

# MULTI-ANALYTE SINGLE MOLECULE DETECTION BY PARALLEL SINGLEPLEX REACTIONS IN A COMPACT ARRAY

W. Hampton Henley and J. Michael Ramsey\*

University of North Carolina, Chapel Hill, NC, USA

## ABSTRACT

We report a simple, massively parallel nucleic acid (NA) extraction, amplification, and detection method using an encoded microbead-array format to perform thousands of isolated, singleplex real-time and digital PCR reactions for different target sequences simultaneously. Singleplex Reactions in a Compact Array (SiRCA) uses primer sets attached to fluorescent dye-encoded, superparamagnetic microbeads. Mixtures of different bead types can be prepared wherein each type has a different primer set. Beads capture sample from the matrix by sequence-specific hybridization before each bead is sealed in an individual microwell where the target is amplified and detected, reducing amplification bias and enabling digital quantification.

**KEYWORDS:** Multiplex Nucleic Acid Detection, Digital Quantification, Superparamagnetic Microbeads

## INTRODUCTION

PCR amplification in real-time or digital modes can provide exceptional sensitivity and dynamic range; however, simplification of sample preparation and simultaneous, quantitative multiplexed analysis has proven challenging. Multiple probes provide low-level one-pot multiplexing spectrally limited to approximately 4-6 targets, but additional constraints are needed for compatibility between probe sets. Currently available multiplexed-in-space approaches such as the Biofire FilmArray, Life Technologies OpenArray, or Fluidigm BioMark require preprinted or hand-loaded primer sets and/or extensive sample preparation. The sample must also be split between each reaction, reducing sensitivity. Other bead-based, multiplexed single-molecule approaches require secondary probes and long assay times. [1] SiRCA avoids many of these problems because the beads carrying the amplification primer sets also selectively capture and purify NAs from complex sample matrices such as lysed whole blood.

## EXPERIMENTAL

The workflow for SiRCA is shown in Fig. 1. Bead sets are prepared by labeling fluorescent dye-encoded, streptavidin-coated superparamagnetic microbeads with 5'-biotinylated primer sets. Bead mixtures are incubated with heat-denatured NA targets, and the attached primers act as hybridization

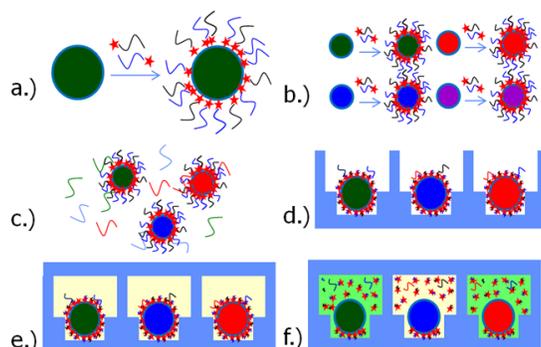


Figure 1: Schematic of SiRCA a) Encoded paramagnetic beads are labeled with forward and reverse primers for a single target. b) Differently encoded beads are labeled with primers for different targets. c) Beads are incubated with sample. d) Beads with hybridized analyte NA are loaded into wells. e) Intercalating dye and other PCR reagents are added to the array and sealed. f) real-time PCR thermocycling releases primers from beads. Target NA is amplified and detected by fluorescence signal.

probes to capture and purify the NA sequence specific to each bead. Extraneous NAs and PCR inhibitors from the matrix are washed from the beads using magnet-based handling for simple and effective sample preparation. The beads are stochastically loaded into individual microwells with a few picoliters of master-mix containing polymerase, dNTPs, and intercalating dye before being sealed with oil to isolate the reactions. The encoding fluorescence is read to determine the identity of the primer set in each reaction well. Thermocycling releases primers from beads during the initiation step of PCR amplification. Intercalating dye fluorescence is recorded in real-time, providing aM LODs and a dynamic range spanning from the digital, single-molecule regime to the analog, multiple copies per bead domain.

## RESULTS AND DISCUSSION

Early, proof-of-concept work with encoded beads showing the delivery of different primer sets to isolated reaction wells was performed in chips where 1,500 reactions (each 25 pL in volume) were carried out on samples spiked with gDNA from methicillin-resistant *Staphylococcus aureus* (MRSA) (Table 1). Positive signals were observed for beads with primers targeting *nuc* and *mecA* sequences in MRSA. A few false positives in the control wells were attributed to double-loading of multiple bead types in single wells; an issue that bead well geometry optimization has resolved. Importantly, no false positives were observed in single bead-loaded blank wells.

Table 1. 3-plex SiRCA assay for MRSA

	<i>Nuc</i>	<i>mecA</i>	<i>S. mutans</i>
Total Beads added to sample	6,000	6,000	6,000
Beads Loaded into Array	174	440	561
PCR Positive Wells	164	413	34
Negative Wells	10	27	527
Double-Loaded	--	13	13

Sample extraction and digital quantification of *nuc* in MRSA gDNA spiked into buffer (Fig. 2a,b) and *mecA* spiked into whole blood (Fig. 2c) was achieved with subfemtomolar LODs using higher density arrays of 6,000 reactions/chip (5 pL/reaction).

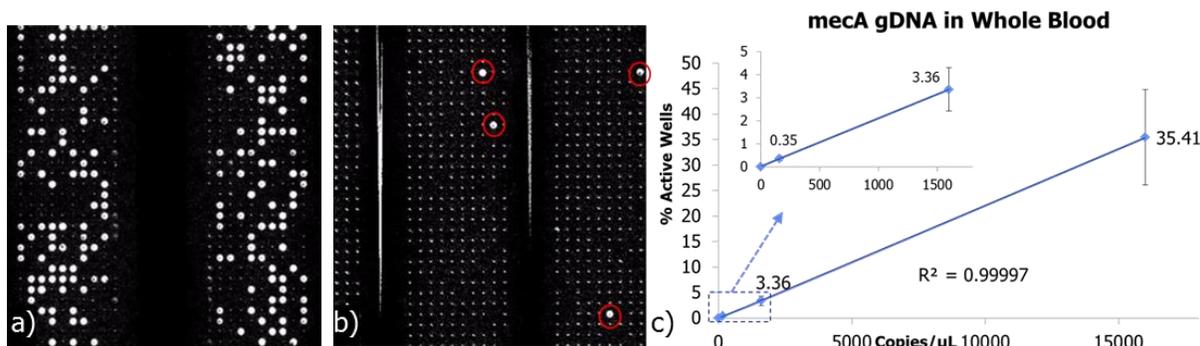


Figure 2: Hybridization sampling of MRSA using beads with primers for the *nuc* gene. a) gDNA at 27 fM or 16k copies/μL b) 270 aM or 160 copies/μL. c) Detection of *mecA* from MRSA gDNA spiked into whole blood ( $n=3$ ). Hybridization is used to capture target gDNA from the sample (heat-lysed whole blood comprised 25% of the incubation volume). A plot of the % active wells vs. copies of DNA per μL shows a linear response from 0 to 16k copies/μL. No false positives were detected in blanks. 270 aM DNA was easily detected.

Fig. 3 shows an example of digital quantification of extracted NA from blood spiked with whole malaria parasites. Sensitivity was greatly enhanced by the addition of a reverse transcription (RT) step for total NA detection, similar to previous reports using bulk RT-qPCR. [2] Use of both digital (positive/negative wells) and analog (real-time cycle threshold) signals allows quantification across a larger dynamic range.

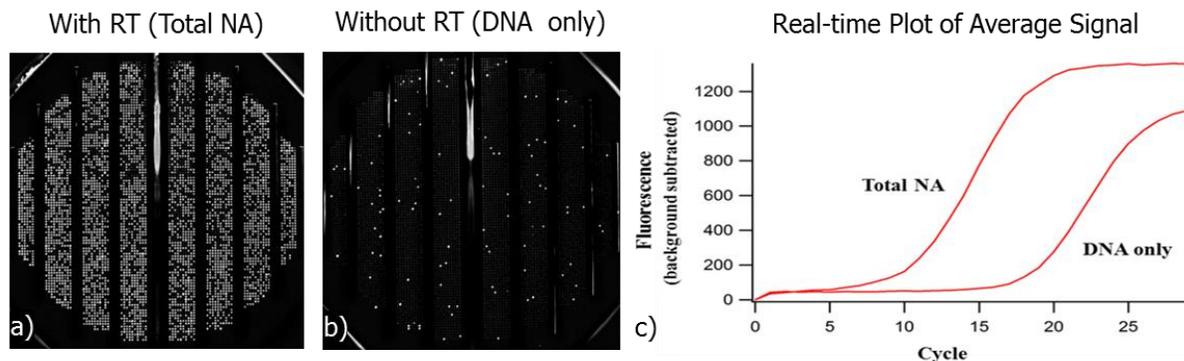


Figure 3: SiRCA with hybridization sampling from a total nucleic acid extraction of a mocked malaria sample in blood a) with RT (Total NA) and b) without RT (DNA only). c) Background subtracted fluorescence signal averaged over the positive wells shows a shift of approximate 8 cycles for reactions incorporating RT, indicating that each bead has  $\approx 250$  copies of RNA. No positives were seen for buffer-only blanks either with or without RT in the master mix.

## CONCLUSION

We have shown that SiRCA allows sub-femtomolar detection of target nucleic acids. No false positives were detected in tens of thousands of blank 5 pL reactions, indicating an ultra-low false positive rate for SiRCA in these volumes. Real-time and digital detection modes expand the dynamic range of these assays while maintaining aM LODs. Sample preparation can be greatly simplified by using the same beads for both NA target capture and amplification, and this advantage will enable future integration of sample preparation on-chip.

## ACKNOWLEDGEMENTS

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## REFERENCES

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## CONTACT

\*J.M. Ramsey, jmramsey@unc.edu