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Enhanced nucleic acid amplification with blood *in situ* by wire-guided droplet manipulation (WDM)

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Abstract

There are many challenges facing the use of molecular biology to provide pertinent information in a timely, cost effective manner. Wire-guided droplet manipulation (WDM) is an emerging format for conducting molecular biology with unique characteristics to address these challenges. To demonstrate the use of WDM, an apparatus was designed and assembled to automate polymerase chain reaction (PCR) on a reprogrammable platform. WDM minimizes thermal resistance by convective heat transfer to a constantly moving droplet in direct contact with heated silicone oil. PCR amplification of the GAPDH gene was demonstrated at a speed of 8.67 sec/cycle. Conventional PCR was shown to be inhibited by the presence of blood. WDM PCR utilizes molecular partitioning of nucleic acids and other PCR reagents from blood components, within the water-in-oil droplet, to increase PCR reaction efficiency with blood *in situ*. The ability to amplify nucleic acids in the presence of blood simplifies pre-treatment protocols towards true point-of-care diagnostic use. The 16s rRNA hypervariable regions V3 and V6 were amplified from *Klebsiella pneumoniae* genomic DNA with blood *in situ*. The detection limit of WDM PCR was 1 ng/μL or 10⁵ genomes/μL with blood *in situ*. The application of WDM for rapid, automated detection of bacterial DNA from whole blood may have an enormous impact on the clinical diagnosis of infections in bloodstream or chronic wound/ulcer, and patient safety and morbidity.

Keywords

PCR; blood infection; 16s rRNA; molecular partitioning

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1. Introduction

The conventional wisdom of molecular biology tells us reagents should be handled with pipettes, and reactions should be conducted in plastic tubes or micro titer plates. A recently emerging technique known as, wire-guided droplet manipulation (WDM), offers a radically different format for conducting experiments, reactions and assays. In WDM, a wire or needle tip manipulates microlitersized droplets in a hydrophobic milieu. The attributes of this revolutionarily different format will address some of the challenges facing the use of conventional techniques and provide solutions toward the development of automated, sample-to-answer, point-of-care systems with potential applications in medicine, life science research, forensics, veterinary diagnostics and disease control in the developing world.

WDM allows for efficient heating and cooling of reactions by maximizing the heat transfer coefficient. The heat transfer mode is convection as the droplet is moved continuously in a temperature controlled hydrophobic fluid. The thermal resistance is also minimized by direct contact of the droplet with the heated fluid. In conventional techniques, the plastic wall of the micro titer plate creates a thermal isolation barrier. Another consequence of the aqueous droplet interfacing with the hydrophobic fluid is molecular partitioning. Molecular partitioning can be leveraged in WDM to separate components of a complex sample matrix such as human whole blood. Erythrocytes will be lysed and release hemoglobin into the extracellular environment when exposed to temperatures above 70°C for longer than 0.3 seconds (Fildes et al., 1998). The phospholipids that make up the erythrocyte membranes will dissociate and become an ideal surfactant to adsorb to the oil-water interface due to their relatively small size, polar head group, and hydrophobic fatty acid tails. Static self-assembly of lipids into membranes is a well-known phenomenon (Whitesides and Grzybowski, 2002; Jones and Chapman, 1995) and has been utilized to create lipid bilayers at the interface of sessile droplets (Poulos et al., 2010). Proteins differentially adsorb and denature at the oil-water interface depending on properties such as size, surface hydrophobicity, and surface charge (Li et al., 2012; Keerati-u-rai et al., 2012, Tubio et al., 2004). Other investigators have shown the utility of this molecular self-assembly at oil-water interfaces to specifically control aggregation of droplets in emulsion (Hadorn and Boenzli, 2012) and to create amphiphilic nanoparticles (Andala et al., 2012).

Controlled manipulations of droplets for the miniaturization of chemical and biological analysis, referred to as droplet or digital microfluidics, can be utilized. Several methods have been demonstrated, most notably magnetofluidics (Egatz-Gómez et al., 2006) and electrowetting (Cho et al., 2003a). While these methods may seem potentially useful, they are associated with complications in fabrication and operation that prohibit their widespread adoption. In magnetofluidics, paramagnetic nanoparticles are necessarily suspended in the reaction mixture to control droplet movement with an external magnetic field (Egatz-Gómez et al., 2006; Ohashi et al., 2007). These particles can cause reaction interference (Yoon and Kim, 2012) and add an extraneous cost to the procedure. Devices designed for control of droplet movement by electrowetting-on-dielectric (EWOD) use a dielectric layer to create charge separation on the surface (Cho et al., 2003a; Yoon and Garrell, 2003). Dielectric breakdown of this layer will lead to electron tunneling and failure of the device after sustained use (Cho et al., 2003b; Chang et al., 2006). There have been some efforts in conducting chemical and biological analysis, including PCR, in straight microchannels (Zhang and Xing, 2007), but these devices are not reconfigurable and must be designed for a single dedicated use. In procedures such as PCR, where cyclical heating and cooling is necessary, plug flow is required to avoid axial dispersion of molecules (Chen et al., 2005) and creation of a heat gradient across microchannels. The speed at which reactions can be performed is limited by the need to maintain plug flow.

WDM can be applied to standard protocols and is easily reprogrammable for different uses. Serial dilution, vibrational mixing, sample concentration by high-speed centrifugation, DNA extraction (lysing, precipitation, washing and rehydration) and rapid thermocycling can all be automated by WDM (You and Yoon, 2012). The principles of droplet manipulation can be easily integrated into the existing ideology of scientific automation using a commercially available robotic pipetting system. Automation, reprogrammability, ease of use, and robustness are essential features of all-in-one, sample-to-answer systems to be used at the point-of-care, making WDM ideal for use in such rapid molecular diagnostic systems.

We have identified PCR as a preliminary application of WDM and have designed and fabricated a dedicated apparatus for conducting PCR by WDM in an automated manner. We have investigated its potential impact on the diagnosis of infections, such as bloodstream infection (BSI) and chronic wound and ulcer infections, by amplification of hypervariable regions of the *Klebsiella pneumoniae* 16s rRNA gene with blood *in situ*. The 16s rRNA gene is the most commonly used gene in taxonomic classification of bacteria (Clarridge, 2004). Specific identification of single or multiple 16s rRNA gene hypervariable regions allows phylogenetic placement of bacteria to either the species or genus level (Cole et al., 2009; Wang et al., 2007; Liu et al., 2007; Sundquist et al., 2007; Huse et al., 2007; Dowd et al., 2008; Wang and Qian, 2009; Liu et al., 2008). Amplification of a relatively short PCR amplicon, 200-bp, will enable species to genus level identification of the causative agents in BSI. The extreme sensitivity of PCR eliminates the need for slow blood culture. The automation and rapid heat transfer of WDM minimize the skilled man-hours needed to diagnose the BSI. The molecular partitioning further simplifies the diagnosis by eradicating the need for sample purification and decreases the concern for inefficient extraction procedures.

Clinical diagnosis of infection in blood is currently inhibited by the need for growth-based, time-consuming techniques carried out by trained personnel in a laboratory setting. Blood culture has the tendency to be selective for certain pathogens, is subject to contamination by skin flora, and is unable to provide useful information until 3–5 days later (Weinstein and Doern, 2011). Delayed diagnosis creates the need for empirical, broad-spectrum antibiotic therapy which may be unnecessarily broad or inadequately narrow (Lipsky and Berendt, 2000) and creates a selective pressure towards development of antimicrobial resistance (Baumgart et al., 2010; Lucignano et al., 2011). The lack of information about the presence of antimicrobial resistant bacteria disables the physician to appropriately prescribe antimicrobial therapy (Lipsky and Berendt, 2000).

Rapid diagnostic tools to identify the cause of infection in blood are of extreme clinical importance due to the high mortality rates (49.7% overall for BSI) (Balk et al., 2001; Rice et al., 2013), significant morbidity, extreme cost of patient care (Kollef et al., 1999; Chakravorty et al., 2010; HCUP, 2009), and rising antimicrobial resistance rates (NNIS System, 2004). Rapid, point-of-care detection enables early use of targeted antibiotic therapy, to reduce morbidity and mortality, and timely isolation of colonized patients, to minimize transmission of antimicrobial resistance through the hospital setting (Akova et al., 2012). A recent outbreak of *Klebsiella pneumoniae* has shown that even the most sophisticated medical facilities are not impervious to the spread of antimicrobial resistance and has highlighted the need to quickly isolate the infection (Kolata, 2012).

2. Materials and Method

2.1. Polymerase chain reaction

PCR reaction mixtures were prepared on ice immediately prior to thermocycling. The reaction mixture contained Promega GoTaq® Green Master Mix, forward primer (10 µM),

reverse primers (10 μ M), purified target DNA and nuclease free water (in the ratio 5:1:1:1:2). Purified *K. pneumoniae* strain Z026 genomic DNA (1 ng/ μ L) was obtained from Zyptometrix and a synthetic GAPDH oligonucleotide (50 ng/ μ L) was obtained from Integrated DNA Technologies. For the reactions containing whole blood, one part nuclease free water was replaced with 10% whole blood. Whole blood was stored refrigerated in a BD Vacutainer® CPT™ Cell Preparation Tube with Sodium Heparin^N (BD Bioscience; 8362834). The MJ Research PTC 150 Minicycler was used for conventional thermocycling. A superficial layer of mineral oil (Sigma; M5904) was used to avoid evaporation. Universal primers were chosen to amplify the V3 and V6 hypervariable regions of the 16s rRNA gene from bacteria (Sundquist et al., 2007). The V3 primers, 338F (5'-ACT CCT ACG GGA GGC AGC AG -3') and 534R (5'- ATT ACC GCG GCT GCT GG -3'), amplify a 196-bp region. The V6 primers, 907F (5'- AAA CTC AAA KGA ATT GAC GG -3') and 1073R (5'- ACG AGC TGA CGA CAR CCA TG -3'), amplify a 166-bp region (Sundquist et al., 2007). The PCR conditions for the 16s rRNA gene primers were 98°C for 3 min, 30 cycles of 98°C for 30 s, 58°C for 30 s, and 72°C for 40 s, followed by extension for 10 min at 72°C. A 143-bp sDNA oligonucleotide served as the GAPDH gene target. The concentration of the GAPDH target in the PCR reaction mixture was 10¹⁰ copies/ μ L. The sequence of forward primer was 5'-ACATCGCTCAGACACCATG -3' and the reverse primer was 5'-TGTAGTTGAGGTCAATGAAGGG -3'. The PCR conditions for the GAPDH gene primers were 94°C for 4 min, 30 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 30 s, followed by extension for 10 min at 72°C.

2.2. WDM thermocycling apparatus

The droplet thermocycling apparatus consists of a rotationally translatable syringe mount (Fig. 1). The syringe plunger is automated by a 12V linear stepper motor (MPJA; 18313MS) residing in the rotating frame. Electrical connection is made to the rotating frame by a through bore slip ring (Keyo Electric Co.; KYH12) to avoid tangling of wires. The entire rotational assembly is translated vertically by a bipolar stepper motor (Phidgets; NEMA 17) connected to a lead screw. The vertical movement is stabilized and guided by two 20 mm linear ball bearings on chrome plated shafts (330.2 mm length) (VXB Ball Bearings). The function of the three motors is automated by a reprogrammable Arduino Mega microcontroller and Easydriver stepper motor controllers (Sparkfun Electronics). There are three heated silicone oil (Fisher Scientific; S159) baths; each is independently maintained by a proportional-integrative-derivative (PID) controller within $\pm 0.25^\circ\text{C}$ of the temperatures required for denaturation, annealing or extension (You et al., 2011). The PID controller consists of an Arduino Uno microcontroller, PID algorithm programming, and a custom transistor circuit. The heating element is a flexible heater (All Flex Heaters; P0225-RA100) fixed to the bottom of each chamber. The temperature is monitored using DS18B20 1-Wire temperature sensors and is displayed on a serial-enabled liquid crystal display (LCD) (Sparkfun Electronics). The baths are connected by thin channels, and oil is prevented from mixing by a flexible plastic film (3M transparency sheets) at the entrance and exit of each bath. The oil temperature set points are adjustable by buttons activated by the user. The entire system was powered by a 0–30 V, 3 A bench-top power supply and a 2 A bench-top power supply (MPJA). The WDM thermocycling apparatus was designed using SolidWorks computer aided design software, and custom components were fabricated by rapid prototyping using acrylonitrile butadiene styrene (ABS) polymer (Dimension uPrint SE).

2.3. WDM thermocycling

The sample is loaded into a single-use, Luer-Lok, 15-gauge blunt needle tip (Jensen Global; JG15-0.5X) by the user with a button controlling the syringe plunger movement. First, 15 μ L of mineral oil are drawn into the needle. Subsequently, 15 μ L of the PCR reaction mixture are drawn in. The mineral oil prevents water in the reaction mixture from evaporating into

the chamber of the syringe and helps to minimize the expansion of the air in the syringe chamber during heating. During the PCR protocol, the syringe needle is positioned by the vertical motor so the 304 stainless steel tip is submerged 2.5 mm into the oil. The 8 μ L pendant droplet is then automatically dispensed and moved at an average velocity of 7.2 mm/s to allow for convective heat transfer between the oil and the droplet. The maximum velocity was set below the threshold of the droplet to fall due to excessive drag. Before the syringe moves to the next oil bath, the droplet is drawn into the needle with 5 μ L of silicone oil below the reaction mixture to prevent loss during the movement between oil baths (Supplementary Material 1). A several hundred millisecond pause is necessary due to the delayed response of the droplet to the syringe. Remaining at the same height, the syringe is rotated to the next oil bath and the process continues for n cycles. When all cycles have been completed, the syringe is moved upward by the vertical motor so the sample can be collected in a tube by the user. The oil set point temperatures for amplification of V3 were 95°C, 52°C and 70°C and for V6 they were 98°C, 50°C and 70°C. For both V3 and V6, the reaction mixture was denatured for 6.9 s, annealed for 10.5 s and extended for 18.3 s. The oil set point temperatures for amplification of the GAPDH oligonucleotide were 94°C, 58°C and 69°C and thermocycling speed was 8.67 s/cycle.

2.4. Thermocycling temperature profile measurement

The conventional PCR temperature profile was measured by submersing a thermocouple in water (20 μ L) with a superficial layer of mineral oil (10 μ L) inside a polypropylene PCR Tube (Fisher Scientific; 14230225). The tube was placed in the heater block of the MJ Minicycler. The PCR conditions were 94°C for 30 s, 55°C for 40 s, and 72°C for 40 s. The droplet PCR temperature profile was measured by mounting a thermocouple coaxially inside the syringe needle tip, locating it at the normal position of the reaction droplet. The oil set point temperatures were 104°C, 55°C, and 72°C and the cycle time was 37 s/cycle. In both cases, a temperature reading was taken every second.

2.5. Gel electrophoresis

The PCR products were analyzed using gel electrophoresis. 3% w/v agarose gel (Sigma; A0169) in 1x tris-acetate-EDTA (TAE) buffer (Invitrogen; 24710-030) were prepared and run at 120 V for VC30-50 min with an electrophoresis power supply (Fischer Scientific; FB200). 1 kb-plus ladder (Invitrogen; 10787) was used as a standard for fragment sizing. Gels were stained with ethidium bromide (Sigma; E1510) and imaged under UV light. Gel images were analyzed using ImageJ software (U.S. National Institutes of Health). Gel band intensities were measured and normalized to the background. The one-sample t-test with a 95% confidence interval was used to determine if the gel band intensity was significantly different from the background.

2.6. Interfacial tension measurements

Interfacial tension measurements were made with an FTÅ 200 contact angle and interfacial tension analyzer (First Ten Ångströms). Measurements were taken after the pendant droplet had been hanging for 150 seconds.

3. Results

3.1. Assembly and operation of WDM PCR apparatus

Figure 1 shows the schematic diagram of the WDM PCR apparatus that we have designed, fabricated, and assembled, utilizing 3D printing and Arduino microcontroller technologies. The operation of this apparatus for PCR thermocycling is shown as a video in Supplementary Material 2.

3.2. Convective heat transfer allows rapid heating and cooling of PCR reaction mixture

The differences in heat transfer between WDM thermocycling and conventional thermocycling are illustrated in Figure 2. In WDM thermocycling, the droplet is in direct contact with the heated silicone oil (Fig. 2b). The droplet is continuously moved in the heated oil, and heat transfer is by convection. Heat transfer by convection is more efficient than conduction due to decreased thermal resistance. The thermal resistance of heat transfer to the reaction mixture is also reduced by eliminating the barrier of the plastic tube. Our apparatus (Fig. 1) contains three heated silicone oil baths, all connected with continuous channels, each independently maintained at one of the three temperatures necessary for PCR. The droplet is sequentially moved in between each heated oil bath, and there is no need to wait for heating or cooling of the oil. In conventional thermocycling, a metal heating block is heated up and cooled down to each temperature, serially. Between denaturation temperatures and annealing temperatures, the heater block can take up to 30 seconds to cool (Fig. 2c). This cooling time is completely eliminated in our system, as illustrated by the nearly vertical temperature profile between each reaction phase in Figure 2d. The conventional PCR temperature profile (Fig. 2c) shows that each target temperature is reached momentarily and then heating or cooling to the next temperature resumes. The WDM PCR temperature profile (Fig. 2d) shows that the target temperature is reached almost instantly and then maintained for the majority of that phase of the reaction. The large dT/dt seen by WDM accounts for the ability to thermocycle at extremely fast speeds.

3.3. Blood increases droplet interfacial surface tension (IFT)

Pure nuclease-free water has the highest interfacial tension (71.5 mN/m), shown in Figure 3. The high IFT of pure water reflects the strength of hydrogen bonding forces between water molecules which have a net attractive force pointing towards the center of the droplet. The interfacial tension decreases as contaminants are added to the mixture and the interactions between water molecules at the interface are disrupted. The IFTs for the solutions of bovine serum albumin (BSA) (1.5 $\mu\text{g}/\mu\text{L}$) and sodium heparinized human whole blood (WB) are 60.8 and 53.1 mN/m, respectively. The pure PCR mixture has the lowest IFT (34.1 mN/m). The addition of WB to the PCR mixture causes an increase in IFT from the pure PCR mixture. The relatively high IFTs of BSA and WB result from the presence of proteins (predominantly hemoglobin, albumin, immunoglobulins, and fibrinogen) which are large and partially charged. The relative hydrophobicity of these proteins is much more intense than that of small molecules such as deoxyribonucleotides (dNTPs), DNA primers, and PCR amplicons. When large molecules such as proteins adsorb to the interface, they leave large gaps in between them where water molecules can interact creating a strong, albeit dampened, center pointing force. The PCR mixture on the other hand contains an extraordinarily high concentration of dNTPs and DNA primers. dNTPs contain a negatively charged phosphate group and an aromatic pyrimidine or pyrimidine-imidazole ring which can participate in hydrogen bonds. DNA primers are similarly structured with a phosphate backbone and exposed nucleobases. Adsorption of small molecules, such as dNTPs, DNA primers and ions, to the interface causes complete exclusion of water molecules and disruption of attractive forces responsible for high IFT. The depressed IFT of pure PCR mixture signifies that there is electrostatic repulsion occurring as a result of the negative charge of the phosphate backbone of DNA. The addition of 10% WB to the PCR mixture causes the IFT to rise to 38.6 mN/m. This increase in IFT is evidence that components of blood are replacing dNTPs and primers at the interface and decreasing the disruptive interfacial effect. The PCR components that are replaced by the blood components at the interface become free in solution and available to participate in the PCR reaction.

3.4. Whole blood increases the reaction efficiency in WDM thermocycling

Rapid species level identification of bacteria can be accomplished with a single hypervariable region of the 16s rRNA gene (Chakravorty et al., 2007; Bertilsson et al., 2002; Stohr et al., 2005; Yang et al., 2002; Wada et al., 2010) and the V1, V3 and V6 regions are the most frequently used for analysis of clinical specimens (Liu et al., 2008; Siddiqui et al., 2011; Claesson et al., 2009). We demonstrated amplification of the V3 and V6 regions, the combination of which provides increased specificity of downstream phylogenetic placement (Chakravorty et al., 2007). The V3 hypervariable region of the 16s rRNA gene was detected from *K. pneumoniae* genomic DNA by WDM and conventional thermocycling. Samples of pure DNA, DNA with 10% WB, DNA with 10% WB augmented with 1.5 µg/µL BSA, or DNA with 1.5 µg/µL BSA were thermocycled (Fig. 4a). (Since 1 µL of sample is mixed with 9 µL of PCR reagent, the effective WB concentration in the droplet was effectively 1%.) The DNA concentration for amplification of the V3 region was 1 ng/µL. The *K. pneumoniae* genome is 5.26 Mbp (Shin et al., 2012). The calculated concentration was 10⁵ genomes/µL. All samples are diluted to 1:10 in the final PCR reaction mixture volume.

For the amplification of the V3 region by WDM thermocycling, the reaction efficiency was increased with 10% WB *in situ* compared to pure DNA (Fig. 4a & 4c), as quantified by the normalized band intensity. The samples with 10% WB augmented with 1.5 µg/µL BSA *in situ* and with 1.5 µg/µL BSA *in situ* showed decreased reaction efficiency compared to pure DNA.

For the amplification of the V3 region by conventional thermocycling, the reaction was inhibited with 10% WB *in situ* and with 10% WB augmented with 1.5 µg/µL BSA *in situ* compared to pure DNA (Fig. 4a & 4c).

Whole blood increases the reaction efficiency for WDM thermocycling, while BSA causes a decrease in reaction efficiency. The phospholipids from blood cell membranes act as an ideal surfactant and replace the more polar PCR components, such as primers and dNTPs, at the interface. Albumin, the predominant blood protein, does not provide this beneficial interfacial effect and does not improve reaction efficiency.

The negative template control (NTC) showed no band in either thermocycling method, indicating that cross-reaction contamination is not an issue associated with repeated use of the WDM thermocycling apparatus. The sample is confined to the disposable needle tip, which is not reused, and never comes into contact with the syringe chamber. The silicone oil was reused without causing contamination between reactions due to the unfavorable interaction between DNA and silicone oil.

3.5. WDM thermocycling enables amplification directly from whole blood

The V6 hypervariable region of 16s rRNA gene from *K. pneumoniae* genomic DNA was thermocycled with 10% WB *in situ* (1% of the total reaction volume) by WDM and conventional thermocycling. The DNA concentration for amplification of the V6 region was 1 ng/µL corresponding to a concentration of 10⁵ genomes/µL. Bands for the expected product were detected for all samples by WDM thermocycling while complete inhibition of the reaction was observed for conventional thermocycling with 10% WB *in situ* (Fig. 4b & 4d). The same conventional thermocycling protocol yielded amplification from pure DNA. Multiple samples conducted with the same conditions are shown in Figure 4b demonstrating the reproducibility of WDM thermocycling.

3.6. Detection limit with 10% whole blood *in situ*

The detection limit of WDM PCR method with 10% WB *in situ* was determined by amplification of *K. pneumoniae* genomic DNA with 16s rRNA V6 region primers, shown in Figure 5. While conventional PCR largely failed to amplify the target DNA sequence for the range of genomic DNA concentrations, typically with no detectable bands or with long streaks in the gel images, WDM PCR successfully amplified the target with 10% WB *in situ*, with the detection limit of 1 ng/ μ L genomic DNA (or 10^5 genome copies/ μ L). All band intensities were normalized to the background of gel images, and the average, normalized band intensities were evaluated: 3.0 ± 0.2 (*), 1.52 ± 0.05 (*) and 0.99 ± 0.01 , for 10, 1 and 0.1 ng/ μ L genomic DNA, respectively. An asterisk (*) is used to denote statistical significance by a one-sample t-test where $\alpha = 0.05$ and $t_{\text{critical}} = 3.182$, further confirming the detection limit of 1 ng/ μ L genomic DNA (or 10^5 genome copies/ μ L). These values were also plotted against the genomic DNA concentration, showing a good correlation similar to the exponential function (Figure 5 in the bottom).

3.7. Extremely fast amplification demonstrated for clean and dirty samples

A synthetic, single stranded oligonucleotide representing the GAPDH gene was amplified using extremely fast cycle times of 8.67 seconds from 100% WB (diluted to 10% with PCR reagents in the final reaction volume), and from pure DNA (Fig. 6). This cycle time enables the completion of 20 cycles in 2 min 53 s and of 30 cycles in 4 min 20 s. The GAPDH target oligonucleotide is 143-bp in length, which is much smaller than the *K. pneumoniae* genome. The small size of the GAPDH target and high copy number (10^{10} copies/ μ L) enabled extremely fast amplification from 100% WB and from pure DNA.

4. Discussion

We have investigated the impact of the unique attributes of wire-guided droplet manipulation (WDM) for conducting PCR. Extremely fast, convective thermocycling was achieved by continuous movement of a microliter-sized droplet in direct contact with heated silicone oil. These improvements in heat transfer efficiency enabled the 1–2 hours required for conventional thermocycling to be reduced to 3–5 minutes.

Sodium heparinized whole blood (WB) has a relatively high interfacial tension (IFT), 53.1 mN/m. The high IFT of WB indicates there is ordered self-assembly of blood components at the oil-water (hydrophobic-hydrophilic) boundary. Phospholipids are amphiphilic molecules and an ideal surfactant to interact at the oil-water interface. PCR mixture has a depressed IFT, and when 10% WB is added to PCR mixture, the IFT is increased. This increase in IFT shows that the small negatively charged molecules which are abundant in the PCR mixture are replaced at the interface by blood components.

WDM has increased reaction efficiency with blood *in situ* as a result of molecular partitioning at the oil-water interface. WB acts to passivate the surface of the droplet which minimizes the adsorption of the PCR components to the interface and leaves a higher proportion of the DNA, primers, DNA polymerase, dNTPs and ions free at the soluble core of the droplet where they can react more efficiently. This molecular partitioning enabled us to amplify DNA with WB *in situ*, using WDM, while WB was shown to inhibit the reaction by conventional thermocycling.

The types of molecular interactions at the oil-water interface have a large effect. The reaction efficiency is not improved with the addition of 1.5 μ g/ μ L BSA despite its high IFT, 60.8 mN/m. This can be explained by the large size and surface properties of BSA molecules. When BSA adsorbs to the interface, large spaces are left in between neighboring

molecules because of molecular size (67 kDa) and electrostatic repulsion (BSA is negatively charged at pH = 8.5, the buffering pH of GoTaq® Green Master Mix). In the case of blood, phospholipids and other smaller components will fill gaps between the larger proteins. With phospholipids absent, the PCR reactants are adsorbed to the interface in these gaps reducing reaction efficiency.

The V3 and V6 hypervariable regions of the 16s rRNA gene were successfully amplified from the *Klebsiella pneumoniae* genome, at the detection limit of 1 ng/μL or 10⁵ genomes/μL, with whole blood *in situ.*, while conventional PCR largely failed to amplify these sequences in the presence of whole blood. Extreme speed was demonstrated by amplification of the GAPDH gene at very high copy numbers, 10¹⁰ copies/μL, in the presence of 100% WB, at a speed of 8.67 sec/cycle. This extreme speed highlights the potential for WDM to be used in a rapid, point-of-care system.

We consistently observed blood protein aggregation in conventionally thermocycled reactions to such an extent that the aggregates of denatured blood proteins would obstruct our micropipettes making it challenging to transfer such samples to a gel for electrophoresis. In contrast, the blood formed no such aggregates during WDM thermocycling. Possible explanations for this lack of protein aggregation include continual mixing and the kinetics of heating. Since the droplets are drawn and ejected from the needle tip between each phase of each cycle, the aggregates may be dissociated into smaller pieces that are not observable and allow for pipetting. Since heating is rapid in WDM, the blood is not exposed to the high temperatures for a long time, decreasing the amount of denaturation.

We have demonstrated that WDM can be employed to amplify DNA with whole blood *in situ.* The proteins that usually aggregate in conventional PCR causing inhibition have little effect on WDM PCR. The phospholipids from the cellular membranes act as an ideal surfactant, stabilize the droplet and the reaction efficiency is increased. These characteristics open up new application areas for PCR assays to be used. Potential application areas for WDM PCR include direct amplification and detection from tissue biopsies, preserved tissue specimens, cell cultures, and bacterial cultures. With the additional, inherent benefits of increased speed and automation, these assays can be conducted routinely in clinical care providing physicians with actionable results at the time of initial treatment prescription, leading to improved patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We introduce a new concept (WDM) and apparatus for performing fast PCR for assaying blood sample.
- PCR can be conducted with whole blood through molecular partitioning at oil-water interface.
- The application of WDM will have a huge impact on the clinical diagnosis of infection in blood.

Figure 1.

Important components include the wire-guided droplet manipulation apparatus, the pendant droplet in oil and molecular schematic at the oil-water interface, (a) Mechanical, motor-driven apparatus for automated syringe movement and droplet dispensing. The reaction chamber consists of three independently heated silicone oil baths maintained at each temperature necessary for PCR: 95°C (denaturation), 50–60°C (annealing), and 72°C (extension). (b) The reaction droplet hanging from the syringe needle, continuously moving in the heated oil bath. (c) Schematic of molecular portioning by self-assembly at the oil-water interface.

Figure 2.

Heat transfer schematic highlighting the differences between WDM and conventional PCR, and the temperature profiles for conventional and WDM thermocycling. (a) In WDM PCR, the reaction mixture is submerged in a heated silicone oil bath as a pendant droplet and is moved continuously. Heat transfer is by convection, and the droplet is in direct contact with the heated oil. (b) In conventional PCR, the reaction mixture is contained in a small plastic tube which is in contact with a metal heating block. Heat transfer is by conduction, and the plastic tube thermally insulates the reaction mixture from the heating block, causing inefficiency, (c) The conventional PCR temperature profile, measured by submerging a thermocouple in water in a plastic PCR tube in the heater block. It takes up to 30 seconds for the heating block to cool from the denaturation temperature to the annealing temperature, (d) The WDM PCR temperature profile, measured by placing a thermocouple at the end of the syringe needle and thermocycling as usual. The transition from denaturation to annealing takes less than a second.

Figure 3.

Interfacial tension of pendant droplets in contact with air (an ideal hydrophobic medium). The composition of the droplets was varied and are as follows nuclease-free water, 1.5 µg/µL BSA in nuclease-free water, sodium heparinized WB, PCR mixture with 10% WB, and pure PCR mixture. The PCR mixture contains GoTaq Green MM, primers, *K. pneumoniae* genomic DNA (1 ng/µL), and nuclease-free water.

Figure 4.

(a) Gel electrophoresis results for the amplification of the V3 hypervariable region of *K. pneumoniae* rRNA gene from genomic DNA using WDM thermocycling (left) and conventional thermocycling (right). Lane 1: pure DNA. Lane 2: pure DNA in 10% WB. Lane 3: 10% WB augmented with 1.5 μ g/ μ L BSA. Lane 4: 1.5 μ g/ μ L BSA. Lane 5: negative template control (NTC). Genomic DNA concentration is 1 ng/ μ L added to all PCR mixtures (lanes 1–4). (b) Gel electrophoresis results for the amplification of the V6 hypervariable region of *K. pneumoniae* rRNA gene from 1 ng/ μ L genomic DNA with 10% WB *in situ*. Lanes 1–4: four replicates by WDM thermocycling. Lanes 5–8: four replicates by conventional thermocycling. (c) Normalized band intensities from the gel images in (a) for comparison between WDM and conventional thermocycling under different conditions. (d)

The averaged, normalized intensity for the four replicates by WDM and conventional thermocycling in (b), shown with standard errors. The WDM intensity is significant ($t = 11.54$) and the conventional intensity is not significant ($t = 1.01$) by the one-sample t-test ($\alpha = 0.05$, $t_{\text{critical}} = 3.182$).

Figure 5. Detection limits of WDM PCR determined with V6 region primers and serial dilutions of *K. pneumoniae* genomic DNA. Three replicates were thermocycled and analyzed for each concentration to determine significant detection. Each bar represents the average, normalized band intensities, shown together with standard errors. WDM PCR detected 10 ng/μL or 10⁶ genomes/μL ($t = 9.6$), 1 ng/μL or 10⁵ genomes/μL ($t = 11.54$), but failed to detect 0.1 ng/μL or 10⁴ genomes/μL ($t = -0.50$) with 10% WB *in situ*, indicating the detection limit of 1 ng/μL or 10⁵ genomes/μL with 10% WB *in situ*.

Figure 6.

Gel electrophoresis results for the amplification by WDM thermocycling of the GAPDH gene (50 ng/ μ L) in 100% WB (left) and purified DNA (right). The GAPDH gene (10^{10} copies/ μ L in the PCR reaction mixture) was amplified in 4:20/30 cycles with WB diluted to 10% v/v of the final reaction volume. GAPDH gene was amplified from pure DNA in 2:53/20 cycles and 4:20/30 cycles. These results were obtained at a speed of 8.67 sec/cycle.