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ARTICLE

Spatially Isolated Reactions in a Complex Array: Using Magnetic Beads to Purify and Quantify Nucleic Acids with Digital and Quantitative Real-time PCR in Thousands of Parallel Microwells

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Quantitative real-time PCR (qPCR) has been the standard for nucleic acid quantification as it has a large dynamic range and good sensitivity. Digital PCR is rapidly supplanting qPCR in many applications as it provides excellent quantitative precision. However, both techniques require extensive sample preparation, and highly multiplexed assays that quantify multiple targets can be difficult to design and optimize. Here we describe a new nucleic acid quantification method that we call Spatially Isolated Reactions in a Complex Array (SIRCA), a highly parallel nucleic acid preparation, amplification, and detection approach that uses superparamagnetic microbeads in an array of thousands of 100 fL microwells to simplify sample purification and reduce reagent dispensing steps. Primers, attached to superparamagnetic microbeads through a thermo-labile bond, capture and separate target sequences from the sample. The microbeads are then magnetically loaded into a microwell array such that wells predominately contain a single bead. Master mix, lacking primers, is added before sealing the reaction wells with hydrophobic oil. Thermocycling releases the primer pair from the beads during PCR amplification. At low target concentrations, most beads capture, on average, less than one target molecule, and precise, digital PCR quantification can be derived from the percentage of positive reactions. At higher concentrations, qPCR signal is used to determine the average number of target molecules per reaction, significantly extending the dynamic range beyond the digital saturation point. We demonstrate that SIRCA can quantify DNA and RNA targets using thousands of parallel reactions, achieving attomolar limits of detection and a linear dynamic range of 10^5. The work reported here is a first step towards multiplexed SIRCA assays.

Introduction

Despite much interest and clear need, inexpensive, high sensitivity, and highly multiplexed quantitative molecular assays with integrated, automatable sample preparation remain unrealized.[1-5] Multiplexed PCR usually requires the use of multiplexed chemistries, such as hydrolysis probes, and assay design can be challenging due to primer interactions and competition between primer pairs. Such problems can be obviated in qPCR by printing or dispensing the primers into different reaction wells.[6] Hydrolysis probes can support low-level multiplexing in a single reaction tube, but the extent of multiplexing is limited to approximately four- to six-plex due to dye label fluorescence emission spectral overlap.[7] Methods that achieve multiplexing by loading different reagents into numerous, isolated locations often result in expensive and difficult to manufacture consumables.[8] Additionally, splitting the sample amongst independent qPCR reactions reduces the number of target molecules in each reaction, which can negatively affect sensitivity and is often prohibited due to limited sample volume.

Despite a limited dynamic range and relatively complicated and expensive consumables, digital PCR has several advantages over traditional real-time PCR.[9] Digital assays partition the sample into numerous identical, parallel reactions.[10-23] In order to be effective, each reaction volume should contain, on average, between 0 and about 4 molecules of target.[24] Rather than determining the number of amplification cycles needed to detect a PCR positive signal, the percentage of positive reactions at the endpoint is used with Poisson statistics to determine the initial target concentration. Segmentation of the reaction into discrete positive or negative units improves quantitative precision and reduces the uncertainty associated with low-amplitude analog measurements, often benefiting the limits of detection (LODs). Consequently, there is growing interest in these assays as their advantages are realized.[9, 21, 25, 26] The desired small-scale reaction volumes can be segmented as aqueous droplets in an immiscible oil [5, 17, 18, 20, 21, 23, 27-29]. However, droplet generation is a serial process that can be difficult to integrate with sample preparation on a single device. Alternatively, small reaction volumes can be compartmentalized in a reaction well array analogous to a microtiter plate with numerous wells at a high density.[5, 6, 10, 11, 13, 19] Additionally, methods to isolate the reaction wells on a substrate are typically based on either mechanical separation[5,

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[10, 13, 19, 27, 30] or an immiscible sealing oil.[11, 31] Rigid microwell arrays have the advantage of a defined geometry and location, allowing reactions set up as digital PCRs to be imaged in real-time, enabling quantification of more than one copy of target per reaction, i.e., qPCR.

Digital PCR can be somewhat more tolerant to inhibitors than qPCR, but apposite sample preparation is critical to attain sufficient signal to noise to discern positive and negative reactions. Traditional approaches to sample preparation and purification often use reagents or conditions that are incompatible with downstream nucleic acid (NA) amplification, resulting in expensive or difficult-to-automate processing steps. For example, the separation of NA from PCR inhibitors such as heme in blood samples usually requires solid phase extraction via a silica stationary phase, ethanol washes, and buffer elution.[32] Magnetic microbeads are easily manipulated by automated systems and have been integrated into multiple PCR-related workflows. Approaches using hybridization of target DNA to probes on beads have been described for sample cleanup[33, 34] or to detect PCR amplicons generated from a multiplex, qualitative PCR.[35-37] Streptavidin coated beads have also been used to purify PCR amplicons generated with biotinylated primers, either from a single-pot reaction or from emulsion PCR with a solid phase.[38-40] A technique that combines the benefits of encoded magnetic particles for both sample cleanup and the distribution of specific primers and specific target molecules to independent, isolated reaction wells would generate arrays of parallel, multiplexed-in-space, digital PCRs without having to split the bulk of the sample amongst the reaction volumes.

Assay Strategy

The goal of Spatially Isolated Reactions in a Complex Array (SIRCA) is to use an encoded microbead-array format to combine massively parallel nucleic acid (NA) extraction, amplification, and detection in thousands of isolated, singleplex reactions for different target sequences. Superparamagnetic microbeads can be encoded with a unique dye combination and then functionalized with primers for a target NA sequence (Figure 1a). Mixtures of different bead types for different targets can be combined to form assay panels for tens of different analytes. The primers tethered to the bead capture target NAs from samples, acting as hybridization probes to purify and concentrate the target NA sequence for each bead type (Figure 1b). High levels of multiplexing can be achieved with small sample volumes (10-100 µL) because the entire sample is incubated with the ensemble of beads making up the panel. A magnet is used to separate the beads from the sample matrix and wash away PCR inhibitors (Figure 1c) before loading the beads into a microwell array, with one bead loaded per well (Figure 1d). Once the array is sealed by flowing oil over the wells, each reaction well contains ≈100 fL of aqueous master mix with dNTPs and polymerase, making it the smallest qPCR reaction reported to the best of the authors' knowledge (Figure 1e). Heating during thermocycling breaks a thermo-labile streptavidin-biotin bond and releases the primers and targets from the beads. Growth in amplicon concentration in each microwell resulting from PCR thermal cycling is detected using intercalating dye fluorescence. Here we describe the development of the SIRCA assay and microfluidic array and explore the sample preparation and quantitative abilities of the technique.

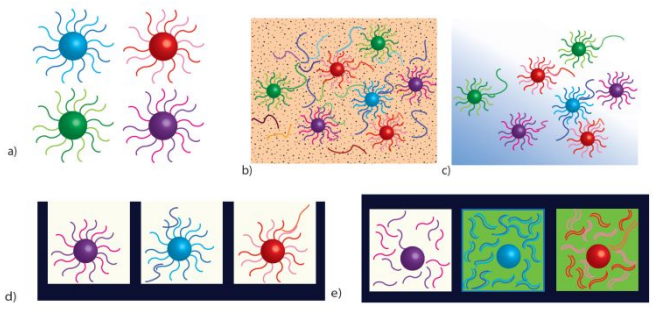


Figure 1. A schematic of the SIRCA assay strategy; a) Sets of streptavidin-functionalized beads are labeled with unique primer pairs and fluorescent dye combinations. b) A mixture of bead sets is incubated with sample to capture their complementary nucleic acid targets before, c) being washed to remove the sample matrix. d) The beads and captured targets are magnetically loaded into isolated microwells with master mix before e) sealing the wells from one another. Thermocycling releases the primers from the beads, and amplicons are detected by intercalating dye fluorescence.

Experimental

Microchip Fabrication Procedure

Several different PCR reaction volumes were evaluated during assay development. Chips with larger volumes from 75 pL down to 4.2 pL were initially used to obtain optimum primer concentrations upon release from the bead (≈60 – 850 nM). Later work focused on condensing the footprint of the array to obtain a higher density of microwells/unit area and to reduce the manufacturing complexity by design simplification. Ultimately, the best assay performance was obtained using ≈100 fL microwells, as more reactions/unit array area increases the number of beads that can be interrogated for each target.

Microchips for 25 and 4.2 pL reactions (Supplementary Figure S1) were fabricated using photolithography techniques described in detail in the Supplemental Information. Briefly, chips were made from glass and silicon substrates coated with a thin layer of hydrophobic alkylthiol-treated gold patterned to form an array of hydrophilic regions. An ≈ 4-4.2 µm diameter, 4.5-5 µm deep, cylindrical pit was etched into the silicon in the center of the hydrophilic region, forming a bead well. Epoxy was patterned to form the sidewalls that sealed the microfluidic chip as well as flow guides that partition areas of the array into a series of parallel channels.

Microchips for 100 fL reactions were fabricated in a different manner, also described in more detail in the Supplemental Information. Briefly, a high density array of microwells were etched into silicon, with each well containing a bead retention region and a reaction volume region. SU8 epoxy was patterned to form sidewalls and flow control channels before bonding a glass coverslip with vias using a 2-part epoxy. The entire chip was treated with alkylsilane to render it hydrophobic.

SIRCA Reagents and Samples

ProMag® 3 Series - Streptavidin superparamagnetic beads (Bangs Laboratories, Inc., Fishers, Indiana, part# PMS3N) were functionalized with 5'-biotinylated forward and reverse primers. Primer sets were purchased from IDT (Coralville, IA) with a 5'-biosg linkage and a 12 bp flap sequence preceding the priming sequence.[41] The flap sequence was used to space the priming sequence away from the surface of the bead to lower steric hindrance when the primer was used as a hybridization probe. Primer sequences are given in the Supplementary Information. Briefly, 1 mg of bead stock (100 µL by volume) was decanted and washed with 200 µL of 100 mM Tris-HCl, 0.1% Tween 20, and 1 M LiCl (TTL buffer). The beads were resuspended in 20 µL of TTL buffer and mixed with 5 µL of TTL buffer containing 0.2 nmol/µL of each of the forward and reverse primer. The solution was vortexed and incubated for at least 15 min at room temperature on a plate shaker at ≈1 krpm. After incubation, the beads were magnetically pelleted and washed twice with 100 µL of TT buffer (250 mM Tris-HCl, 0.1% Tween 20, pH 8.0). Beads were then resuspended in 100 µL of loading buffer (50 mM KCl, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 1% BSA, and 0.1% Tween 20) and stored at 4 °C until use.

Hybridization buffer (typically): 20% dextran sulfate, 10x SSC buffer (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0), and 0.2% Tween 20 was diluted 1 to 1 with the sample; target concentrations reported are the concentrations during hybridization. For the adenovirus work, the dextran sulfate concentration of the hybridization buffer was 13%.

Master mix formulations for early work were typically 1 µL of Platinum Taq (5 units/µL), 22.5 µL of 2x Real-time PCR Platinum Taq Supermix (Life Technologies), 2.5 µL of 10% bovine serum albumin, 6.8 µL of 13.3x SYBR Green I (Life Technologies), 4 µL of the gDNA solution (omitted in hybridization experiments), and water to a final volume of 45 µL. Master mix for 100 fL reactions was typically 1 µL of KAPA 2G polymerase, 20 µL of KAPA SYBR FAST 2x master mix (KAPA Biosystems, Inc.), 0.5-1% bovine serum albumin, 5X SYBR Green I dye, diluted in water to a total volume of ≈40 µL.

Early work was performed with extracted and purified genomic DNA purchased from ATCC (Manassas, Virginia): methicillin-susceptible *Staphylococcus aureus* (MSSA, ATCC 25923), methicillin-resistant *S. aureus* (MRSA, ATCC 700699), and *Streptococcus mutans* (ATCC 25175). The gDNA was digested with restriction enzymes to fragment the DNA into smaller lengths (as described in more detail in the Supplemental Information). Malaria parasite samples were provided by the Juliano and Meshnick labs, as parasites cultured and then diluted in purchased whole blood. The adenovirus sample was purchased as a gDNA fraction from cell culture (ATCC number VR-846D Human adenovirus 2, strain Adenoid 6, Lot 58067104). The adenovirus copy number was determined using droplet digital PCR (BioRad ddPCR QX200 using the manufacturer's protocol).

Sample capture and purification

Samples of bacterial gDNA in buffer were used during early development work. Briefly, samples in buffer were heated to 95 °C for ≈5 min, flash cooled or immediately added to primer-functionalized beads suspended in hybridization buffer. The mixture was vortexed and heated to 37-60 °C for 30 min. The mixture was diluted and washed in 40 µL of loading buffer, then twice in 20 µL of loading buffer before resuspending the beads in 5 or 10 µL of loading buffer and placing them on ice.

Blood samples used for the MRSA work (5 µL) were spiked with digested gDNA and mixed with hybridization buffer at a 1/1 ratio by volume and frozen at -20 °C until needed. Samples were thawed, heated to 95 °C for 10 min, and added to an equal volume hybridization buffer in which beads were suspended. The mixture was placed into a heat block set to 37 °C. After 30 min, the hybridization mixture was diluted with 100 µL of loading buffer, and the beads were magnetically pelleted and the supernatant removed. The beads were washed in 40 µL of loading buffer, then twice in 20 µL of loading buffer before resuspending the beads in 5 or 10 µL of loading buffer and placing them on ice.

Cultured malaria parasites were spiked into whole blood at ≈80,000 parasites/µL and serially diluted from 8000 to 0.8 parasites/µL. Samples were further diluted by 50% into hybridization buffer for a total volume of 40 µL before being frozen at -78 °C. Samples were thawed, 2 µL of proteinase K (Invitrogen, 20 mg/mL) was added, and the samples were incubated at 55 °C for 5 min before heating to 95 °C for 10 min to denature the NA and the proteinase K. Bead slurry in 40 µL of hybridization buffer was then added and incubated for 30 min at 55 °C. After the incubation, 120 µL of loading buffer was added, the beads were decanted and then washed twice with 100 µL aliquots of loading buffer before being resuspended in 20 µL of loading buffer. Primers used for the reverse transcription and PCR amplification were those described by Kamau et al.,[42] incorporating the 5' biotinylated flap.[41]

Loading the bead array

Preliminary experiments were performed on 4.2 pL devices that had 6,000 reaction well sites per device. Chips were wetted with ethanol, flushed with deionized water, and then loading buffer. The chip was placed onto a plate containing an ≈ 3 mm by 3 mm by 1.5 mm neodymium magnet (K&J Magnetics, Inc., Pipersville, PA, part # B221) positioned under a via on the chip. Bead slurry (≈ 1-5 µL containing ≈18k to 150k beads, depending on the experiment) was pipetted into the via. The beads coalesced at a point on the bottom of the via directly above the magnet. The chip was then translated over the magnet such that the mass of beads was pulled towards the array. As the beads were transported over the array of microwells, they fell into the bead wells where typically a single bead was trapped in each well, as the bead well geometry was sized to hold only one bead. In this manner, the array could be loaded in only a few minutes. After loading, the array was washed with loading buffer to remove extraneous beads not residing in wells. Loading buffer was evacuated from the bead loading via, and master mix (≈ 2.5 µL) was added. Vacuum was applied to the second via to pull master mix through the array chamber. When the first via was almost empty, another ≈ 2.5 µL aliquot of master mix was added to the via and pulled into the array chamber. Mineral oil was then

added to the via and pulled through the chip. Oil flowed into the array chamber, wetting the alkylthiol-treated gold surfaces while leaving aqueous droplets of master mix trapped between the hydrophilic regions. In this way, arrays of 1,500 to 6,000 reactions in an ≈5 mm diameter array chamber were formed in less than 2 min.

Loading of the 100 fL devices that contained >30k reaction wells was performed in a similar manner with a few key differences. The chips were sequentially wet with ethanol, water, and loading buffer before addition of the beads. The smaller footprint and significantly higher well density enabled faster bead loading of many more reaction wells using the magnet. After loading, master mix was added to the via and pulled into the array using vacuum over the course of ≈60 to 90 s. Typically, two 2.5 µL portions were added before filling the via with Krytox GPL 104 perfluoropolyether oil (DuPont). The oil was pulled into the chip such that it traversed the array in ≈60 – 90 s. In this device, the top edge of the hydrophobic microwell formed a sharp edge with the top of the silicon substrate. As the oil flowed over the array, it flowed over the microwell and trapped a small (≈100 fL) aqueous droplet in the well with the bead.

Thermocycling and Imaging

An AZ100 Multizoom fluorescence microscope (Nikon Instruments Inc., Melville, NY) with an OptiScan II Three-Axis Stage System (Prior Scientific, Rockland, MA) was fitted with a high-temperature, 40 mm x 40 mm Peltier device (MCPF-127-14-25-E; Newark element14, Chicago, IL) covered with a pyrolytic graphite heat spreader (EYGA091203V, Panasonic) and a 100 ohm RTD (F3102, Omega Engineering, INC., Norwalk, CT) to form a thermocycling stage. The device was driven using an MPT5000 temperature controller (Wavelength Electronics, Inc., Bozeman, MT) controlled via custom software written in LabVIEW. The 4.2 pL reaction arrays were imaged with a frame transfer camera (Model NTE/CCD-512-EBFT, GR-1, Roper Scientific, Trenton, NJ) with a Nikon Intensilight excitation source and a model 49002 ET-GFP filter cube (Chroma Technology Corp., Bellows Falls, VT). The 100 fL reaction arrays were imaged with an ORCA-Flash 4.0 V2 Hamamatsu Digital sCMOS camera (Hamamatsu Corporation, Bridgewater, NJ) using a Lumen 200Pro excitation source and a model 49011 ET-FITC filter cube (Chroma). Images were collected using µManager[43] and processed using custom macros with ImageJ software.[44] Images collected before and after thermocycling were compared to determine which wells showed an increase in intercalating dye fluorescence (i.e. were PCR positive). Average target molecules per reaction (TPR) values were calculated according to the following equation:[20]

$$Targets\ per\ Reaction = -\ln\left(1 - \frac{PCR\ Positive\ Wells}{Total\ Wells}\right)$$

Results and discussion

Concept Evaluation

Primer coupling and release was initially tested by adding primer carrying beads to 10 µL bulk PCR reactions in tubes. Identification of amplicons of the correct length in the supernatant was performed using an Agilent 2100 Bioanalyzer and DNA1000 kit (Supplementary Figure S2). Results confirmed functionality (i.e. targets were successfully amplified by primers that were cleaved from the bead and were released into solution). The primer concentration resulting from release from bulk beads and measured by UV absorbance (NanoDrop 2000, Thermo Fisher Scientific) was used to estimate that release from a bead would produce ≈ 250 nM of each primer in a 25 pL reaction.

Initial demonstration of multiplexing capability was performed by adding target DNA (MRSA) to the master mix rather than utilizing the hybridization capture step shown in Fig. 1b. Beads were encoded, in these experiments, by incubating primer-containing bead sets with biotinylated quantum dots as the encoding functionality. Although the beads were nearly saturated with primers, there were sufficient free streptavidin sites to bind biotinylated quantum dots for encoding purposes to distinguish the three bead sets. Briefly, beads labeled with forward and reverse primers for *mecA* (a gene associated with methicillin resistance) were incubated with Qdot605-biotin and beads with *S. mutans* primers (negative control) were incubated with Qdot655-biotin (both dyes at 200 nM, Life Technologies). Beads carrying primers for the *nuc* gene common to both MSSA and MRSA were unlabeled. Approximately 6k beads from each set were mixed together and magnetically loaded into the wells of a 25 pL/reaction chip filled with loading buffer. Excess beads were removed by flowing loading buffer into the chip before fluorescence encoding images were taken to determine the locations of each bead type (Figure 2a). A master mix containing ≈ 1 pM of MRSA genomic DNA (≈ 14.5 copies per 25 pL reaction) was added to the chip before flowing mineral oil to displace the master mix from the hydrophobic areas and leaving 25 pL droplets with each bead. The chip was placed on the thermocycling stage and imaged after each cycle of PCR (Figure 2b shows a section of the chip after 30 cycles). Positive PCR signal was for reactions with either beads carrying *nuc* primers (92%, average of two chips) and beads carrying *mecA* primers (94%, average of two chips), and most reactions containing beads with *S. mutans* primers were PCR negative (96%, average of two chips). Only a few false positive wells were seen for the

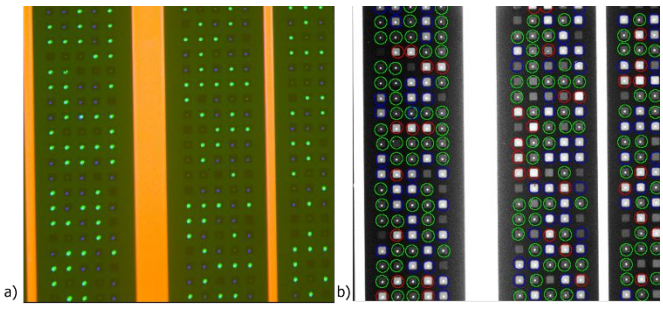


Figure 2. A preliminary three-plex SIRCA assay is shown. a) A composite image from three encoding wavelengths is shown. Beads with primers for a sequence from *S. mutans* (green), the *mecA* gene from MRSA (blue), and the *nuc* gene from *S. aureus* (faint green) are shown. b) PCR results from a sample containing MRSA gDNA show positive reactions (bright squares) for the *nuc* and *mecA* primer beads (circled in red and blue, respectively, with the *S. mutans* beads circled in green). All beads are visible as white dots in the center of the wells.

S. mutans beads, most likely due to double loading of the bead wells with non-encoded *nuc* primer beads that could not be seen in the encoding images. This preliminary assay demonstrated the possibility of using encoded bead sets to deliver unique primers to isolated microreactions and therefore perform multiplex assays in space with each microwell being a singleplex assay. This assay strategy circumvents the interaction of primer pairs for different targets in a multiplex assay from interacting, greatly simplifying the development of primer pairs for multiplexed PCR assays.

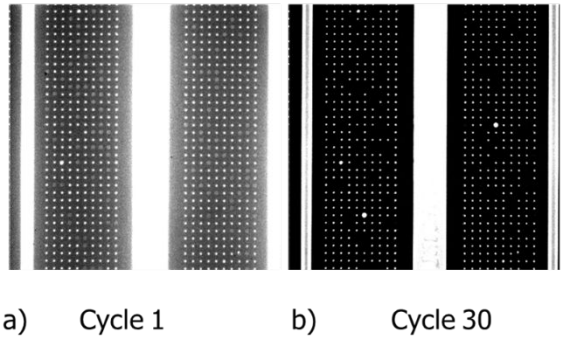
To better utilize the array footprint, smaller reaction wells with only 25 µm circular regions were evaluated with an 8.5 µm tall spacer (nominal well volume of ≈4-5 pL and estimated released primer concentration of 1.5 µM). This configuration increased the reaction well density by a factor of 4 from 5.3k/cm^2 to 21.2k/cm^2 with a total of 6k wells/device. Reducing the reaction well volume also significantly improved the amplitude of the PCR signal above the background by approximately 5-fold, most likely an effect of the higher primer concentration increasing the target amplicon concentration (Supplementary Figure S3).

Sample Preparation Feasibility

The potential of the beads to act as a solid phase for sample preparation *via* hybridization was explored using digested MRSA gDNA. Initial testing with the sample in loading buffer showed low capture efficiency and suboptimum reproducibility. To improve DNA capture, buffer specifically formulated for improved DNA hybridization (10x SSC buffer, 20% dextran, and 0.2% Tween 20)[34] was evaluated and found to perform well in preliminary evaluation. Restriction enzyme-digested whole gDNA from MRSA was diluted into hybridization buffer, heated to 95 °C and then rapidly cooled on ice. Approximately 175k beads carrying primers for the *nuc* gene were then added to form 20 µL final hybridization mixtures containing 0, 270 aM, 2.7 fM and 27 fM gDNA (i.e. 0, 160, 1.6k, and 16k copies/µL). These mixtures were incubated at 37 °C for 1 h, diluted to 60 µL with loading buffer and then magnetically separated and washed twice before suspending the bead slurry in 10 µL of loading buffer. The beads were magnetically loaded into the array chips and master mix containing 1x Pt Taq Supermix, 0.5% BSA, and 1x SYBR Green (no primers) was added and sealed with mineral oil before thermocycling and imaging.

Figure 3a show a comparison of images of one region of interest containing ≈600 reactions at PCR cycle 1 and cycle 30 for beads incubated with 160 copies/µL. Two positive wells can be seen in Figure 3b. Over the entire chip, thirteen positives out of 4752 beads (0.27% positive with an occupancy of ≈79%) were noted for the whole chip. A calibration curve plotting TPR vs. concentration at incubation was generated (Figure 3c). The linearity (r^2=0.999) was excellent over this range. Most importantly, it demonstrates sampling at low, clinically relevant concentrations of gDNA. An average of 0.24%±0.03% positives (n=3) was seen at the lowest hybridization concentration tested (160 copies/µL or 270 aM), in this 6k-well array chip. No false positives were observed in negative control reactions, indicating that adding more array wells should correspondingly improve the LOD.

At higher concentration of target (160k copies/µL), almost every reaction well was positive. The real-time PCR signal (i.e. the average of ≈20 representative positive reactions) was plotted against the cycle number for each concentration tested (Figure 4). Lower concentrations of target yielded arrays with stochastic distributions of positives, where Poisson statistics indicated a TPR of less than 1. In this case, the amplification plots are similar and all exhibit approximately the same threshold cycle, indicating a single copy per reaction as expected, although there is some variation in the observed cycle threshold. At 160k copies/µL, where the majority of the beads are positive, the cycle threshold (C_t) is shifted earlier by about 2 to 3 cycles when compared to C_t values for the lower concentration targets. This difference is expected and indicates a TPR of ≈ 6. These preliminary observations provided evidence that it would be possible to use the analog assay (i.e., qPCR signal) to extend the quantitative range of the technique.



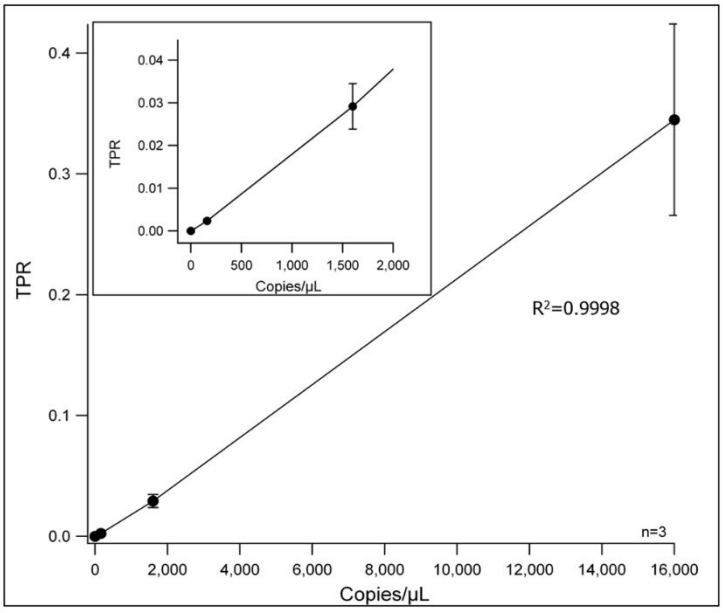


Figure 3. a) Cycle 1 and b) cycle 30 for a SIRCA assay with beads carrying primers for the *nuc* gene hybridized with 160 copies/µL of MRSA gDNA in buffer. c) A calibration plot for 0, 160, 1.6k, and 16k copies/µL is shown with a linear fit line. Error bars represent +- one standard deviation.

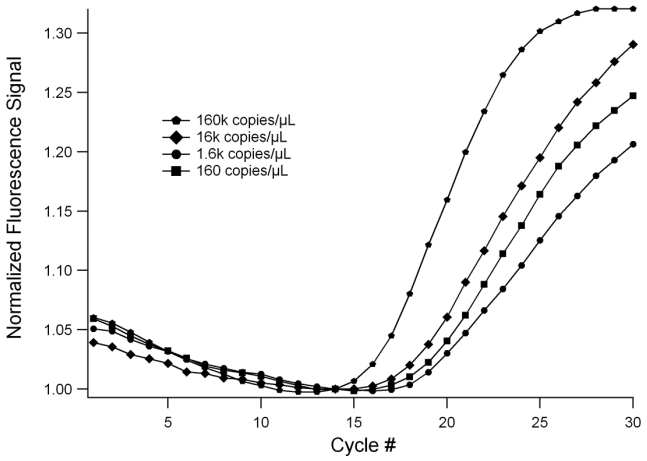
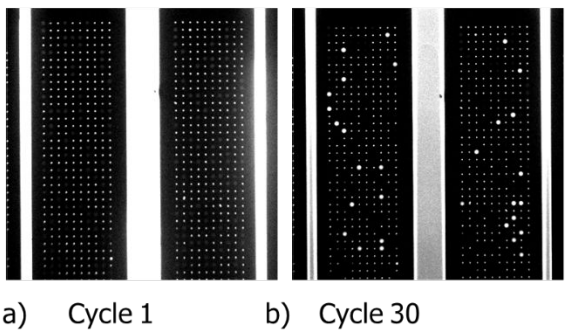
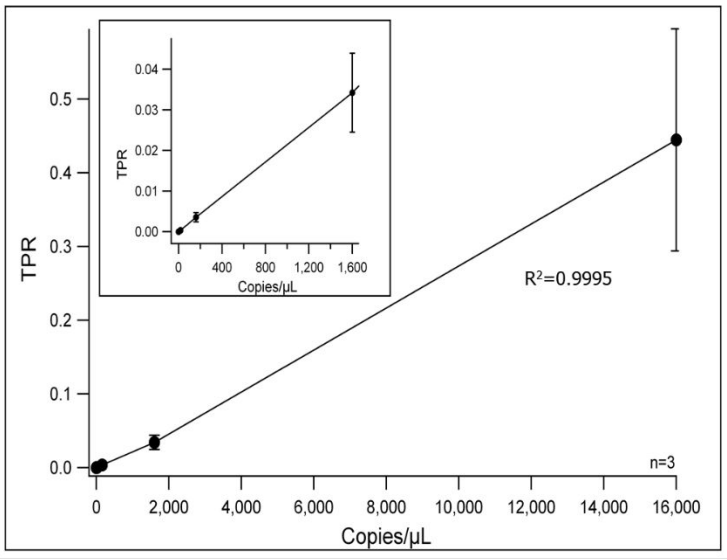


Figure 4. Real-time PCR signal averaged from several positive reactions for beads incubated with 160, 1.6k, 16k, and 160k copies/µL is shown. A decrease in cycle threshold at the higher concentrations indicates that the TPR is greater than one at these concentrations.

Experiments were then performed using a complex sample matrix, whole blood spiked with MRSA gDNA, using beads containing primers for *mecA* and dextran sulfate/SSC hybridization buffer. Whole blood (5 µL) was mixed with an equal volume of hybridization buffer that was spiked with MRSA gDNA. The sample was mixed and heated to 95 °C for 10 min to lyse the cells and denature the dsDNA. The sample was cooled on ice and 10 µL of hybridization buffer containing ≈ 175k beads with primers for *mecA* were added and mixed to yield a total volume of 20 µL (concentrations are reported as copies/µL at incubation). The 20 µL incubations were then diluted to 100 µL with loading buffer, vortexed to suspend the beads, magnetically separated, and washed with 20 µL volumes of loading buffer. The beads were resuspended in 10 µL of loading buffer, loaded magnetically into the c hip, sealed with master mix, and thermocycled. Figure 5a and b show a region of a chip from a 1.6k copies/µL sample at cycles 1 and 30, respectively. A calibration curve is shown in Figure 5c, where the concentration of MRSA gDNA is given at hybridization (75% hybridization buffer, 25% whole blood). Excellent linearity can be seen over the entire range (r^2=0.999), and no indications of PCR inhibition were noted when using this simple and straightforward sample preparation protocol. Of great significance is the lack of observed nonspecific amplification products (false positives) in the negative control whole blood samples (n=3). Out of a total of ≈ 27.5k negative control reaction wells, no false positives were detected, indicating that larger arrays should correspondingly lower the LOD, assuming the false positive rate remains low.

Figure 5. a) Cycle 1 and b) cycle 30 for a SIRCA assay with beads carrying primers for the *mecA* gene hybridized with 1.6k copies/µL of MRSA gDNA spiked into whole blood. c) A calibration plot for 0, 160, 1.6k, and 16k copies/µL is shown with a linear fit line.





Higher Density Arrays

The low false positive rate observed in the above experiments indicated that significant performance enhancements might be achieved with regards to LODs if more reactions could be interrogated on-chip. In order to increase the number of reactions per device, the reaction well area was reduced. However, the mechanical tolerances required to align smaller dimension hydrophilic/hydrophobic patterns on two substrates are not easily achieved. Traditional cylindrical bead/reaction wells were tested, but bead background fluorescence was significant and obscured the PCR amplification signal. A novel microwell geometry was developed that separated the bead retention and assay readout regions. These microwells (Figure 6) had a cylindrical bead loading region with a fluidically connected channel or "tail" that is too narrow to load a bead and where the assay signal could be observed. The well dimensions are roughly 3.5 µm diameter at the bead retaining portion of the well, 1.8 µm wide at the slit, and approximately 4.9 µm deep (Figures 6a). Figure 6b shows a brightfield image of an array loaded with beads. While the majority of the wells loaded a single bead, some wells loaded a smaller bead in the tail region of the reaction well. Additionally, a small number of the bead containing regions can occasionally load a second bead on top of the first bead, resulting in a signal that is usually >50% brighter than the other wells. Due to the relatively homogeneous staining of the beads by the intercalating dye, it is easy to ascertain which wells are occupied by more than a

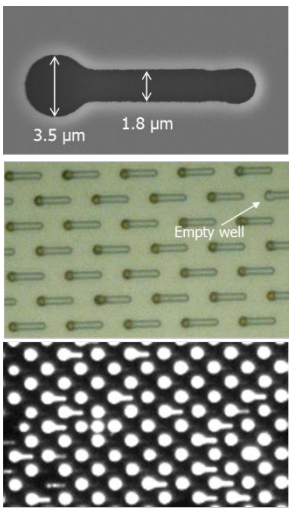


Figure 6. a) An SEM image of the ≈100 fL reaction well is shown. b) A brightfield image showing beads loaded into the bead-retaining portion of the wells is shown. c) A fluorescence microscopy image of an array after PCR thermocycling is shown. Wells with bright signal in the "tail" portion of the well indicate positive PCR results. A misloaded bead can be seen in the middle, left of 6c.

single bead, and these wells can be excluded from analysis. Each array contains >30k reaction wells in an approximately 3 mm by 3 mm square area. The chip was evenly coated with an alkylsilane to render all surfaces hydrophobic, and the sealing oil traps a droplet of master mix in the well by surface tension and droplet pinning.[31] Figure 6c is a fluorescence microscopy image of a microwell array that has been loaded with beads that had been incubated with sample and thermal cycled. The bright circular fluorescence features indicated loaded beads; only those microwells with fluorescent tails indicated a positive PCR result.

Testing of these arrays was performed using malaria samples in whole blood. Sample concentrations from 0.8 to 8,000 parasites/µL were digested with proteinase, heat denatured, and incubated with beads in 75% hybridization buffer and 25% whole blood. The primers used for this experiment[42] were capable of amplifying either gDNA under normal PCR conditions or total nucleic acid (TNA) if reverse transcriptase was added prior to PCR. Preliminary experiments showed that reverse transcription could be performed directly on the bead, without release of the primers. While Kamau et al. reported an approximate 10-fold improvement in sensitivity for quantitative, reverse transcriptase PCR (qRT-PCR) as compared

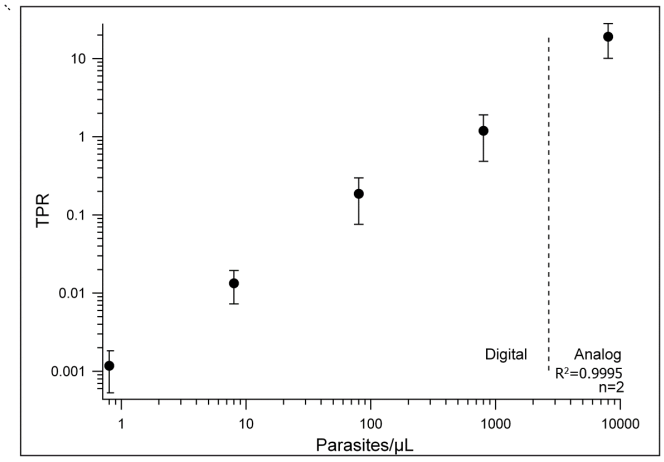


Figure 7. A total nucleic acid assay for malaria parasites diluted into whole blood is shown. The signal is linear from <1 parasite/µL to at least 8,000 parasites/µL. Digital and analog quantification was used to determine the TPR. Error bars represent +- one standard deviation.

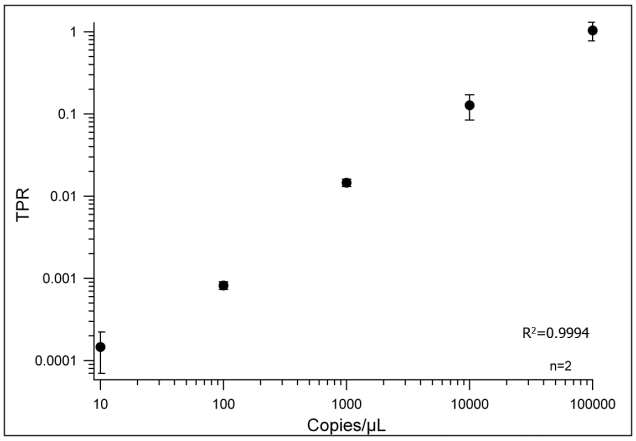


Figure 8. gDNA from an adenovirus culture was hybridized in buffer from 10 to 100k copies/µL. Refinements in sample preparation and bead hybridization reduce the variance seen in these assays.

to qPCR, we noted an improvement of >1,000 fold with the addition of reverse transcriptase. Although this could be due to poor hybridization of the gDNA relative to rRNA, the relative abundance of 18s rRNA in our samples may account for the difference in signal. A calibration curve (n=2) shown in Figure 7 for TNA demonstrates excellent linearity (>0.99) across five orders of magnitude. Most values were calculated from the digital signal using Poisson statistics; however, the highest concentration was calculated based on the C_t shift that indicated a TPR of ≈ 20. These results demonstrate the ability of SIRCA to process and quantify TNA from a complex sample, such as whole blood, with a simple protocol. Future work to improve the reproducibility and efficiency of the RT step may reduce the observed variance between samples if the number of RT enzymes/well is limited. One possible approach includes performing the RT step in bulk solution, before isolating the beads in individual wells as the cDNA is extended from a tethered primer and will remain bound to the beads.

In order to determine the sensitivity of SIRCA with undigested gDNA, a sample of adenovirus DNA with background host gDNA (A549 cells) was examined as a dilution series in buffer. Aliquots were prepared by heating samples to 95 °C in TE buffer for 10 min before removing from the heat source and adding beads suspended in hybridization buffer. Samples were placed into a thermoshaker (Multi-Therm, Benchmark Scientific, Inc. Edison, NJ) held at 45 °C for 30 min. After incubation, the mixture was diluted with 20 µL of loading buffer and washed twice before resuspending in 10 µL of loading buffer. Figure 8 shows a calibration curve of concentrations from 10 to 100,000 copies/µL. Linearity is excellent (>0.99) as is the sensitivity, which we estimate to be better than 10 copies/µL under these conditions. We determined that the hybridization efficiency (TPR / [Molecules of sample per # of beads]) was 12 +/- 2.8% under the conditions studied. Future work will explore ways in which to improve both the efficiency and the speed of the hybridization. The reduced variance as compared to the malaria samples may be due to the relative complexity/purity of the samples or inefficiencies in the RT step. Additionally, a reduced concentration of dextran sulfate was used along with a

thermoshaker, allowing for significantly better mixing of the samples during the hybridization step.

Conclusions

SIRCA is a simple, rapid sample preparation and nucleic acid amplification technique that allows sub-femtomolar (copies/µL) detection of target nucleic acids. We have shown that the technique achieves a broad linear dynamic range by combining a high precision, digital PCR mode with an analog, real-time PCR mode for higher concentrations significantly above the digital signal saturation limit. Initial proof-of-principle was demonstrated in larger volume chips before reducing the volume of each reaction to only ≈100 fL to achieve a high density array. Future work will include the use of fluorescent dye-encoded beads to create arrays of singleplex PCR assays that are multiplexed in space.

Conflicts of interest

W.H. Henley and J.M. Ramsey are listed as inventors on patents related to SIRCA and its implementation.

Acknowledgements

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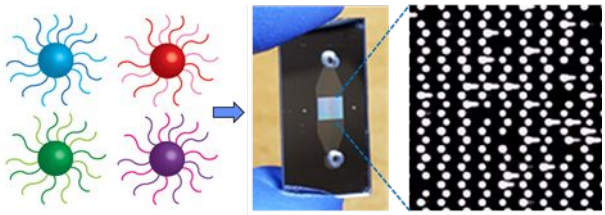
Table of Contents Graphic:



Encoded beads carrying primer pairs for nucleic acid targets are used for sample preparation and multiplexed-in-space digital PCR quantification.