount of starting genetic material. Rapid amplification and quantitation of DNA within droplets in a petri dish using IR heating has been performed demonstrating that this method is feasible.^{15,16} In the future, this droplet-based amplification strategy may be incorporated within microfluidic structures to fully take advantage of the ability to integrate with other pre- and post-PCR processes. In this report, we describe a quantitative IR-PCR (IR-qPCR) method within a microfluidic system that integrates low background fluorescence detection while thermal cycling with IR radiation. This method should be applicable to other IR-PCR systems that incorporate other sample handling steps besides PCR.^{4,17,18}

MATERIALS AND METHODS

Chemicals and reagents

HNO₃, HCl, NaOH, and ethylenediaminetetraacetic acid (EDTA), and H₂SO₄ were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). HF and sodium borate were from Fisher Scientific (Pittsburgh, PA). Fluorescein, SigmaCote, and tris(hydroxymethyl)aminomethane (Tris), were from Sigma-Aldrich (St. Louis, MO). Low molecular weight DNA ladder (N3233S) was from New England BioLabs (Ipswich, MA). DNA primers and YO-PRO-1 were from Invitrogen (Carlsbad, CA). PCR mastermix was SsoFast EvaGreen Supermix with SYBR Green (Bio-Rad, Hercules, CA). All solutions were made with Milli-Q (Millipore, Bedford, MA) 18 MΩ·cm deionized water.

Microfluidic device

The device was fabricated in glass as previously described.¹⁹ Two identical 2 cm channels were fabricated, one for the thermocouple and the other for PCR. In the center of each channel was an ellipse that was 0.75 × 3 mm width × length prior to etching. The two ellipses were separated by 1.7 mm (center-to-center). The design was etched 300 lm deep using a stirred HF/HNO₃/H₂O solution. Fluid access holes were drilled with diamond-tipped drill bits (Tripple Ripple, 1.1 mm diameter, Crystalite Corp., Lewis Center, OH) at the end of each channel. The etched glass piece and another piece of glass were cleaned for 30 min with a 3:1 (v:v) solution of H₂SO₄/H₂O₂ followed by 30 min with a 5:1:1 (v:v:v) solution of H₂O/NH₄OH/H₂O₂. The two pieces of glass were then thermally annealed at 640 °C for 8 h.

Temperature control system

The temperature control system consisted of a tungsten (W) lamp (CXL, 8 V, 50 W, Ushio, Inc., Tokyo, Japan) to provide the infrared radiation, a pressurized air source for cooling, and a thermocouple (T-240C, Physitemp Instruments, Inc., Clifton, NJ) for temperature measurement, all controlled by a custom program (LabView, National Instruments, Austin, TX). The W-lamp was positioned 3.5 cm above the device so that the ellipses were in the focal spot of the lamp. An 8 V power supply was used to power the lamp via a TTL-

controlled solid-state relay (CMX60D10, Crydom Co., San Diego, CA). When the output from the data acquisition (DAQ) card (PCI-6221, National Instruments, Austin, TX) was 5V, the relay closed, completing the circuit to the lamp and turning it on. The TTL line was actuated at 1000 Hz with the duty cycle controlled by the program as a function of the temperature measured by the thermocouple. A 715 nm longpass colored glass filter (FGL715S, 2 inch square, ThorLabs, Inc., Newton, NJ) was placed 2.5 cm above the microfluidic device to block the majority of the visible light from the W-lamp. The signal from the thermocouple was digitized and amplified 25-fold using a commercially available circuit (TAC80B-T, Omega Engineering, Stamford, CT) and further amplified 75-fold using a homemade circuit prior to input into the DAQ card. The thermocouple output voltage was calibrated with a two-point calibration using deionized water at room temperature and the boiling point of water. The air pump delivered pressurized air (~10 psi) through a computer-controlled solenoid valve (Model A00SC232P, Parker Hannifin Corp., Cleveland, OH) to the top of the microfluidic device. The timing of this solenoid valve was controlled by the program and corresponded to the cooling from denaturation to annealing.

Fluorescence detection

The microfluidic device was placed on a hot mirror (25 mm diameter, 0° angle of incidence, Edmund Optics, Barrington, NJ) on the stage of a Nikon TS100F microscope. Light from a halogen lamp (Intracellular Imaging, Inc., Cincinnati, OH) was made incident on a dichroic cube (XF93, Omega Optical, Brattleboro, VT, USA) in the microscope, and the 488 nm excitation light was focused on the PCR ellipse via a 10X, 0.25 NA objective. Fluorescence was collected by the same objective, passed through the dichroic cube, a bandpass filter (520DF40, Omega Optical), and a spatial filter prior to detection by a photomultiplier tube (PMT). Data was collected at 10 Hz using the same DAQ card and LabView program used for the temperature control system. Unless stated otherwise in the text, the fluorescence signal in all figures is given as the voltage (V) recorded from the PMT.

PCR protocol

The two channels were rinsed with 1 M NaOH, 1M HCl, H₂O, dried, and coated with SigmaCote according to manufacturer's directions. The PUC19 PCR template (kindly donated by Rani Dhanarajan, Department of Biological Sciences, Florida State University) and primers were diluted in the PCR mastermix to a final concentration of 0.6 μ M primers. The PCR channel was filled with 10 μ L of this mixture and 5 μ L of mineral oil was used to cover each of the fluidic access holes. A similar procedure was performed for the thermocouple channel except no PUC19 template was included in the mastermix. The amount of DNA template in the PCR channel was 10⁷, 10⁶, 10⁵, or 10⁴ molecules. Amplification of a 304 bp fragment of PUC19 consisted of an initial denaturation step at 95 °C for 60 s, followed by 40 cycles of 95 °C for 5 s, 55 °C for 5 s, and 72 °C for 10 s. The threshold cycle (C_T) was defined as the cycle number at which the fluorescence emission exceeded a threshold, which was set at the average of the baseline plus 10 times its standard deviation. A Bio-Rad C1000 thermocycler with a CFX96 real-time module was used to compare amplification and melting curve results with the microfluidic system.

To verify the size of the amplified DNA, the solution from the PCR channel was removed and separated on a 2.5% agarose gel at 100 V for 30 min using a Tris-Borate-EDTA buffer at pH 8.

RESULTS AND DISCUSSION

IR-PCR is a rapid amplification method that has the potential to be applied to a range of applications from forensic analysis to point of care diagnostics.⁴ Meeting this potential

depends on the ability to integrate amplification with other analytical processes such as solid phase extraction, electrophoresis, or real-time measurements for quantitative determination of the starting copies of DNA. In this report, we describe a method that can be used for successful IR-qPCR.

Heating and fluorescence systems

For accurate fluorescence measurements and to prolong the lifetime of the fluorescence optics, an effort was made to minimize the amount of stray light from the IR heating system to the fluorescence system. Figure 1 details the setup which utilized a longpass IR filter in front of the IR-lamp to reduce the amount of visible radiation that passed to the microfluidic device and to the epifluorescence system under the device. A short-pass filter was placed under the microfluidic device to transmit visible radiation used during fluorescence detection and block stray IR. To further safeguard the fluorescence optics, a shutter was used to block the objective during the initial denaturation step. The shutter was removed after this initial step and was not used again throughout the experiment. The temperature of the objective was measured using an infrared photometer and not found to increase significantly during thermal cycling.

Fluorescence from the PCR chamber was monitored throughout the duration of the experiment. Figure 2A contains both the temperature (black) and fluorescence (blue) traces for 40 temperature cycles using 10^7 starting copies of DNA. The fluorescence fluctuates throughout the cycles because of background from the IR lamp, the temperature-sensitive nature of SYBR Green dye, and the different stages of PCR that had either dsDNA or ssDNA. The overall trend of the fluorescence signal increased beginning at cycle 14 and began to plateau at cycle 32. The entire thermal cycling took 35 minutes.

A zoomed in view of one thermal cycle with the corresponding fluorescence curve is shown in Figure 2B. The vertical dashed lines separate the cycle into stages (1, 2, 3, etc.) listed above the figure. Stage 1 was denaturation at 95°C that produced a relatively low and noisy signal in the fluorescence system. The noise in the system was due to fluctuations in the IR lamp intensity, while the signal is low because dsDNA has melted and the fluorescence was low. Stage 2 occurred as the temperature dropped from 95 to 55 °C. As can be seen, once the IR lamp turned off, the noise in the fluorescence system decreased and the fluorescence began to increase as dsDNA was formed. In stage 3, the temperature was held at 55 °C for 5 s producing a high and relatively stable fluorescence signal. In stage 4, the IR lamp intensity increased to bring the temperature to 72 °C for extension of the primers. In stage 5, the extension temperature was held for 10 s and a stable fluorescence signal was observed with most noise coming from small fluctuations in the temperature. In stage 6, the temperature was raised to the denaturation temperature and the fluorescence signal decreased as all dsDNA melted. The fluorescence in this stage was used for determination of melting temperatures, which will be discussed later. Stage 7 was the denaturation step of the next cycle.

As described previously²⁰, a great deal of information on the strand status during DNA amplification can be obtained by plotting the fluorescence vs. temperature during the experiment (40-cycles). As shown in Figure 3A, when the data is plotted in this manner, the fluorescence intensity alternates between low and high values during each cycle. For an easier visualization, the first 14 cycles, prior to significant accumulation of dsDNA, are colored black, the next 18 cycles (exponential phase) are colored blue, and the last 8 cycles (plateau phase) are colored grey. During the initial cycles, there was only a slight difference in the fluorescence signal during heating and cooling. We believe that this slight difference was due to background signal from the IR lamp since the signal is higher during the heating phase compared to the cooling phase when the lamp was off. During the exponential

amplification stage, the observed fluorescence signal in each cycle was still greater during heating than during cooling, similar to what was observed with SYBRGreen using a different heating method.²⁰ As the sample was heated, the fluorescence signal decreased from a relatively high initial value, but decreased due to melting of nonspecific dsDNA and with decreased fluorescence intensity of SYBRGreen at elevated temperatures. The fluorescence signal increased while the temperature was held at 72 °C. At approximately 85 °C, a sharp drop in fluorescence signal was observed due to the melting of specific dsDNA. As the sample cooled, the fluorescence increased due to accumulation of dsDNA. During the plateau phase, the melting and annealing of dsDNA and ssDNA was observed without any further increases in the overall fluorescence signal.

The melting temperature can be deduced from a cycle within the fluorescence vs. temperature plots. As can be seen, the dsDNA melted at ~85 °C during each cycle, but a traditional plot of the change in fluorescence vs. temperature from a single cycle (38th cycle) is shown in Figure 3B. Because of noise in the fluorescence system, an accurate melting curve could not be made directly from the fluorescence data. A 6-point moving average was made to the fluorescence data (blue line) as shown in Figure 3B. The maximum in the change of this fitted curve (black line in Figure 3B) was then used as an indicator of the melting temperature of the amplified product. The melt temperature was 85 °C which correlated well with the melting temperature of 85 °C obtained in a commercial instrument. One important note is that the melting temperature was obtained from a cycle within the entire thermal cycling program in our system. A more accurate melt temperature could have been generated by slowing the temperature increase to obtain a higher resolution scan, but we did not feel this was necessary due to the similarities of the melting temperatures obtained in the IR-mediated amplification and that found in the commercial instrument.

Quantitative PCR

Due to the presence of nonspecific dsDNA during stage 3, the average fluorescence measured in stage 5 during all thermal cycles from the data shown in Figure 2A are shown in Figure 4A. The expected trend was observed where the average fluorescence intensity increased exponentially followed by a plateau phase. Gel electrophoresis was used to confirm the purity of the amplified product as shown by the inset of Figure 4A. A fragment at 304 bp was observed with no detectable DNA at other sizes, confirming specific amplification of the template. Amplifications at other concentrations of DNA starting copies (10^6 , 10^5 , and 10^4) and three replicates of each concentration were then performed. The average intensity during stage 5 from all PCR reactions are shown in Figure 4B as a function of cycle number. Negative controls were also performed in a similar manner using sterile water instead of DNA template. As can be seen, as the starting copy number decreased, more cycles were required for amplification above the fluorescence threshold. The negative control also produced an amplified signal above cycle 35, which is not uncommon to observe using SYBRGreen dye.

The inset of Figure 4B shows the linear trend of the cycle threshold vs. log starting copy number. The best-fit line was $y = -3.9x + 42.2$, $r^2 = 0.9909$ which was used to extract a value of 80% for the PCR efficiency. Although there have been no other reports of PCR efficiency in IR-mediated PCR, similar efficiencies have been described with different template DNA and heating methods in microfluidic devices.²¹⁻²³ The negative control showed amplification above 35 cycles, corresponding to a limit of detection of 70 copies of DNA. This value is similar to limits of detection found in other microfluidic PCR systems.⁵ Combining the low limits of detection reported here with the ability to perform rapid thermal cycling by IR-PCR (< 1 min/cycle), and ease of integration of multiple pre- and post-PCR steps^{4,13,14}, the IR-qPCR method should be useful for many practical applications.

CONCLUSION

The combination of IR-mediated heating with fluorescence detection was successfully used for quantitative PCR amplifications in a microfluidic device. Using the proper filter combinations, the fluorescence background was minimally affected by the IR lamp allowing the fluorescence generated during the entire thermal cycling process to be observed. PCR efficiency was calculated and correlated well with other microfluidic amplification reports indicating that the heating method and treatment of the microfluidic substrate were effective. This system should be applicable to other amplification strategies such as multiplex PCR, isothermal amplification, or in combination with numerous pre- and post-PCR processes allowing a highly integrated system.

Acknowledgments

This project was supported with a grant from the National Institutes of Health (R01 DK080714).

REFERENCES

1. Kopp MU, de Mello AJ, Manz A. *Science*. 1998; 280:1046–1048. [PubMed: 9582111]
2. Burns MA, Johnson BN, Brahmasandra SN, Handique K, Webster JR, Krishnan M, Sammarco TS, Man PM, Jones D, Heldsinger D, Mastrangelo CH, Burke DT. *Science*. 1998; 282:484–487. [PubMed: 9774277]
3. Hashimoto M, Chen PC, Mitchell MW, Nikitopoulos DE, Soper SA, Murphy MC. *Lab Chip*. 2004; 4:638–645. [PubMed: 15570378]
4. Easley CJ, Karlinsey JM, Bienvenue JM, Legendre LA, Roper MG, Feldman SH, Hughes MA, Hewlett EL, Merkel TJ, Ferrance JP, Landers J. *Proc. Natl. Acad. Sci. USA*. 2006; 103:19272–19277. [PubMed: 17159153]
5. Liu P, Li X, Greenspoon SA, Scherer JR, Mathies RA. *Lab Chip*. 2011; 11:1041–1048. [PubMed: 21293830]
6. Liu P, Mathies RA. *Trends Biotechnol*. 2009; 27:572–581. [PubMed: 19709772]
7. Burns MA, Mastrangelo CH, Sammarco TS, Man FP, Webster JR, Johnson BN, Foerster B, Jones D, Fields Y, Kaiser AR, Burke DT. *Proc. Natl. Acad. Sci. USA*. 1996; 93:5556–5561. [PubMed: 8643614]
8. Lagally ET, Medintz I, Mathies RA. *Anal. Chem*. 2001; 73:565–570. [PubMed: 11217764]
9. Oda RP, Strausbauch MA, Huhmer AFR, Borson N, Jurens SR, Craighead J, Wettstein PJ, Eckloff B, Kline B, Landers JP. *Anal. Chem*. 1998; 70:4361–4368. [PubMed: 9796420]
10. Huhmer AFR, Landers JP. *Anal. Chem*. 2000; 72:5507–5512. [PubMed: 11080907]
11. Giordano BC, Ferrance J, Swedberg S, Huhmer AFR, Landers JP. *Anal. Biochem*. 2001; 291:124–132. [PubMed: 11262165]
12. Roper MG, Easley CJ, Legendre LA, Humphrey JAC, Landers JP. *Anal. Chem*. 2007; 79:1294–1300. [PubMed: 17297927]
13. Legendre LA, Bienvenue JM, Roper MG, Ferrance JP, Landers JP. *Anal. Chem*. 2006; 78:1444–1451. [PubMed: 16503592]
14. Easley CJ, Karlinsey JM, Landers JP. *Lab Chip*. 2006; 6:601–610. [PubMed: 16652175]
15. Kim H, Dixit S, Green CJ, Faris GW. *Opt. Express*. 2009; 17:218–227. [PubMed: 19129891]
16. Kim H, Vishniakou S, Faris GW. *Lab Chip*. 2009; 9:1230–1235. [PubMed: 19370241]
17. Hagan KA, Meier WL, Ferrance JP, Landers JP. *Anal. Chem*. 2009; 81:5249–5256. [PubMed: 19514712]
18. Hagan KA, Reedy CR, Uchimoto ML, Basu D, Engel DA, Landers JP. *Lab Chip*. 2011; 11:957–961. [PubMed: 21152489]
19. Zhang X, Roper MG. *Anal. Chem*. 2009; 81:1162–1168. [PubMed: 19178342]
20. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. *Biotechniques*. 1997; 22:130–138. [PubMed: 8994660]

21. Walsh EJ, King C, Grimes R, Gonzalez A. *Biomed. Microdevices*. 2006; 8:59–64. [PubMed: 16491332]
22. Chen L, West J, Auroux P-A, Manz A, Day PJR. *Anal. Chem*. 2007; 79:9185–9190. [PubMed: 17979297]
23. Wang J-H, Chien L-J, Hsieh T-M, Luo C-H, Chou W-P, Chen P-H, Chen PJ, Lee D-S, Lee G-B. *Sens. Actuat. B*. 2009; 141:329–337.

Figure 1. Layout of the infrared heating and fluorescence detection systems

The temperature control system consisted of a W-lamp as the IR source, a thermocouple for temperature measurement, a compressed air source for cooling, and electrical relays used to turn on and off each of these components. The fluorescence system used a halogen lamp for excitation and a photomultiplier tube (PMT) for detection. A 775 nm longpass IR filter and hot mirror were used to decouple the two optical systems.

Figure 2. Simultaneous collection of temperature and fluorescence

In all graphs, temperature is plotted as the black line and corresponds to the left y-axis and PMT voltage from SYBRGreen fluorescence is plotted as the blue line and corresponds to the right y-axis. **A.** 10^7 starting copies of PUC19 were amplified by PCR in 40 cycles. The fluorescence intensity varied as a function of temperature and cycle, but the overall trend indicated that the intensity increased starting at approximately the 14th cycle and reached a plateau at approximately cycle 30. **B.** A single temperature cycle and fluorescence data from **A** is shown. As explained further in the text, the stages are shown at the top of the figure and correspond to different times during the thermal cycling, such as denaturation (stage 1), annealing (stage 3), or extension (stage 5).

Figure 3. Continuous monitoring of fluorescence during amplification

A. The fluorescence from Figure 2A is plotted against its corresponding temperature. The different colors represent the cycles that belong to the initial cycling (black), exponential phase (blue), and plateau phase (grey). During the exponential phase, the fluorescence signal increased during each cycle. Within each cycle, the fluorescence signal slowly declined as the temperature was increased until specific dsDNA melted at ~85 °C. During cooling, the fluorescence signal increased due to formation of dsDNA. **B.** The melting curve of PUC19 product was obtained from the 38th cycle in **A**. The fluorescence (blue line) shown was smoothed with a 6-point moving average and shown on the left y-axis. The melting temperature was determined as the temperature that produced the largest rate of change in fluorescence (black line, right y-axis).

Figure 4. Quantitative PCR of PUC19

A. The average fluorescence intensity during stage 5 (extension) from all 40 cycles from Figure 2A are plotted. Each data point represents the average of three replicates with the error bars corresponding to one standard deviation. The inset is an image of a gel separation of the amplified product with the 300 bp fragment of the ladder indicated on the right. Lane 1: DNA ladder, lane 2: microfluidic IR-PCR sample using 10^7 starting copies, lane 3: a second microfluidic IRPCR amplification using 10^7 starting copies, and lane 4: DNA ladder.

B. The average fluorescence intensities during the extension (stage 5) from all PCR reactions for PUC19 at starting concentrations of 10^7 (■), 10^6 (◆), 10^5 (▲), 10^4 (○), and the negative control (*). The grey horizontal line indicates the threshold that was used for determination of C_T . The inset is a plot of C_T vs. log starting copy number, which was used to determine amplification efficiency.