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Digital Assays – Project Overview

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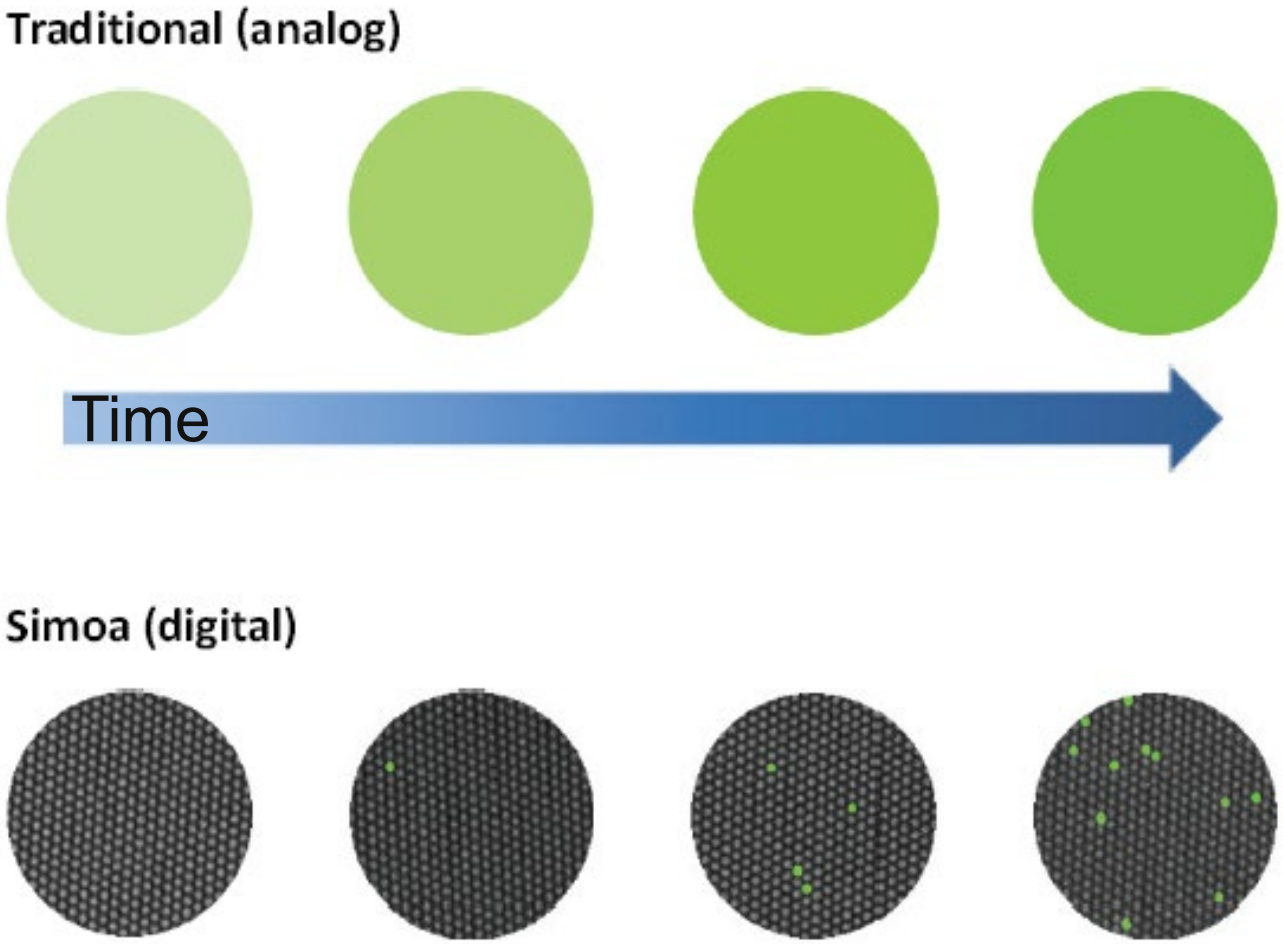
Executive Summary

This project introduces a novel approach to digital assays by simplifying droplet generation and analysis by using polydisperse droplets and advanced statistical modeling, eliminating the need for complex and costly microfluidic systems traditionally used in digital droplet assays.

Comparing bulk and digital droplet assay detection methods

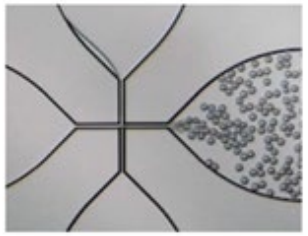


+ Advantages
  - Quantification without a standard curve
    - (or thresholding without calibration)
  - Accurate quantitation when amplification efficiency is low
  - Improved precision
  - Improved sensitivity
  - Robustness to presence of inhibitors

Improved Statistics Allow Simpler Droplet Generation and Analysis Systems

+ Monodisperse Droplets
  - Expensive chips and equipment
  - Requires trained user
  - Limited sample processing (0.1 mL)

i.



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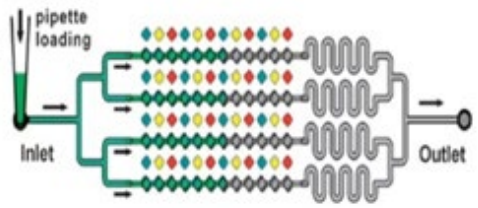

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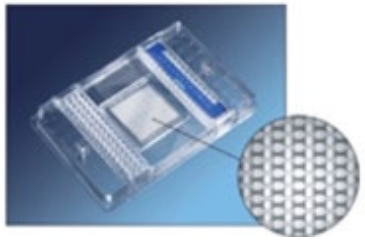
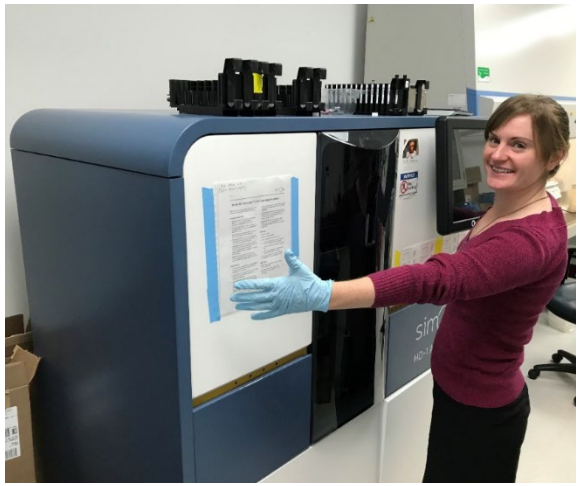


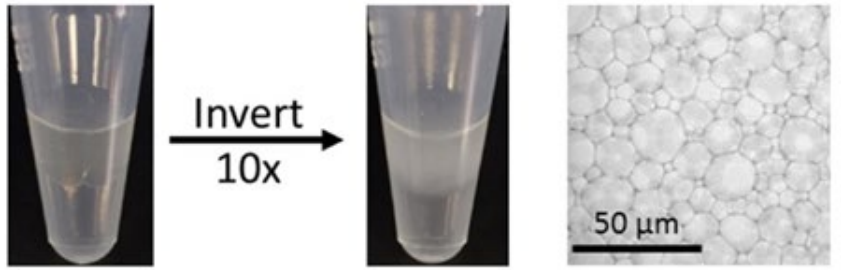
+ Simoa, $200k



+ Biorad ddPCR, ~$150K

+ Polydisperse Droplets
  - Requires no specialized equipment
  - Requires no specialized training
  - Potential for large volume sample processing (µL – mL)

i.



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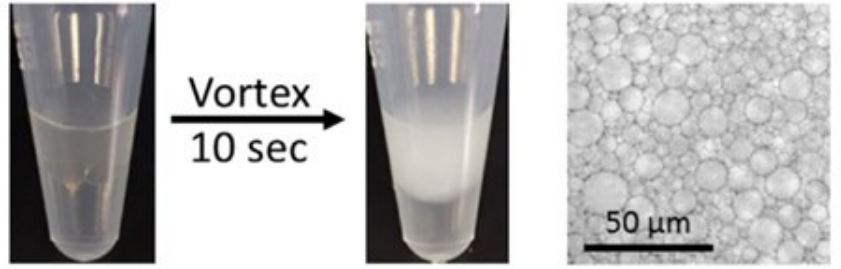

i. *Dolomite: http://www.dolomite-microfluidics.com/*

ii. *SlipChip: Du W, et al. SlipChip. Lab Chip. 2009;(16)*

iii. *Fluidigim: New Products. Science (80-). 2009;324(5924):280*

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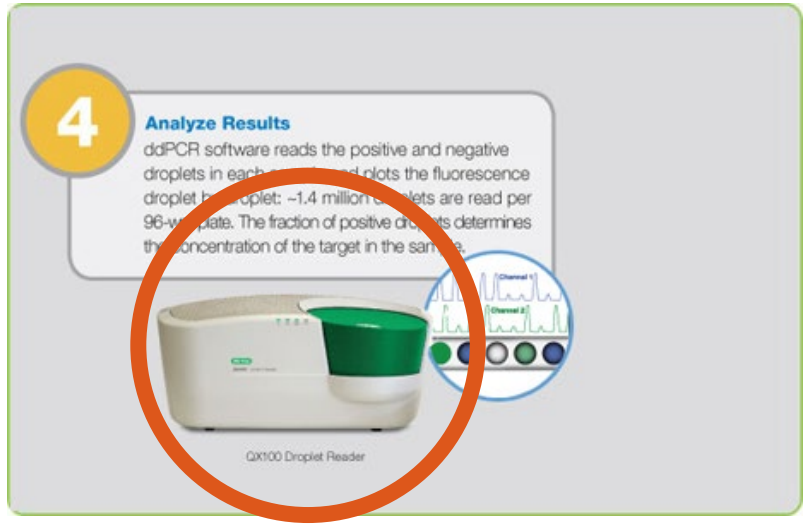
BioRad digital droplet PCR (ddPCR)

Individual Units Necessary

Droplet Maker
$50,000

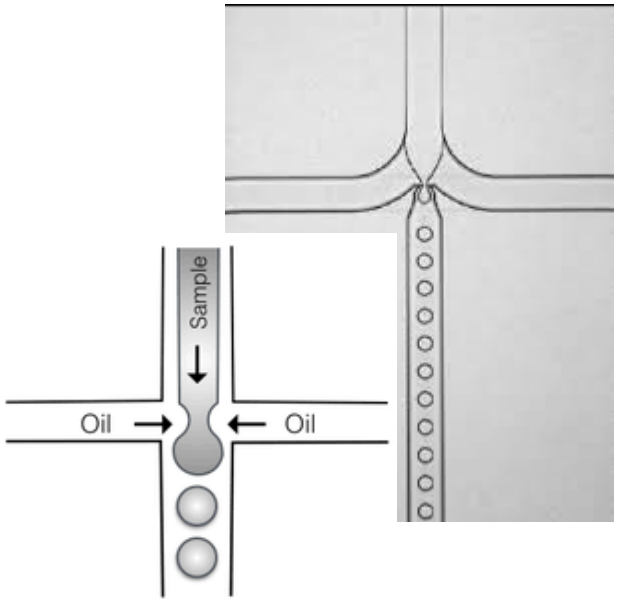
Thermal Cycler
$10,000



Biorad ddPCR Reader
$100,000

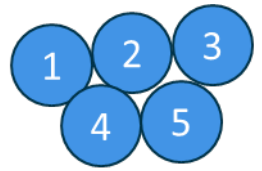
Existing Digital Droplet Assays use Monodisperse Droplets

- In monodisperse systems, a target has an equal chance of being in any given droplet
  - Quantitation is easy, you just count!
  - (with a Poisson correction…)
- Tradeoff is that microfluidic systems are typically required to get monodisperse droplets
  - Microfluidics are relatively expensive and high maintenance
    - Precise, and they clog
  - You inevitably lose dynamic range
    - Limited total addressable volume
    - Droplet rejection rates as high as 50% are common

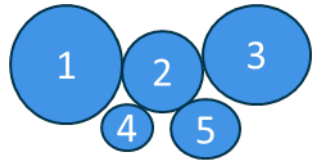


Polydispersed droplets (polydroplets): Leapfrogging monodisperse droplet complexities with statistics

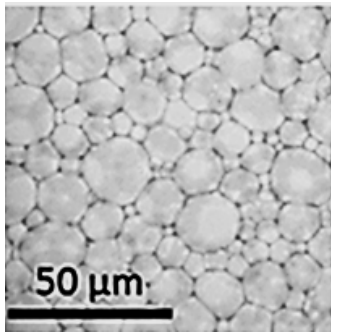
- In polydisperse systems, a target has a higher chance of being in a larger droplet compared to a smaller one
  - Range of droplet sizes = range of probabilities
  - Need to develop a new statistical approach to account for polydispersity
- Goal: determine sample (bulk) concentration of a target from a digital assay result
- Need to correct for under-counting from droplets with multiple targets
  - bulk concentration, λ → what we want to calculate
  - a positive droplets out of m droplets → data we have
  - droplet volumes: $v\_1, v\_2, \ldots, v\_m$

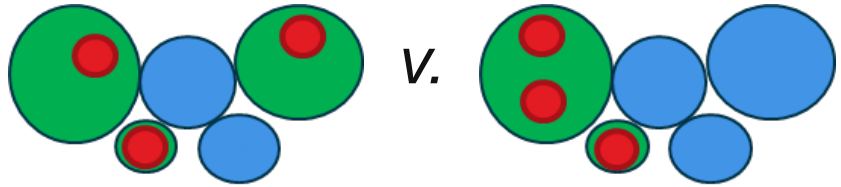


P(1) = P(2) = P(n)



P(1) > P(3) > P(4)





= target
Green = positive
Blue = negative

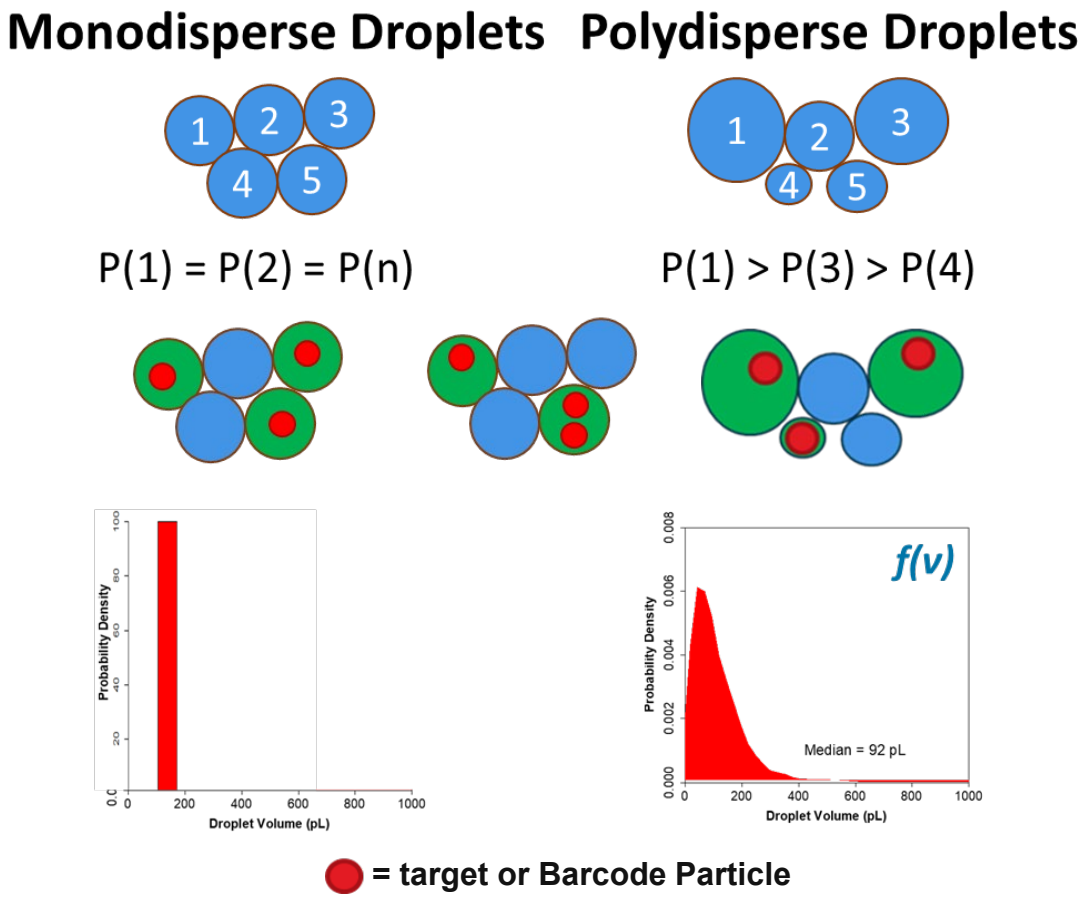
Reducing droplet preparation complexity by removing the need for monodispersity and developing new statistical corrections

- A randomly chosen droplet of volume v_1 (from m total droplets):
  - Probability of volume v_1: $\frac{1}{m}$; probability of having target: $1 - e^{-v_1\lambda}$
  - …
  - Probability of volume volume v_m: $\frac{1}{m}$; probability of having target: $1 - e^{-v_m\lambda}$
- Take all the individual droplet probabilities , $1 - e^{-v_i\lambda}$, and sum them to get the overall probability of positive droplets
  - $P_{on}(\lambda) = \sum_{i=1}^{m} \frac{1}{m}\left(1 - e^{-v_i\lambda}\right) = 1 - \frac{1}{m}\left(\sum_{i=1}^{m} e^{-v_i\lambda}\right)$
- Solve numerically for λ, concentration, using common method of maximum likelihood approximation
  - $\frac{1}{m}\left(\sum_{i=1}^{m} e^{-v_i\lambda}\right) - 1 + \frac{a}{n} = 0$ → know $m, v_i, a,$ and n

Patent application filed and published

Partitioning statistics to determine target and reagent encapsulation in droplets

+ Mathematics of a droplet system are dependent on multiple distributions that can be measured: droplet size, target, reagents



+ Goal: correct for under-counting of droplets with multiple targets
  - want to calculate → λ (bulk concentration)
  - data we have:
    - α → # of pos droplets
    - m → total # droplets
    - $v_1 \ldots v_m$ → droplet volumes
+ Poisson statistics to solve numerically for λ using droplet volume distribution, $f(v)$
  - Mono: sum over $\mathbf{1 - e^{-v_m \lambda}}$ using total # droplets
  - Poly: sum over individual probabilities $\mathbf{1 - e^{-v_i \lambda}}$ for each droplet volume (v_i): $\sum_{i=1}^{m} \frac{1}{m}\left(1 - e^{-v_i \lambda}\right)$
  - $f(v)$ does not need to be measured every time, **pre-measured values** can be used

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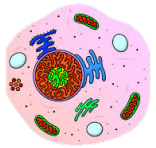
We have developed and demonstrated multiple detection modes in polydisperse digital assays

- Inexpensive digital PCR

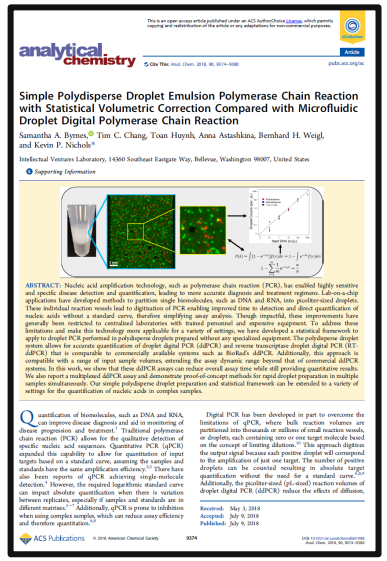


- rapid cell culture in droplets

- One pot digital immunoassays

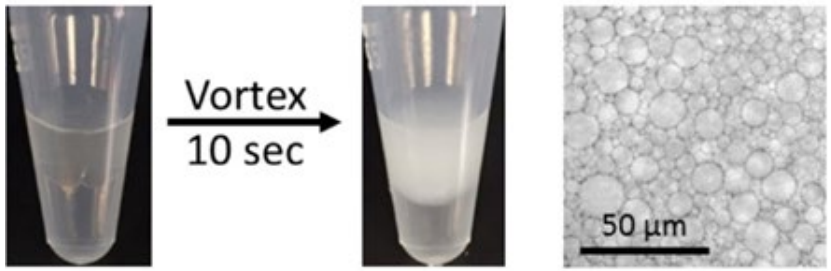
We have demonstrated quantification of PCR in droplets

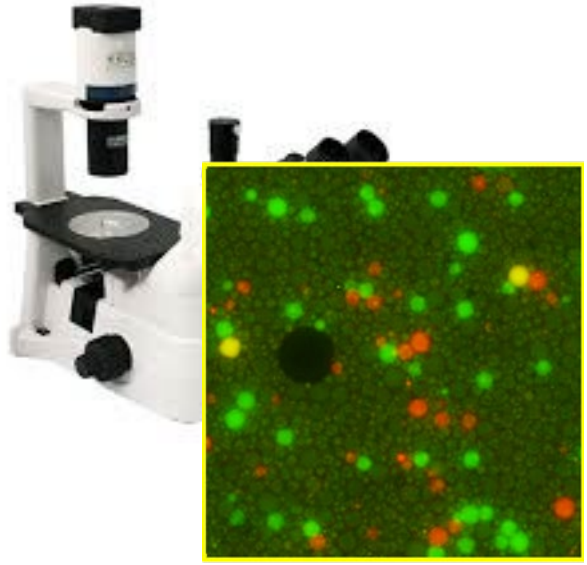
+ Droplet NAATs enable quantification without a standard curve
  – Published manuscript in Analytical Chemistry
+ Demonstrated results for ddPCR, RT-ddPCR, and multiplexed ddPCR consistent with a gold-standard commercial



doi: 10.1021/acs.analchem.8b01988

ddPCR



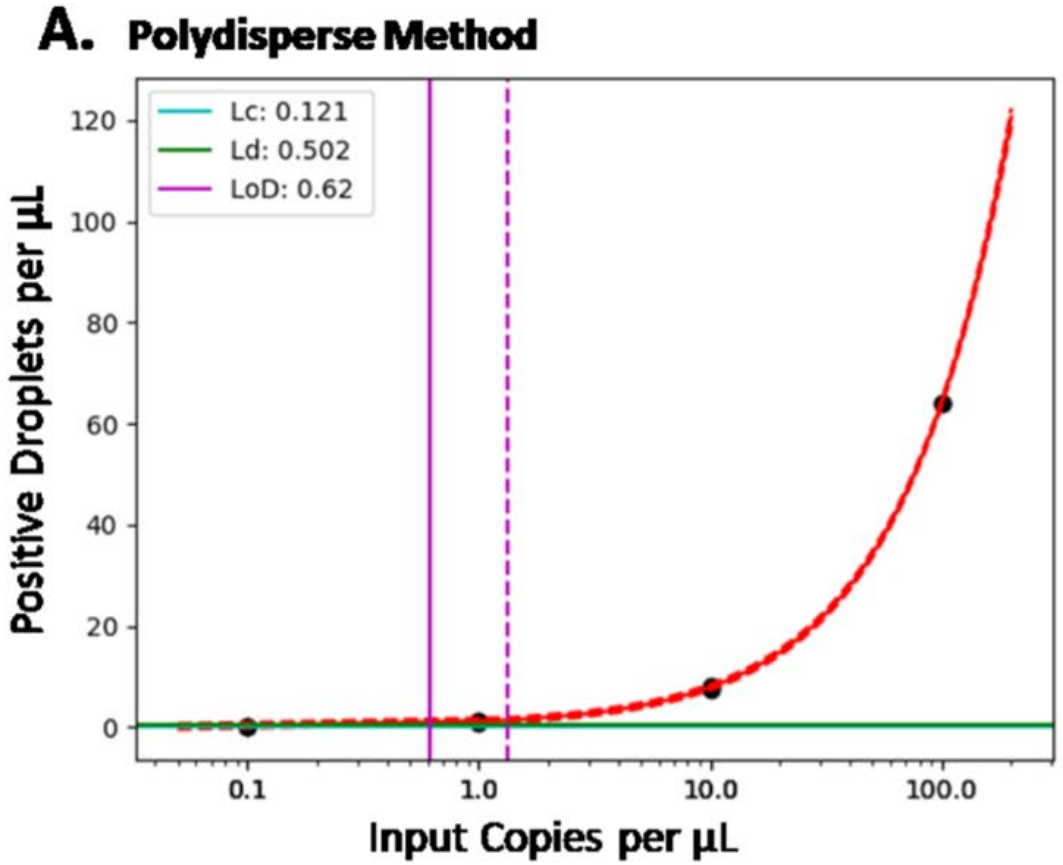


Vortexing (Droplet Generation) **Thermocycling** **Microscopy**

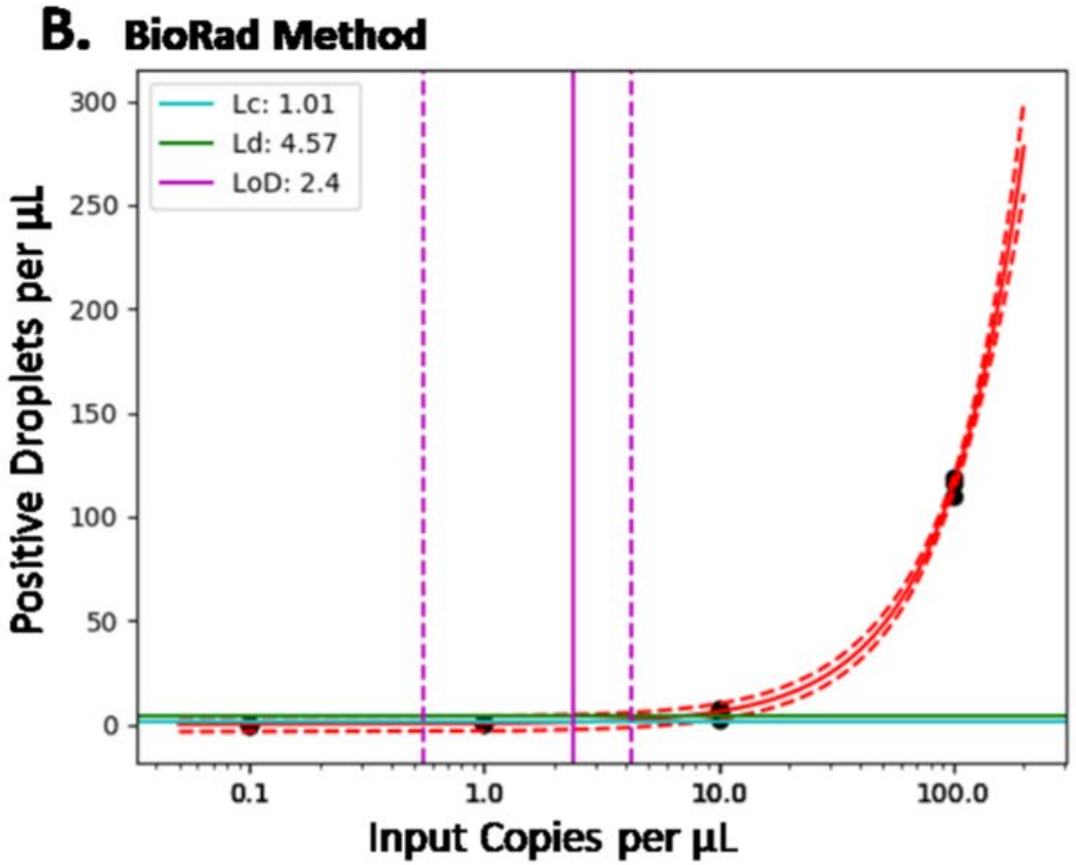
We have demonstrated quantification of PCR in droplets

PCR

LOD = 0.6 c/uL (95% CI: 0.53 – 0.67)



LOD = 2.4 c/uL (95% CI: 1.9 – 3.0)



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PCR

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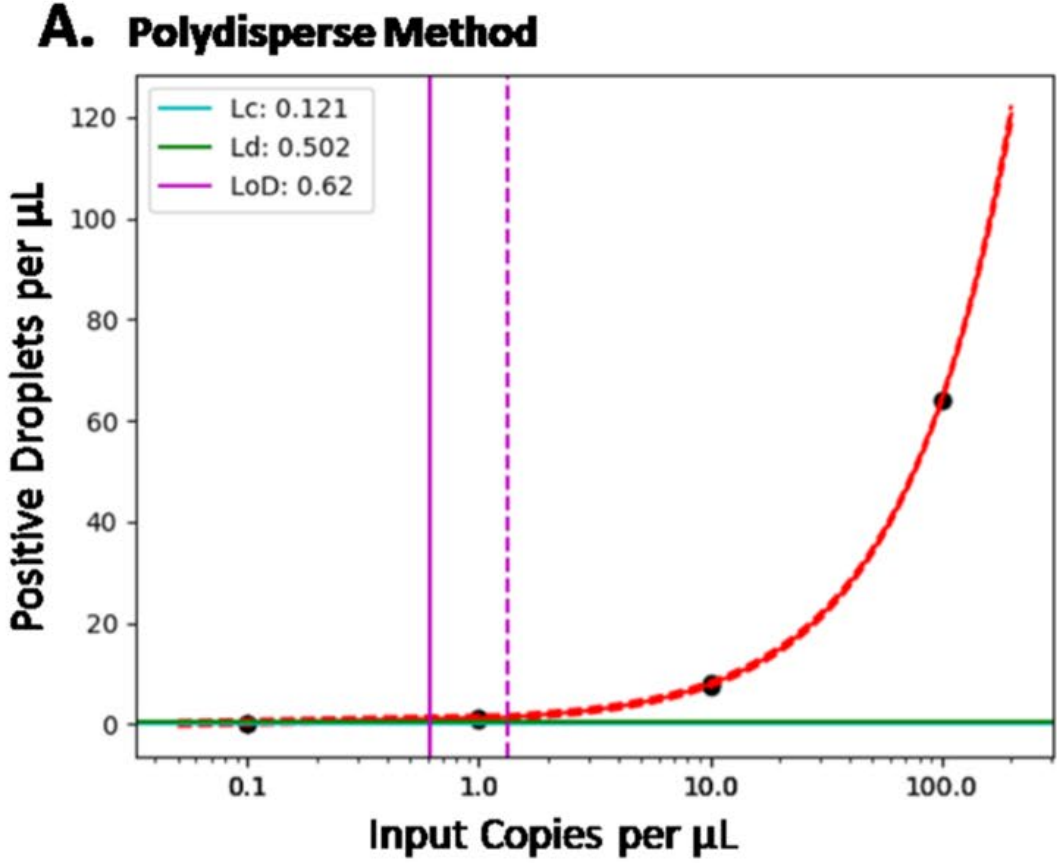

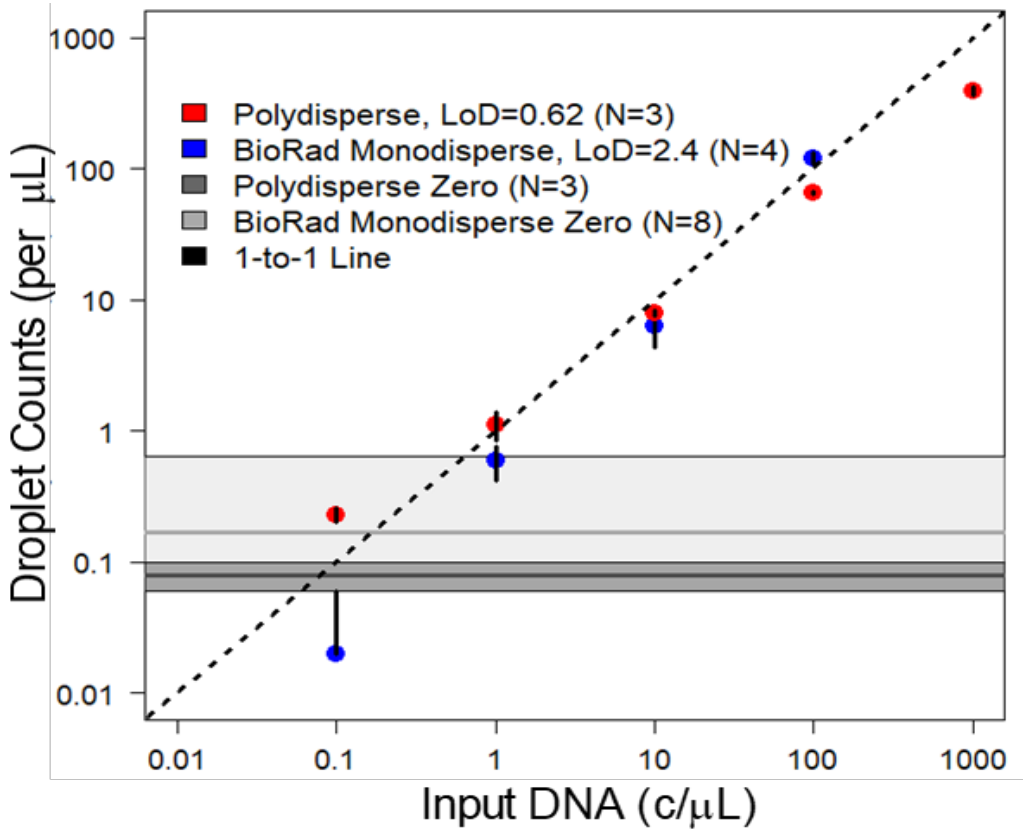

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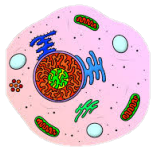
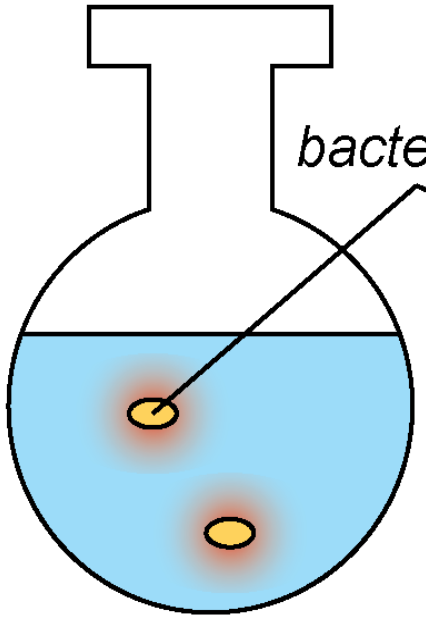


We have demonstrated rapid cell culture in droplets

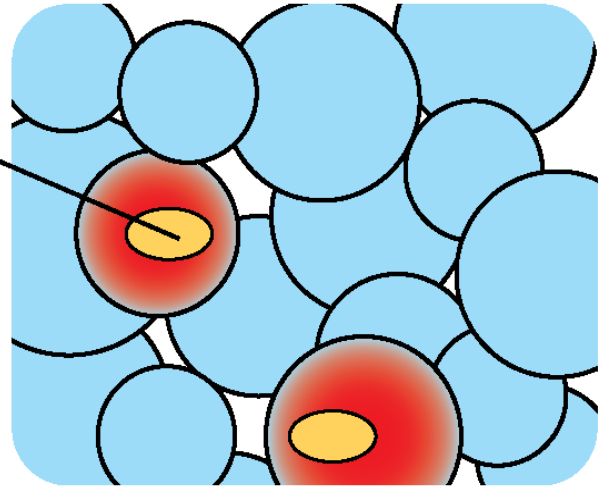
centimeter scale flask



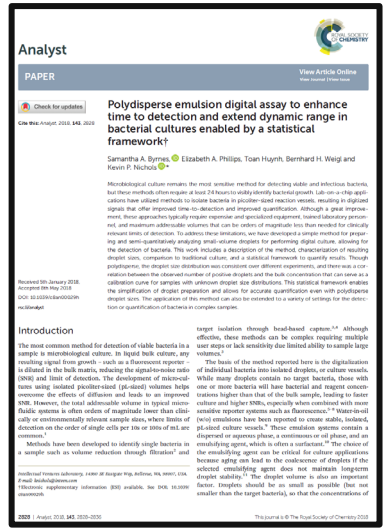
generation of bacterial products in bulk solution: **diffusion reduces conc.**

picoliter scale droplets

micron scale water in oil droplets



generation of bacterial products in droplets: **concentration is kept high**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PAPER

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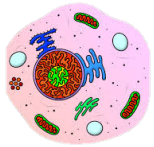
Polydisperse emulsion digital assay to enhance time to detection and extend dynamic range in bacterial cultures enabled by a statistical framework†

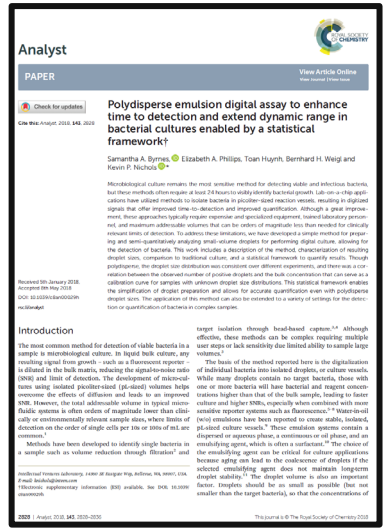
Samantha A. Byrnes, Elizabeth A. Phillips, Toan Huynh, Bernhard H. Weigl and Kevin P. Nichols*

Introduction

doi: 10.1039/c8an00029h



We have demonstrated rapid cell culture in droplets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Introduction

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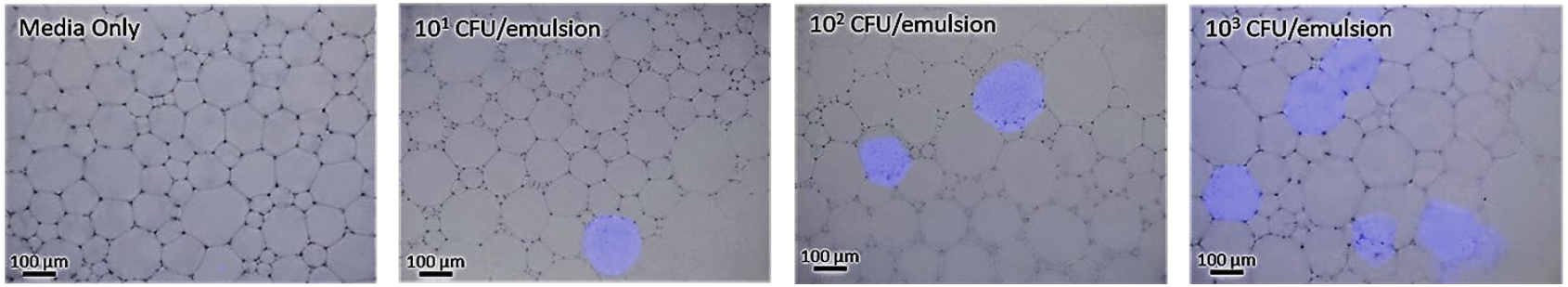
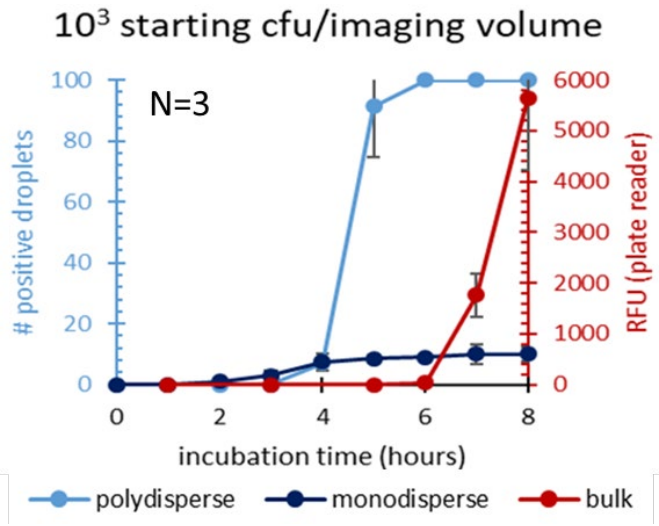
+ Proof-of-concept detection and quantification of E. coli
  – Published manuscript in Analyst



+ Demonstrated improved time-to-detection and LoD in droplets compared to standard bulk methods



We have demonstrated one-pot digital immunoassays


Wash-Free, Digital Immunoassay in Polydisperse Droplets

Samantha A. Byrnes[†], Tona Huynh[†], Tim C. Chang, Caitlin E. Anderson, James J. McDermott, Cata I. Oncina, Bernhard H. Weigl, Kevin P. Nichols*

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KEYWORDS: digital assays, droplet microfluidics, immunoassay

ABSTRACT: Immunoassays are important for the detection of proteins to enable disease identification and monitor treatment, but many immunoassays suffer from sensitivity limitations. The development of droplet-based digital assays has enabled highly sensitive biomarker detection and quantification, but devices used to perform digital assays typically require precisely controlled volumes to reduce biases in concentration estimates from compartment size variation. These constraints have led to systems that are often expensive, cumbersome, and challenging to operate, isolating many immunoassays and droplet-based assays to centralized laboratories. To overcome these limitations, we have developed a simplified digital immunoassay performed in polydisperse droplets that are prepared without any specialized equipment. This polydisperse digital droplet immunoassay (ddIA) uses proximity ligation to remove the need for wash steps and simplifies the system to a single reagent addition step. Using interleukin-6 (IL-6) in an example analyte, we demonstrated the concept with samples in buffer and diluted whole blood with limits of detection of 0.424 pM and 0.969 pM, respectively. The development of a one-pot, wash-less assay greatly improves usability compared to traditional immunoassays or digital-based systems that only have dry or wash steps. We also developed a stochastic model with physically meaningful parameters that can be utilized to optimize the assay and enable quantification without standard curves, offer initial characterization of the parameters. Our polydisperse ddIA assay serves as an example of sensitive, lower-cost, and simpler immunoassays suitable for both laboratory and point-of-care applications.

INTRODUCTION

Immunoassays are important for the detection of proteins to enable disease identification and monitor treatment. Since its introduction in the 1950s, immunoassays have been widely used in many areas including medical diagnostics and drug monitoring.[1] While there are many immunoassay variations, they can be generally grouped into two categories: bulk immunoassays[2–4] and digital immunoassays.[5–7] They both share steps to form immunocomplexes involving a target molecule and a reporter that results in a measurable signal.

The most commonly utilized bulk immunoassay is the ELISA, which relies on target-specific capture molecules bound to a surface to detect a desired antigen.[2] ELISAs have reported sensitivities in the low pM range, but this is not sensitive enough to detect many clinically-relevant targets, especially in human whole blood.[8] Additionally, ELISAs usually require an independent standard curve to quantify the amount of target in a sample and account for run-to-run variations.[9–11]

High-sensitivity digital immunoassays were developed to address the limitations of bulk systems. In digital assays, the signals from a single or a few reporters are compartmentalized and read independently, enabling antigen quantification without the need for a standard curve in each assay run.[5,6] Due to the ability to effectively detect single binding events, digital immunoassays generally have better sensitivity than their bulk counterparts.[5,12,13]

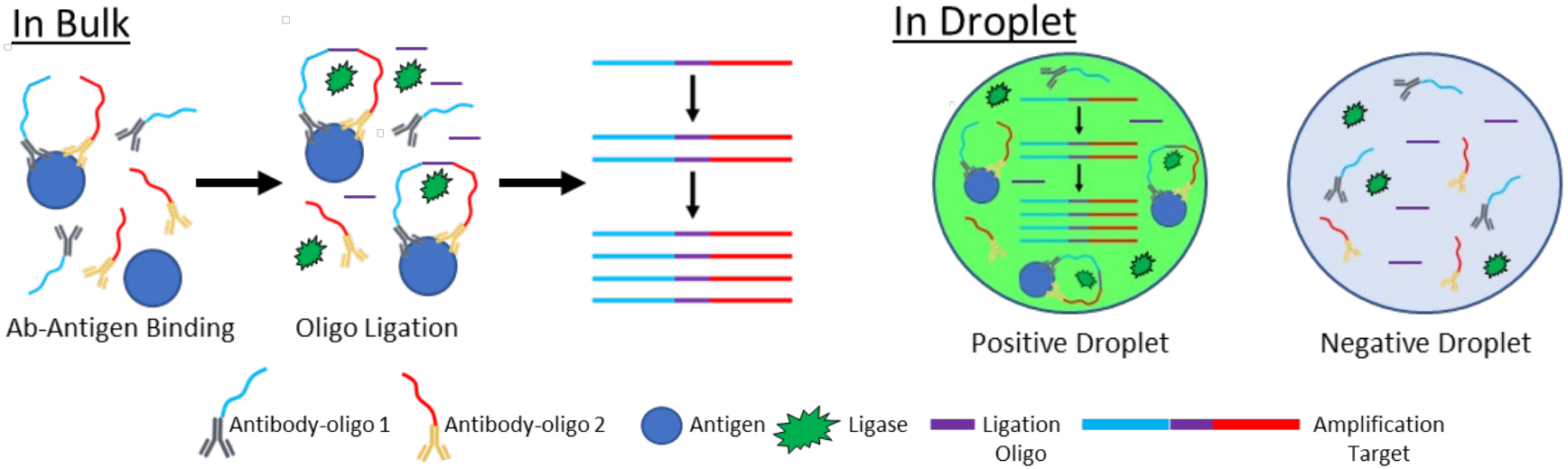
Current digital immunoassay platforms partition target molecules using two different approaches. In the first approach, immunocomplexes are physically separated into individual compartments, such as microwells, and signal from positive binding events is observed only in wells that contain the target complex. This results in bright (ON) and dim (OFF) wells.[5,7] Signal from a positive reaction within these small volumes is highly confined and concentrated. These isolated signals can then be counted to quantify the amount of target in a sample.[5,6] Although effective and very sensitive, the implementation of the microwell approach requires microfabrication and complex operation (by a robotic instrument[14] or a human operator), which is not suitable for most point-of-care or resource-limited settings. As alternative to microwells for compartmentalization is droplets,[15,16] but they are not amenable to wash steps. To address this limitation, methods have been developed for droplet merging and mixing within microfluidic chips, though these methods can be slow or non-uniform[16,17] and add complexity that is not amenable to all locations.

In the droplet-based approach, the reporter groups, such as fluorophores, that result from the immunocomplexes are optically separated.[5] The fluorophores are in a homogenous solution within a droplet, but are separated from the contents of other droplets, effectively counting individual positive reactions. Most droplet-based reactions, however, require specialized microfluidics to create uniform droplets which can require multiple user steps to operate.

To address these challenges, we aimed to develop a digital immunoassay with properties more suitable to point-of-care devices. In particular, the two goals are to reduce the number of user steps (to 1 if possible) and to eliminate the need for complex instrumentation for droplet preparation.[5,6]

To reduce the number of steps, we employed proximity ligation chemistry. Proximity-based assays require two or more reaction components coming into proximity for a signal to be generated. Therefore, only when a binding event takes place (such as antigen-antibody binding) will the reaction components be in close enough

doi: 10.1021/acs.analchem.9b02526



- Two primary drivers of immunoassay complexity
  - Multiple user steps (washing, reagent addition)
  - Equipment
- Goal: build single-pot digital immunoassay
  - Principles of proximity-based assays
  - ProQuantum (ThermoFisher)

We have demonstrated one-pot digital immunoassays


Wash-Free, Digital Immunoassay in Polydisperse Droplets

Samantha A. Byrnes[*], Tova Heyrak[*], Tim C. Chang, Caitlin E. Anderson, James J. McDermott, Cata I. Oncina, Bernhard H. Weigl, Kevin P. Nichols[*]

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Immunoassays are important for the detection of proteins to enable disease identification and monitor treatment. Since its introduction in the 1950s, immunoassays have been widely used in many areas including medical diagnostics and drug monitoring.[1,2] While there are many immunoassay variations, they can be generally grouped into two categories: bulk immunoassays[3,4] and digital immunoassays.[5,6] They both share steps to form immunocomplexes involving a target molecule and a reporter that results in a measurable signal.

The most commonly utilized bulk immunoassay is the ELISA, which relies on target-specific capture molecules bound to a surface to detect a desired antigen.[3] ELISAs have reported sensitivities in the low pM range, but this is not sensitive enough to detect many clinically-relevant targets, especially in human whole blood.[4] Additionally, ELISAs usually require an independent standard curve to quantify the amount of target in a sample and account for run-to-run variations.[3,7]

High-sensitivity digital immunoassays were developed to address the limitations of bulk systems. In digital assays, the signals from a single or a few reporters are compartmentalized and read independently, enabling antigen quantification without the need for a standard curve in each assay run.[5,6,8] Due to the ability to effectively detect single binding events, digital immunoassays generally have better sensitivity than their bulk counterparts.[5,6,9]

Current digital immunoassay platforms partition target molecules using two different approaches. In the first approach, immunocomplexes are physically separated into individual compartments, such as microwells, and signal from positive binding events is observed only in wells that contain the target complex. This results in bright (ON) and dim (OFF) wells.[5,7] Signal from a positive reaction within these small volumes is highly confined and concentrated. These isolated signals can then be counted to quantify the amount of target in a sample.[5,10] Although effective and very sensitive, the implementation of the microwell approach requires microfabrication and complex operation (by a robotic instrument[11] or a human operator), which is not suitable for most point-of-care or resource-limited settings. As alternative to microwells for compartmentalization is droplets,[6,12] but they are not amenable to wash steps. To address this limitation, methods have been developed for droplet merging and mixing within microfluidic chips, though these methods can be slow or non-uniform[13,14] and add complexity that is not amenable to all locations.

In the droplet-based approach, the reporter groups, such as fluorophores, that result from the immunocomplexes are optically separated.[6] The fluorophores are in a homogenous solution within a droplet, but are separated from the contents of other droplets, effectively counting individual positive reactions. Most droplet-based reactions, however, require specialized microfluidics to create uniform droplets which can require multiple unit steps to operate.

To address these challenges, we aimed to develop a digital immunoassay with properties more suitable to point-of-care devices. In particular, the two goals are to reduce the number of user steps (to 1 if possible) and to eliminate the need for complex instrumentation for droplet preparation.[5,6]

To reduce the number of steps, we employed proximity ligation chemistry. Proximity-based assays require two or more reaction components coming into proximity for a signal to be generated. Therefore, only when a binding event takes place (such as antigen-antibody binding) will the reaction components be in close enough

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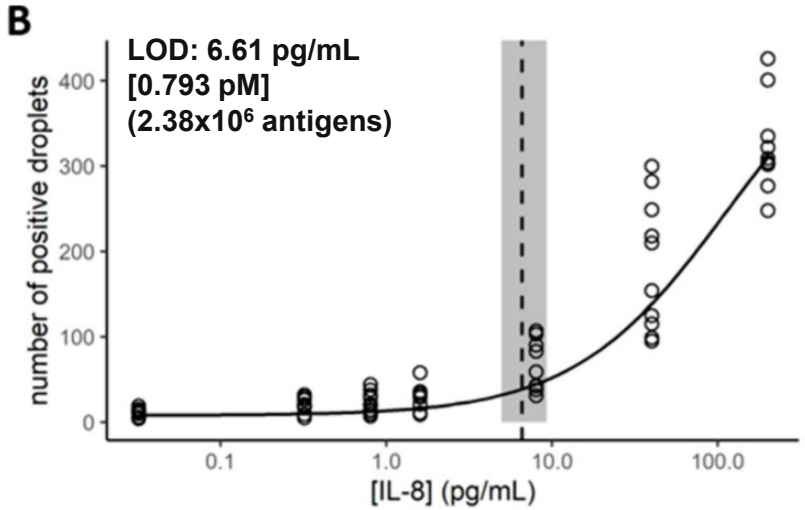
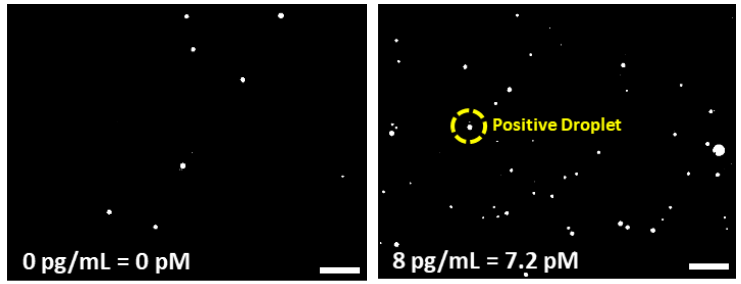
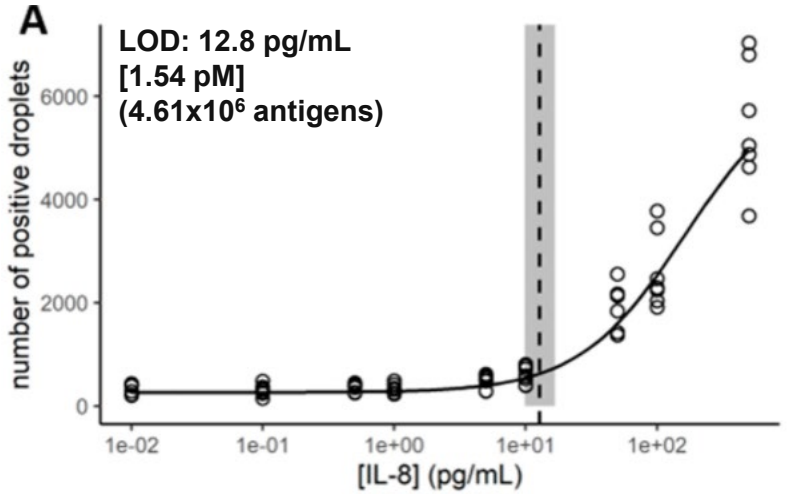
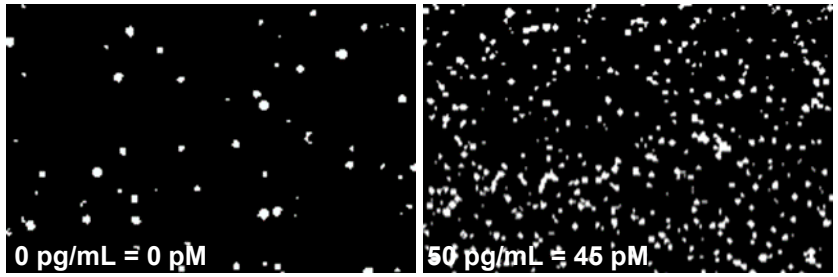
ddIA in Buffer





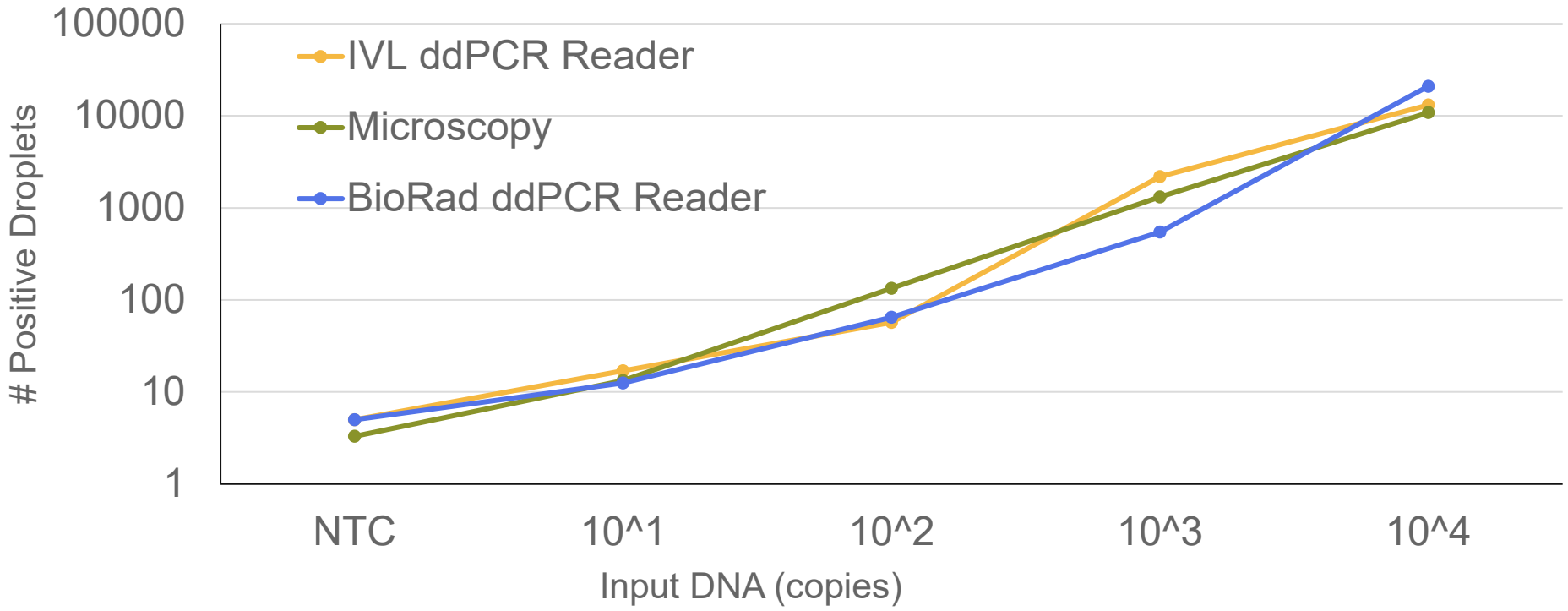
ddIA in Diluted Whole Blood





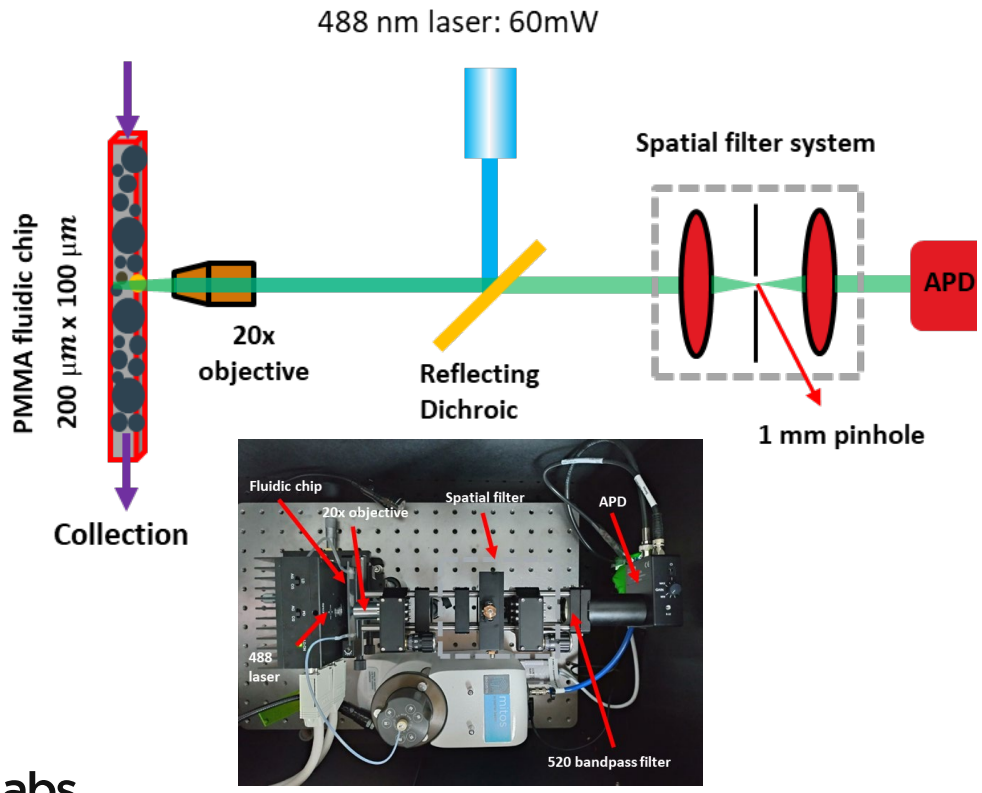
We are on a path to a low-cost digital assay reader

- Recording volume: 100 µL ± 10 µL
  - Droplets were collected at the end of each sample recording and the volume was measured by pipets
  - The droplet count from both microscopy and biorad reader are converted to 100 µL for the comparison



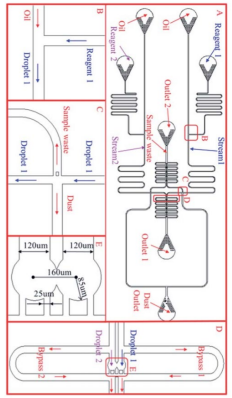
Simple polydroplet digital assay reader as development platform

+ No need to resolve individual droplets (Biorad required <2% CV)
+ High sample volume capability 1 mL/min read rate
+ Low-cost optics and sensors

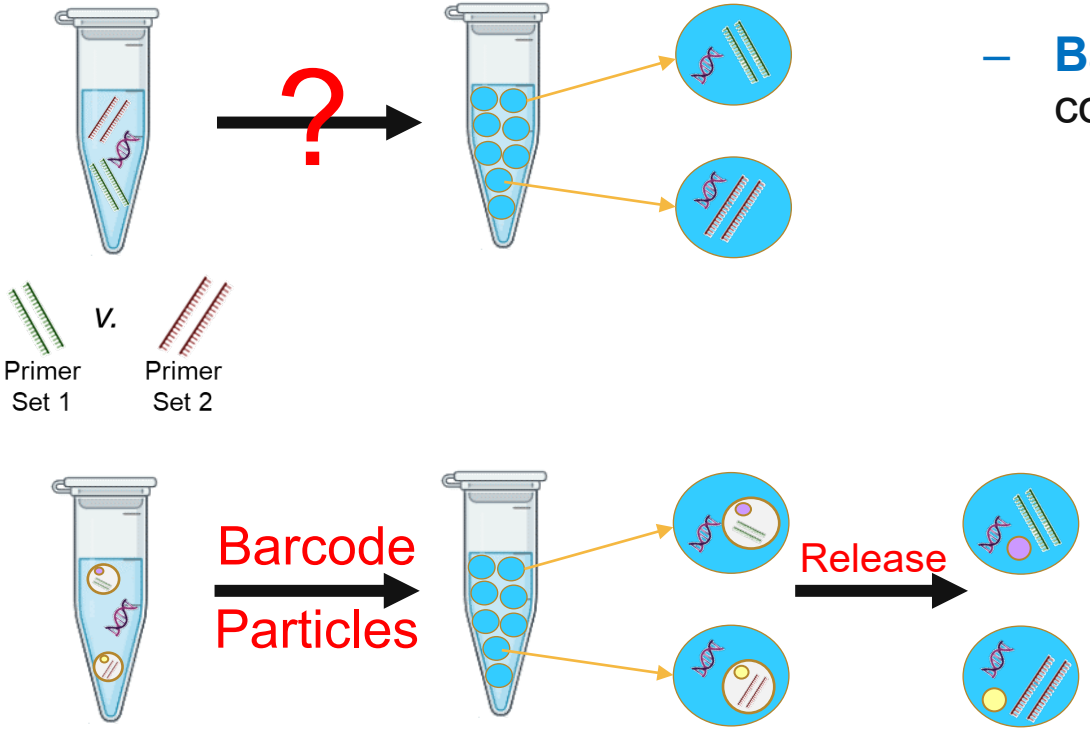


GHL Droplet Assays: multiplexed reactions using barcode particles

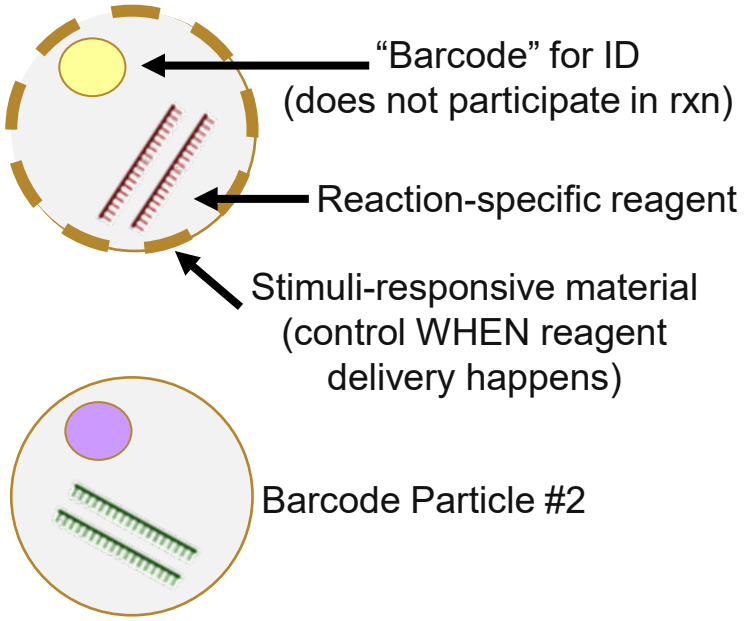
+ Barcode particles enable multiplexed, simultaneous, independent, unique reactions without complex microfluidics and minimal user steps



Chen, Ren. *RSC Advances*, 2017.



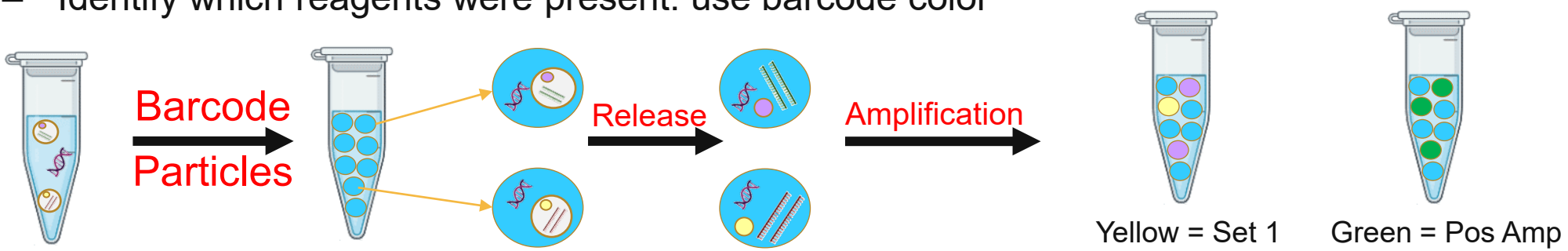
– **Barcode Particle**: controlled-release particle containing reagents unique to a reaction



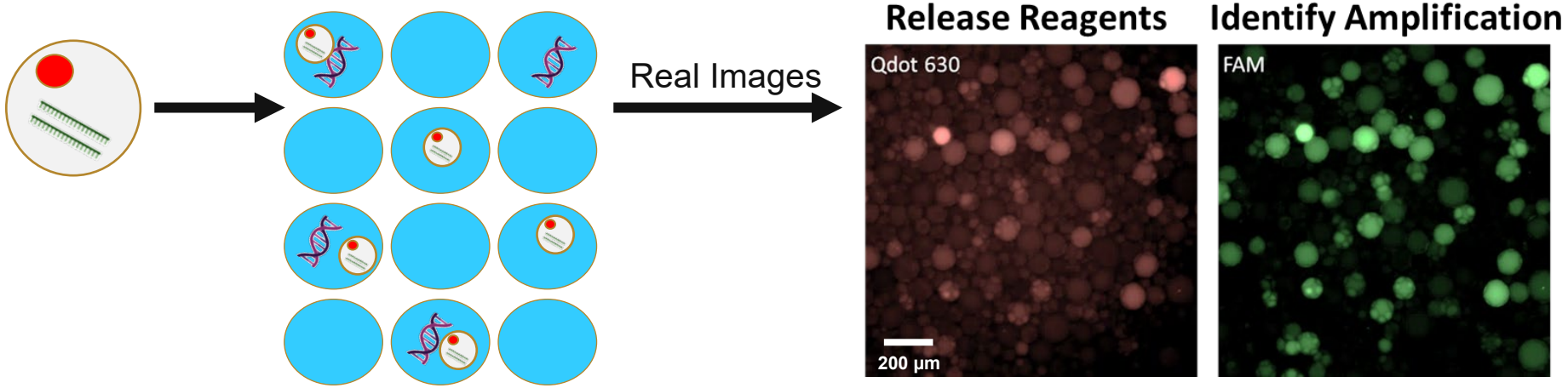
Patent application filed

Example of barcode in practice

+ Run amplification use two(+) colors to identify positives and which primers were present
  – Positive Amplification: EvaGreen or sequence-specific probe
  – Identify which reagents were present: use barcode color



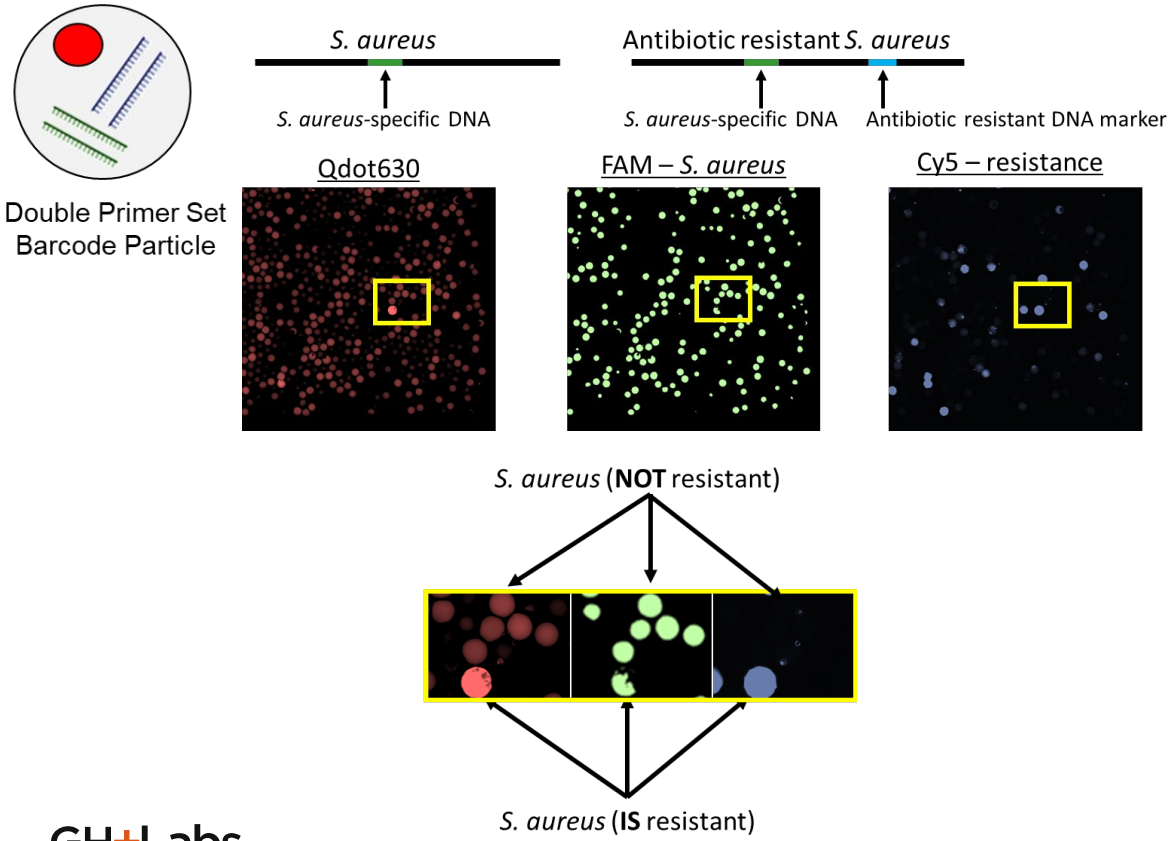
+ Example: Red barcode particle + FAM probe



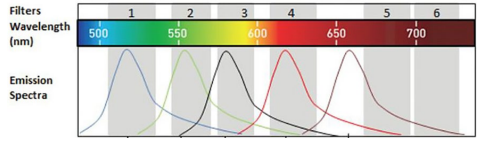
Patent application filed

Barcode particles for expanding multiplexing capabilities for digital applications

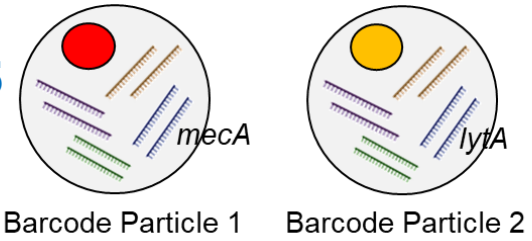
- Barcode particles can also be used to link primer sets in the same reaction
  - Ex: Identify antibiotic resistance assay by detecting multiple targets on a single piece of DNA (or within a single cell)

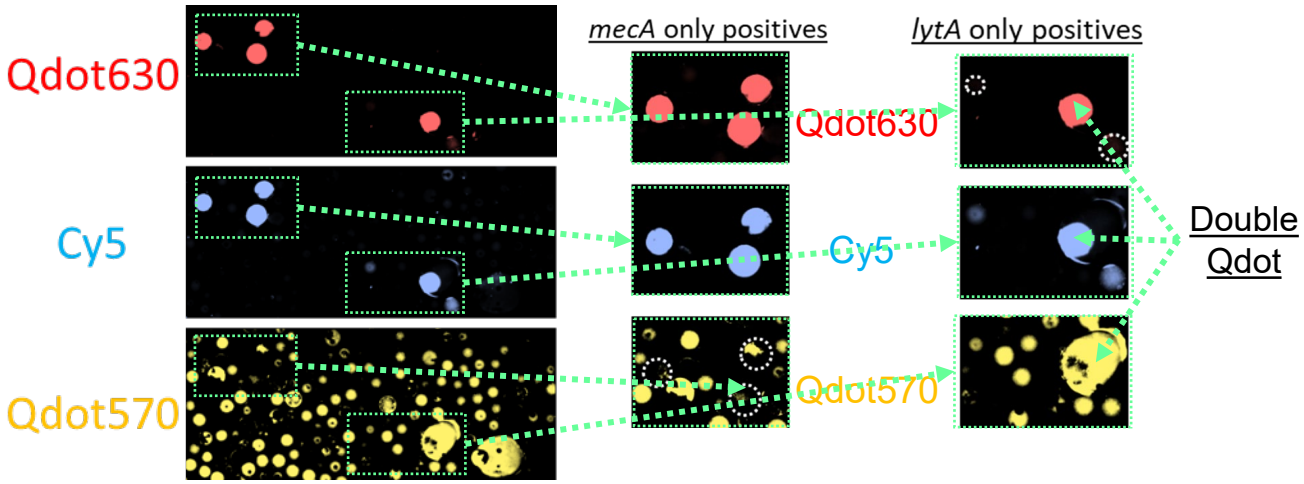


- We've demonstrated a 4-plex ddPCR, but we're nearing the limitations of traditional and readily-available fluorescent-probes



- Demonstrated an 8-plex ddPCR with two barcode particles
  - BP1: Qdot 630 + *mecA*-**Cy5**
  - BP2: Qdot 570 + *lytA*-**Cy5**





GH+Labs

Project Status

- Outcomes
  - Multiple peer-reviewed publications
  - Multiple patents filed and dedicated to public domain
  - Open-source tools and technical documentation available on GitHub
- Next Steps
  - Further development of low-cost reader hardware needed to enable scaling this technology in low-resource settings
  - Further expansion of assay applications to support adoption of this technology

References

+ Publications
  - Byrnes SA, Huynh T, Chang TC, Anderson CE, McDermott JJ, Oncina CI, Weigl BH, Nichols KP. Wash-Free, Digital Immunoassay in Polydisperse Droplets. Anal Chem. 2020 Mar 3;92(5):3535-3543. doi: 10.1021/acs.analchem.9b02526. Epub 2020 Feb 12. PMID: 31999432.
  - Byrnes SA, Chang TC, Huynh T, Astashkina A, Weigl BH, Nichols KP. Simple Polydisperse Droplet Emulsion Polymerase Chain Reaction with Statistical Volumetric Correction Compared with Microfluidic Droplet Digital Polymerase Chain Reaction. Anal Chem. 2018 Aug 7;90(15):9374-9380. doi: 10.1021/acs.analchem.8b01988. Epub 2018 Jul 26. PMID: 29985594.
  - Huynh T , Byrnes SA , Chang TC , Weigl BH , Nichols KP . General methods for quantitative interpretation of results of digital variable-volume assays. Analyst. 2019 Dec 2;144(24):7209-7219. doi: 10.1039/c9an01479a. PMID: 31663521.
  - Byrnes SA , Phillips EA , Huynh T , Weigl BH , Nichols KP . Polydisperse emulsion digital assay to enhance time to detection and extend dynamic range in bacterial cultures enabled by a statistical framework. Analyst. 2018 Jun 11;143(12):2828-2836. doi: 10.1039/c8an00029h. PMID: 29781480.

+ Droplet Analysis Software
  - GitHub.com/Global-Health-Labs/DropletID_python
  - GitHub.com/Global-Health-Labs/DropletID_matlab