Supplementary Materials

Supplementary Materials and Methods:

Strains and Media

The low copy (approx. 5 – 15 copies/bacterium) number plasmid pBR322 $bla_{CTX\text{-}M\text{-}9}$ kan^r, containing a 160-bp truncated synthetic $bla_{CTX\text{-}M\text{-}9}$ target gene and kanamycin resistance marker, was transformed into a WT background *E. coli* strain for use in this evaluation. Engineered *E. coli* were grown overnight on LB agar containing 50 µg/ml Kanamycin in a 37 °C incubator. A single, isolated colony was selected and grown in LB broth containing 50 µg/ml Kanamycin overnight. Frozen aliquots of culture were kept in -80 °C for additional studies. The night prior to the day of the experiment, frozen aliquots of bacteria were used to inoculate growth media and cultured overnight. The OD_{600} of the overnight *E. coli* culture was measured the next day and serially diluted to prepare stock concentrations for spiking PCR mixtures.

Microinjector growth study

Prior to this experiment, the possibility of growth in the overnight storage condition was investigated by preparing 9 x 5 CFU samples. Three of these samples were plated immediately after preparation; the remaining samples were stored at 4 °C and plated over the following 2 days (day 1: n=3, day 2: n=3). Colonies were counted for each plate on the following morning. Results confirmed both that a high degree of precision is obtainable by using the microinjector to prepare low CFU samples and that the samples did not grow under the described conditions (**Figure S8**).

CFU verification assays

CFUs in culture stocks used in **Figures 4 and 5** were verified by plate assays. LB agar plates were made using standard petri dishes. All plates were incubated overnight at 37 ^{0}C prior to counting. Specifically, 0.1 ml of a stock dilution (2,000 CFU/ml) was spread onto agar plates in triplicate. Plates were counted the next day. The average CFU count was multiplied by a factor of 10 to give the CFU/ml of the stock dilution. To verify bacterial stock concentrations used in IC3D and bulk real-time PCR samples, CFU verification plates were prepared as described above and predicted stock concentrations (CFU/ml) of bacteria were based on OD_{600} values obtained with a Thermo Scientific™ NanoDrop™ Microvolume Spectrophotometer (1 OD = 8 X 10^8 CFU/ml). While expecting approximately 200 CFU/plate, consistently fewer colonies were present on plates from the two separate biological replicates (41, 45 and 27 colonies were counted for the 2,000 CFU/ml stock concentration from Day 1 and 43, 58 and 35 colonies were counted from the stock concentration from Day 2. Based on the CFU verification results, though the actual number of bacteria in 100 CFU/ml - 1,000 CFU/ml samples were slightly lower than anticipated, the relative order of magnitude between different concentrations was maintained and a linear correlation was observed between predicted sample concentrations and IC3D hit rates.

Blood Bulk PCR (real time PCR)

A master mix for real-time PCR was prepared containing 10% whole blood (EDTA-treated), inhibitor-resistant Taq (VitaNavi Technologies), PCR buffer (1x), PEC buffer (1x), dNTP (0.2 mM), BSA (5 mg/ml), forward and reverse primers (1 µM each), and target-specific probe (0.5 µM) and Rox (ThermoFisher Scientifics), and subjected to filtration with 10-micron filter. For each condition, bacteria (or PBS for negative control samples) were spiked into corresponding amount of PCR mix to reach final concentrations of 0, 50, 100, 500, 1,000, 5,000, 10^4, 10^5, 10^6

and 10^7 CFU/ml. Each sample condition was run in quadruplicates (20 µl in each well). The sample plate was covered with sealing film and placed into the 7900 HT Fast Real-Time PCR system (Applied Biosystems). Samples were thermocycled using the same PCR protocol as the droplet PCR, described previously.

Rapid-PCR proof-of-concept study

Though most studies presented here utilized standard PCR protocols, a demonstration was performed to illustrate the capability of IC 3D blood ddPCR system to report a sample-to-answer readout in under one hour. For this demonstration, the droplet generation rate was increased from 50 µl/min (1ml sample = 20min) to 100 µl/min (1ml sample = 10min), while maintaining uniform droplets at an average diameter of approximately 100 µm. With our standard-length PCR thermocycling protocol of 40 cycles of 30s at 95°C for denaturation and 30s at 55°C for annealing/extension, the average duration is approximately 1 hour and 40 minutes. By shortening the denaturation and annealing/extension steps of each cycle to 10 seconds, and only using a total of 35 cycles, a shortened PCR protocol was obtained, resulting in a total time of 42 minutes. Since scanning samples on the 3D particle counter only takes less than 5 minutes, the total time from sample encapsulation to analysis readout is approximately 57 minutes. To assess the effect of compressing the PCR assay, fluorescent microscopy and particle counting analyses were performed on positive (high concentration of 10^4 CFU/ml) and negative controls prepared with the standard-length protocol and rapid-PCR protocol. Raw fluorescence IC3D data was translated into "hits/60s" by applying the following shape-fitting algorithm settings: Gaussian width = 33 sdv, amplitude > 350, $X^2 < 0.005$.

Blood droplet PCR optimization

1. *Fluorescence signal detection*

Due to the unique challenges of using whole blood in a fluorescence-based diagnostic instrument, strategies were developed to maximize performance by avoiding any compromising effects of blood autofluorescence, especially after thermocycling. Since the multi-channel IC 3D instrument is calibrated to perform multiplex detection at 3 standard wavelengths (469, 532, 635nm: FAM/HEX/Cy5), potential fluorophores were investigated using fluorescence microscopy SNR measurements. Not surprisingly, blood autofluorescence significantly interfered with fluorophores emitting in the green region of the visible spectrum (e.g. Alexa-Fluor 488) (**Figure S3**). Therefore, the far-red-emitting fluorophore, Quasar 670 (LGC Biosearch Technologies), was selected for the majority of TaqMan probes designed for the following studies (SNR of 5.5 compared to SNR < 2.5 for Alexa-Fluor 488 and CAL Fluor Gold 540) (**Figure S3**). Another parameter affecting the fluorescence detection sensitivity is the relative size of the droplets within the cuvette. We theorized that smaller droplets, while critical for certain ddPCR applications where increased sample partitioning can enable higher sensitivity (i.e. liquid biopsy applications, where smaller droplets enable loading more background gDNA[1,2]), may result in weaker signal detection since the observation volume would be comprised of more autofluorescent "negative" droplets. By controlling the concentration of Cy5 dye within droplets, a simulation indeed found that stronger fluorescence signals could be detected from larger droplets (on the order of 100µm diameter), particularly in situations with a high background fluorescence signal from negative droplets. Therefore, microfluidic droplet generators designed and used in the following studies typically involved droplets with average diameters between 85 and 110 µm (size controlled by different flow rates used).

2. *Primer and probe sequence design*

First, PubMed searches were performed to select candidate primer pairs and probes for targets of each AST panels, and NCBI blasting was conducted to confirm the species-specificities of selected candidates. Traditional PCR followed by gel electrophoresis was implemented to double check PCR amplification and sequence specificity. After validation of sequence specificity for each target, we then carefully evaluated the specificity and efficiency of bulk qPCR performance in the presence of 20% whole blood using either engineered bacteria or clinical isolates. As shown in **Supplementary Figures 1,2** with *bla*$_{KPC}$*, bla*$_{VIM}$*, vanA, vanB, bla*$_{CTX-M-2}$*, and* MRSA *(mecA, nuc)* as representative examples**,** we successfully optimized qPCR condition that enables efficient amplification of bacterial spiked whole blood sample without DNA purification.

3. *Blood droplet PCR SNR optimization*

After specificity was confirmed in bulk PCR, each genetic panel was transitioned to droplet PCR, where additional parameters were modified to maximize the SNR of positive droplets.

As the IC 3D BSI assay detects specific genetic sequences from single bacteria within droplets, the efficiency of different bacterial lysis strategies to expose genetic targets was evaluated including incubation at 95°C, sonication (30s on/30s off for a total of 2 min), and UV exposure (2 min) (**Figure S5**). The optimal condition that resulted in the highest SNR and lowest background signal via fluorescence microscopy was a 5-minute incubation at 95°C, which is performed as the initial step in the thermocycling protocol. Gram-positive MRSA isolates (**Figure 7.b**) were efficiently lysed using a proprietary protocol.

Next, primer and probe concentrations were optimized to achieve the highest possible signal while minimizing background fluorescence. By using fluorescence microscopy imaging, different primer/probe ratios were assessed by varying the concentration of each from 100 nM to 1 μM. Final concentrations of 1 μM primer and 500 nM probe were selected due to optimal fluorescence signal from positive droplets while maintaining low background signal of negative droplets (data not shown).

In addition to blood droplet stability, blood concentration within droplets was also evaluated to determine if increasing concentrations of blood compromised PCR efficiency. While stable amplification was observed for blood concentrations up to 30% (**Figure S6.a, b**), a final blood concentration of 10% was selected for the following studies as a conservative approach to achieve robust detection across a broad panel of molecular targets.

BSA is an established PCR amplification facilitator often applied to overcome inhibition(3). In order to evaluate if addition of BSA could potentially enhance the efficiency of droplet PCR, droplets were encapsulated in 10% whole blood with PCR mixtures that contain different amount of BSA. The highest SNR was observed with BSA concentrations between 5-14 mg/ml. Since there was no noticeable increase in SNR beyond 5mg/ml BSA (**Figure S6.c**), this was selected as the final concentration for remaining experiments.

To evaluate the optimal concentration of magnesium chloride ($MgCl_2$), a common reagent in standard PCR that serves as a cofactor to DNA polymerase, we performed a titration of $MgCl_2$ from 0.5 mM to 2 mM into PCR mixtures. As shown in **Figure S6.d**, addition of $MgCl_2$ had minimal effect on enhancing positive fluorescence signals SNR and therefore determined that the $MgCl_2$ level within the PCR reagent buffer was optimal for droplet PCR performance.

PCR cycle number was also investigated to identify the appropriate number of cycles that resulted in the highest possible positive fluorescence signal while not increasing the overall background fluorescence signal or leading to droplet coalescence **Figure S6.e, f**. After a final protocol was established for robust droplet generation and thermal stability, we determined that droplets could be thermocycled for 40 PCR cycles without compromising droplet integrity or significantly increasing the background fluorescence signal.

Head-to-head comparison with conventional 1-D droplet digital PCR

Using the same model bacteria as the experiments demonstrating IC3D instrument sensitivity (*E. coli* with synthetic $bla_{\text{CTX-M-9}}$ target gene), bacteria were spiked into whole blood to prepare samples with final concentrations of 50,000, 5,000, 500, 100, 50, 10, and 0 CFU/ 1ml raw blood. DNA from each sample was extracted using the Quick-DNA™ Microprep Plus Kit from Zymo Research (Irvine, CA), which is intended for high-quality, total DNA extraction (eukaryotic and prokaryotic) from biological fluid specimens, including whole blood. Extractions were carried out according to the manufacturer's protocol and optimized as needed to improve the total yield and quality of DNA. DNA extractions were quantified and inspected for quality using a spectrophotometer and were determined to meet standards necessary for our downstream PCR applications (data not shown). Due to the DNA loading limitations specified in the BioRad instrument manual(4), each sample was diluted to a final concentration of 66ng/20µl reaction. Three measurement replicates were analyzed by performing 3 independent extractions from samples with the same initial starting concentrations of bacteria in whole blood.

Using a common definition for limit of detection as the lowest detectable concentration at which the measurement is statistically distinct from the negative control (higher than the upper error bar), the LOD for the BioRad ddPCR system is between 50 – 100 CFU/ml. However, since this experiment involved a synthetic target with roughly 6-10 copies/bacterium, we anticipate that this detection sensitivity will actually translate to a clinical LOD of 500 – 1,000 CFU/ml when adjusting for the additional targets present prior to droplet generation. For comparison, even with a 10% sample dilution, by performing one-step PCR directly from whole blood, the IC3D system is capable of an LOD of 10 CFU/ml and requires no adjustment for target copy number since whole bacteria are encapsulated during droplet generation.

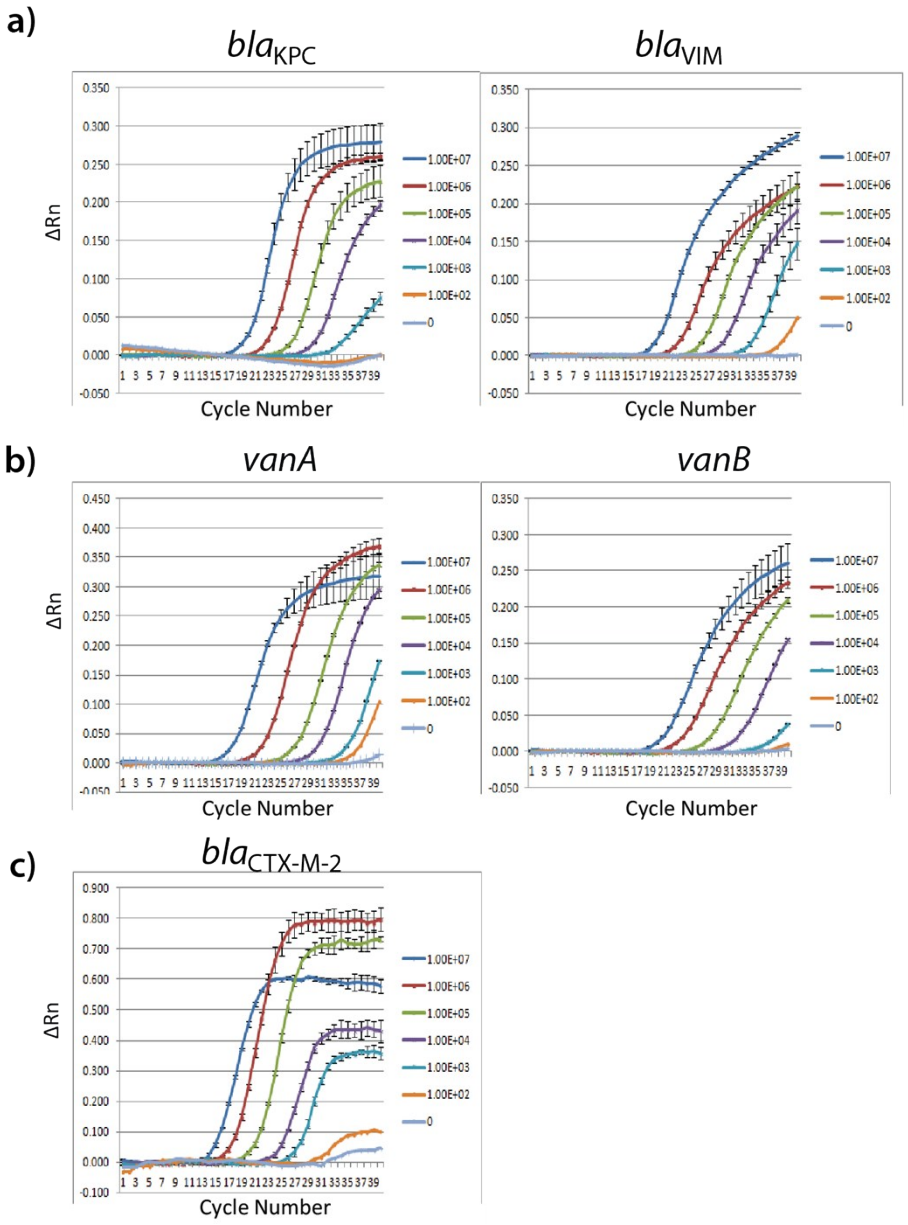


Figure S1. Representative qPCR panel validation against engineered bacteria in 20% blood. **a)** Carbapenem-resistant enterobacteriaceae (CRE) engineered bacteria (bla_{KPC} and bla_{VIM} targets), **b)** VRE engineered bacteria (*vanA* and *vanB* targets), **c)** Cefotaxime resistant (CTX) engineered bacteria ($bla_{CTX-M-2}$ target). Wells were loaded in quadruplicate. Error bars represent the standard deviation of the delta Rn (normalized reporter) value for each target between the quadruplicates.

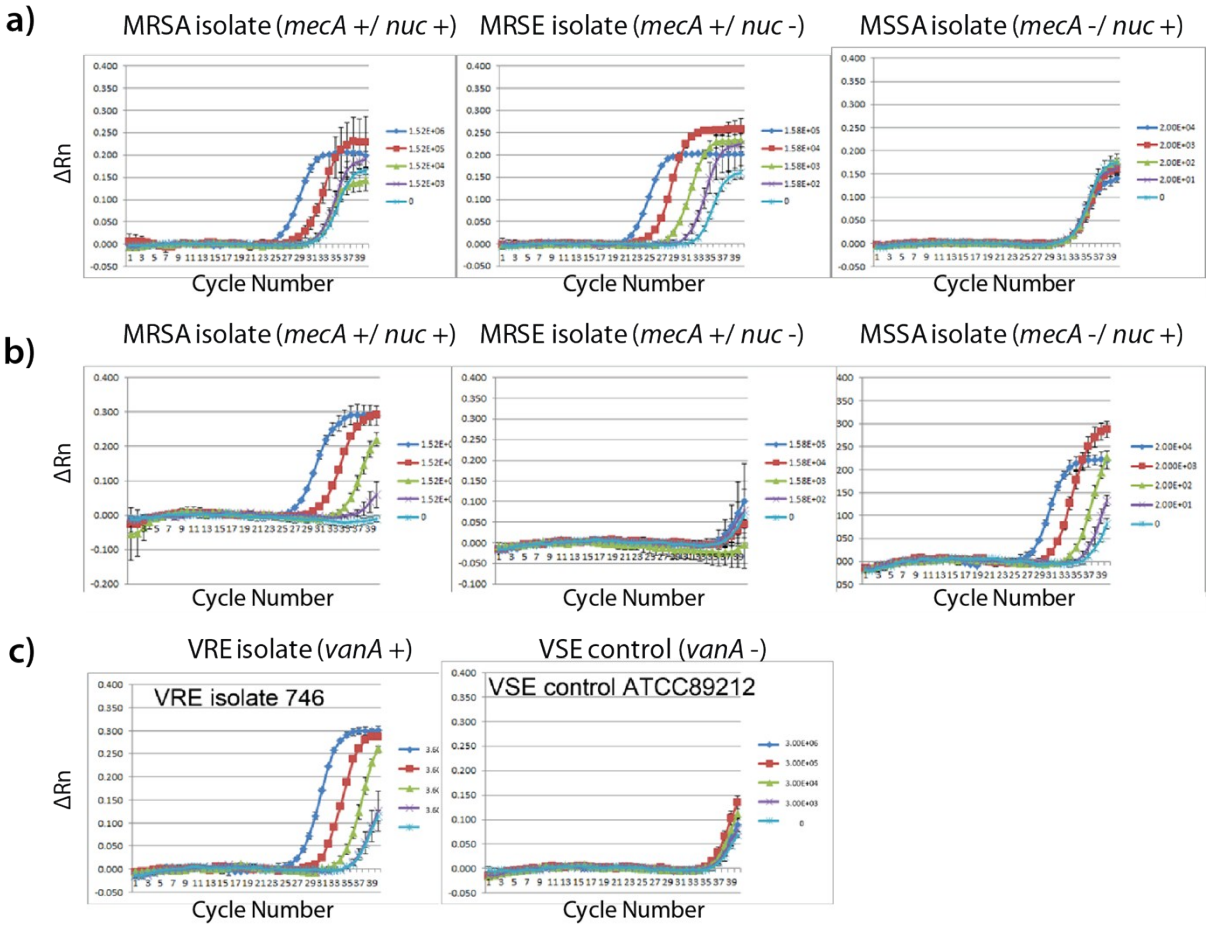


Figure S2. Representative qPCR panel validation against clinical isolates in 20% blood. **a)** MRSA (*mecA*+/nuc+), MRSE (*mecA+/nuc*-), and MSSA (*mecA-/nuc*+) clinical isolates for *mecA* detection, **b)** MRSA, MRSE, and MSSA clinical isolates for *nuc* detection, **c)** VRE and VSE clinical isolates for *vanA* detection. Wells were loaded in quadruplicate. Error bars represent the standard deviation of the delta Rn (normalized reporter) value for each target between the quadruplicates.

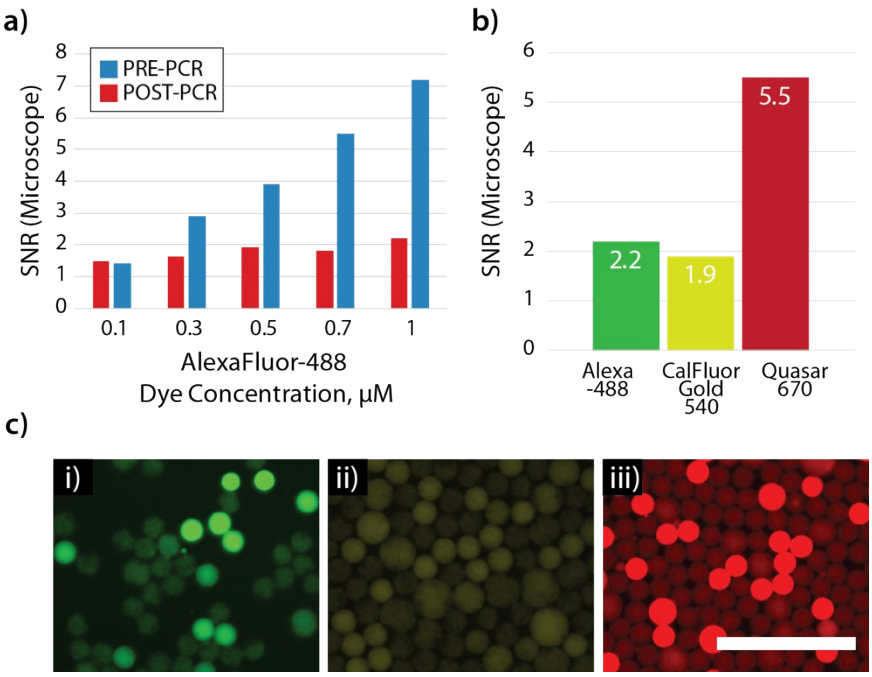


Figure S3. Fluorophore selection for robust signal detection in whole blood. Using a model system of WT *E. coli* (ATCC 23922) and a *16s* pan-bacteria detection chemistry, investigation of signal-to-noise ratio in single bacterium droplet PCR using different fluorescence probes was carried out in 10% whole blood. **a)** Investigation of blood autofluorescence interference with fluorophore detection. Droplets were encapsulated in 10% unprocessed blood with different concentrations of FITC-Biotin dye (0, 0.1, 0.3, 0.5, 0.7, or 1 µM) and thermocycled for 35 cycles (95 ^{O}C for 10s, and 55 ^{O}C for 10s). Quantitative image analysis was used to calculate SNR, defined as average signal from "positive" droplets / average signal from "negative" droplets. Strong interference from blood autofluorescence observed over a range of dye concentrations. **b-c)** Droplets were encapsulated in 10% unprocessed blood with 0.5 µM of indicated probe, 1 µM each of forward and reverse primers, along with PCR buffer and reagents. After thermocycling, droplets were imaged, (**c**, images scaled to same intensity range, scale bar = 500 µm); (**b**) Normalized SNRs of droplets were calculated as described above. Result: far-red emitting fluorophores (e.g. Quasar 670) were selected for the majority of designed probes due to superior signal detection in blood matrices.

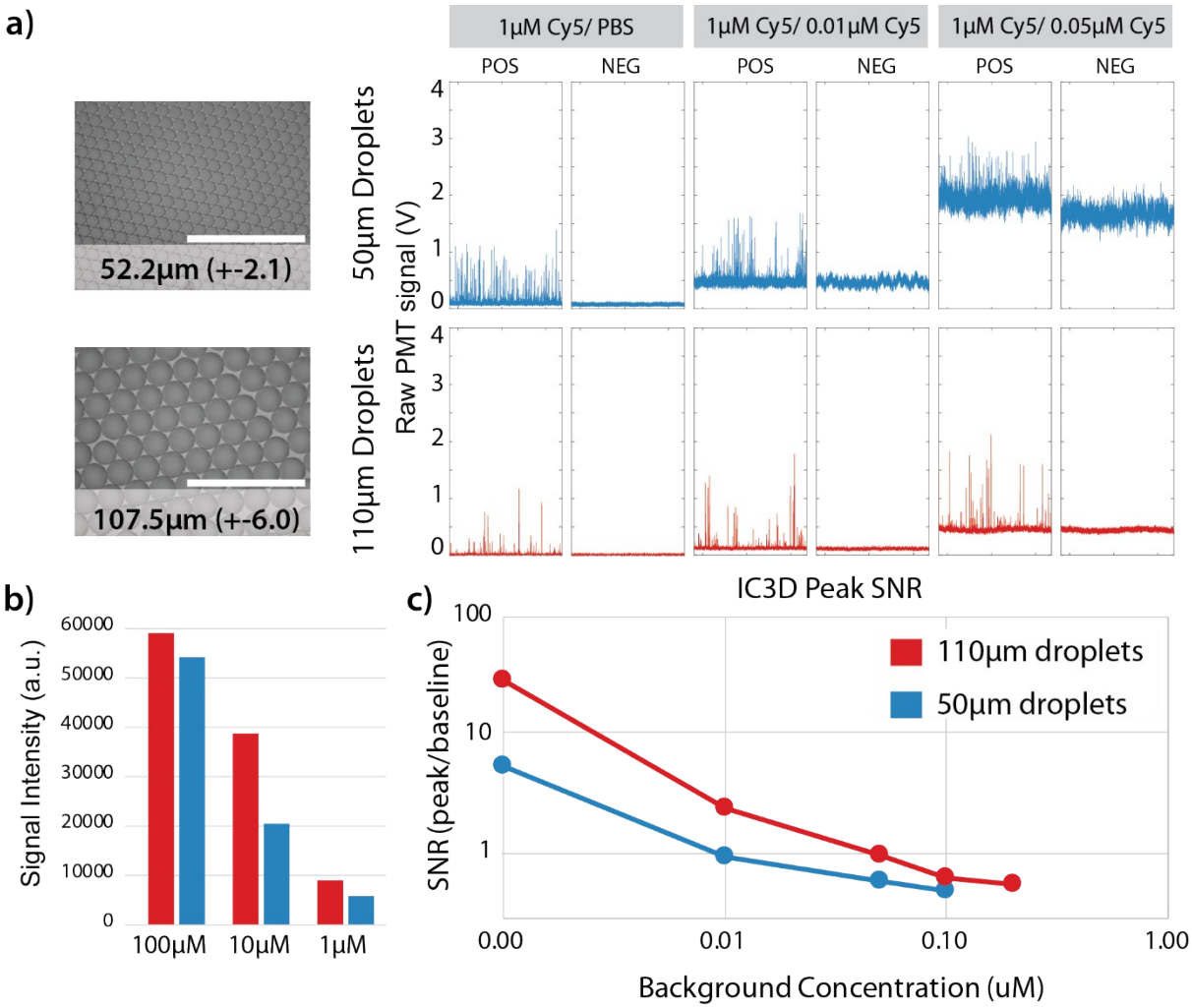


Figure S4. Droplet size optimization for robust particle counter detection. By creating samples with a fixed fluorophore concentration for "positive" droplets and increasing concentrations of fluorophore for "negative" droplets, droplet size (50 vs 100 µm diameter) was evaluated in a controlled experiment to determine optimal detection on the particle counter. **a)** Representative raw PMT signal data (x = 2s), **b)** quantitative microscope fluorescence signal of positive, Cy5 dye-containing droplets at different dye concentrations (demonstrated that droplet size alone did not affect observed median signal intensity), **c)** particle counter SNR (defined as peak amplitude over baseline intensity) for samples with increasing background fluorescence signal. Results indicated that larger droplets (~100 µm diameter) were more easily detected than smaller droplets, especially in cases with a non-negligible baseline fluorescence signal (as in cases of complex matrices, like whole blood).

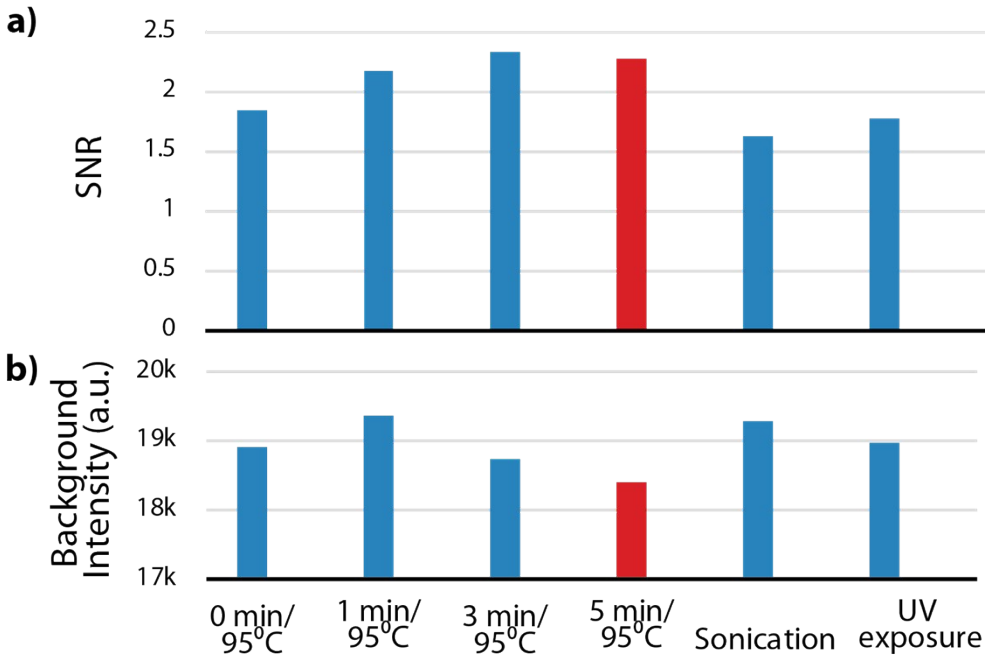


Figure S5. Bacteria lysis fluorescence signal optimization. WT *E. coli* (ATCC 23922) with a *16s* pan-bacteria detection chemistry was used as a representative example here to determine relative lysis efficiency. **a)** Signal-to-noise ratio of positive fluorescent droplets to negative droplets, **b)** average background fluorescence intensity of negative droplets.

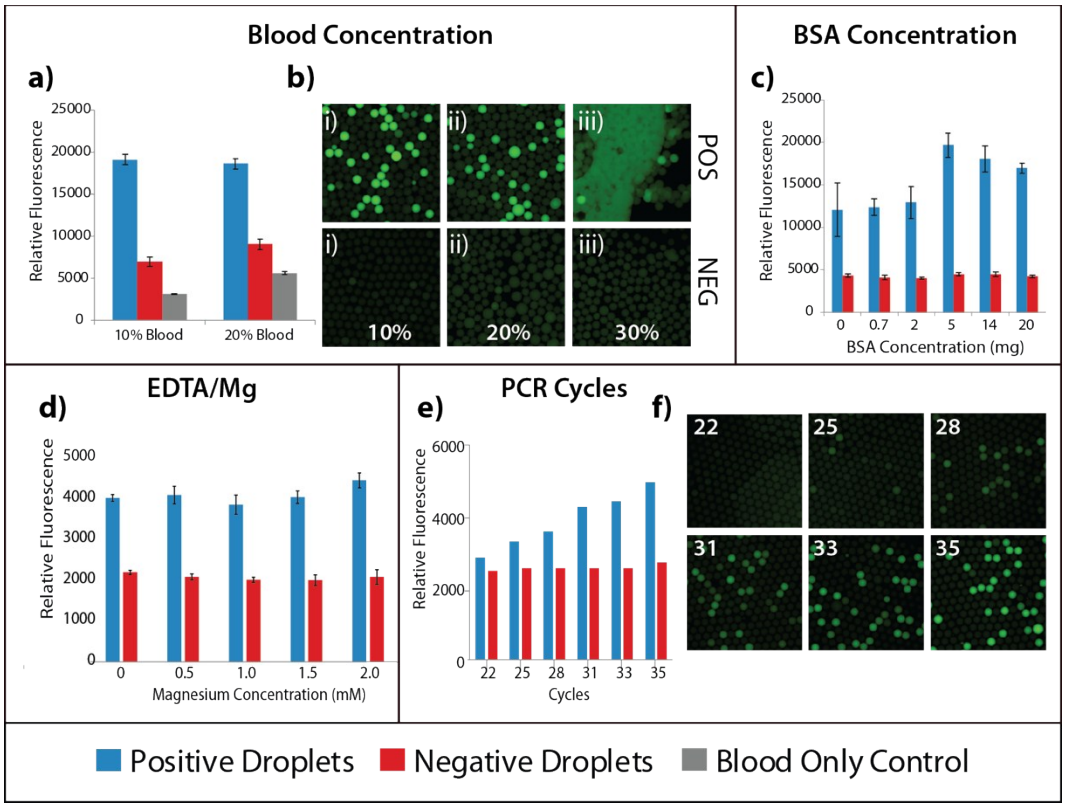
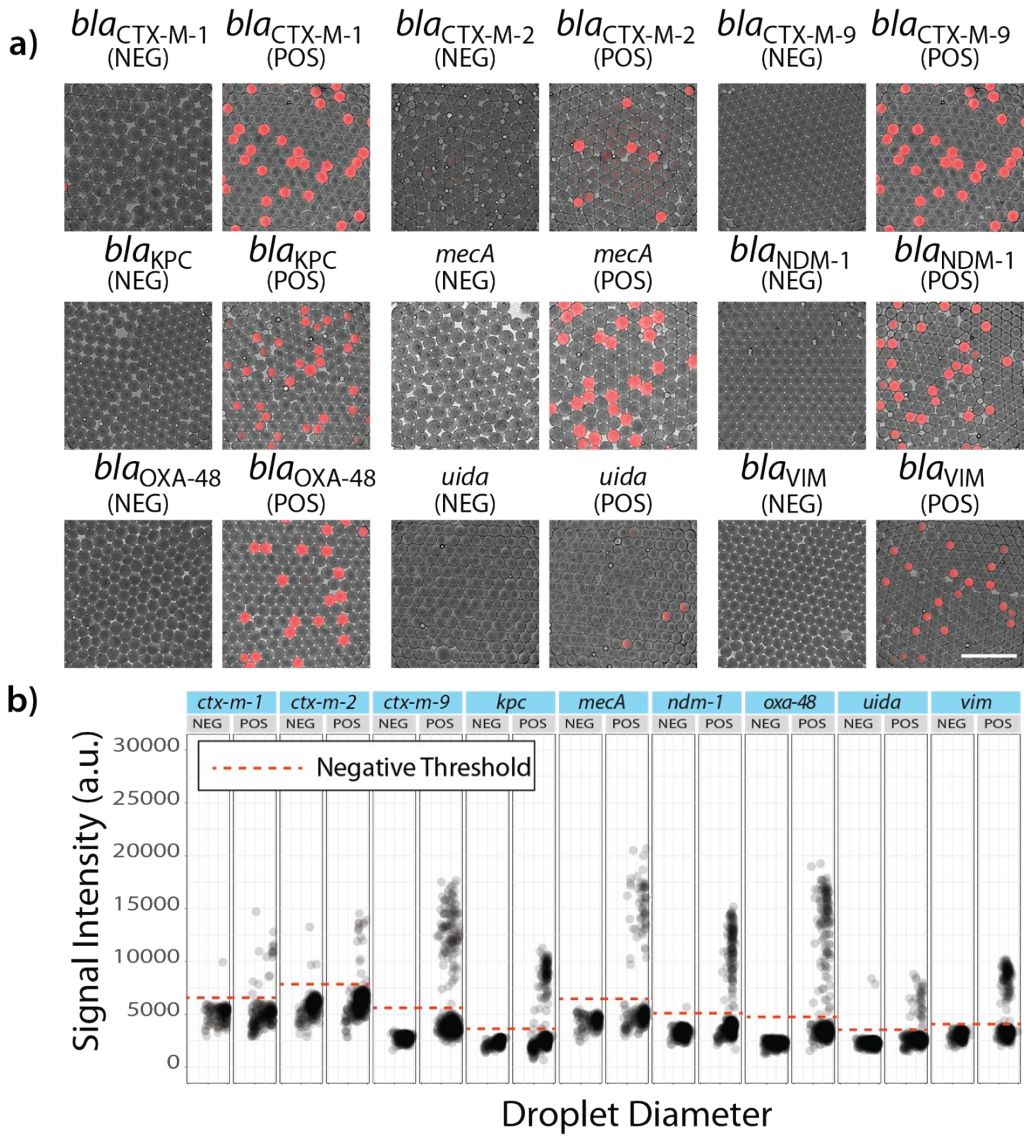


Figure S6. Droplet PCR signal-to-noise ratio (SNR) optimization. Representative examples using engineered *E. coli* with 16s pan detection primers and probes. **a)** Optimization of whole blood concentration from 10 – 30% in the PCR master mix volume. Fluorescence signal from positive droplets does not increase with an increase in blood concentration while background fluorescence of negative droplets does, compromising SNR. Final concentration of 10% blood was selected for experiments due to a decrease in droplet stability at 20% and 30% whole blood. **b)** Thermocycled droplet images used for assessing SNR and droplet stability of droplets from 10-30% whole blood. **c)** BSA optimization in PCR master mix. The highest SNR was observed with BSA concentrations between 5-14 mg. Since there was no noticeable increase in SNR beyond 5mg BSA, this was selected as the final concentration for remaining experiments. **d)** Magnesium chloride ($MgCl_2$) optimization in PCR master mix with 10% EDTA whole blood. 10x PCR reagent buffer used for this study includes 500 mM Tris-HCl pH 9.1, 160 mM ammonium sulfate, 0.25% Brij-58, and 25 mM magnesium chloride. We performed a titration of $MgCl_2$ by adding $MgCl_2$ at a variety of concentrations ranging from 0.5 mM to 2 mM into PCR mixtures, followed by droplet formation and thermocycling. SNR of droplets was calculated as described earlier. Addition of $MgCl_2$ had minimal effect on enhancing positive fluorescence signals SNR; therefore, we determined that $MgCl_2$ level within PCR reagent buffer is optimal for droplet PCR performance. **e)** PCR cycle number optimization in terms of droplet intensity and **f)** representative fluorescence microscope images used for PCR cycle analysis. Background fluorescence (negative droplets) does not increase as number of cycles is increased from 22-35 cycles or from 35-40 cycles (data not shown).



c)

	ctx-m-1	*ctx-m-2*	*ctx-m-9*	*kpc*	*mecA*	*ndm-1*	*oxa-48*	*uida*	*vim*
Avg SNR	2.11	2.26	3.83	3.63	3.54	3.44	5.96	2.72	2.72
90% SNR	2.34	2.41	5.57	4.25	4.05	4.24	7.31	3.34	3.03
Max SNR	2.49	2.44	6.34	4.63	4.76	4.40	7.70	3.63	3.23
Avg Background	5,130	5,787	2,788	2,432	4,405	3,355	2,298	2,300	3,136
N(POS)	475	709	768	886	654	1117	869	639	1054
N (NEG)	367	350	522	573	480	930	681	820	835

Figure S7. Molecular target validation with droplet microscope panel. **a)** Using the same fluorophore for target-specific probe sequences (Quasar 670), droplet images were recorded and scaled to the same intensity range for negative and positive controls of the following genetic targets (engineered *E. coli* with synthetic plasmid): $bla_{CTX-M-1}$, $bla_{CTX-M-2}$, $bla_{CTX-M-9}$, bla_{KPC}, bla_{NDM-1}, bla_{OXA-48}, bla_{VIM}, MRSA (*nuc* (not shown), *mecA), and E. coli (uidA).* Scalebar = 500 µm. **b)** Using the above image panel, quantitative measurements of median droplet signal intensity (y-axis) were recorded from an average of 1,414 droplets per sample and plotted against individual droplet diameters (x-axis) to determine average baseline, SNR, and maximum

fluorescence intensity for each target. **c)** Quantitative measurements from (**b**) for key parameters including SNR and background intensity.

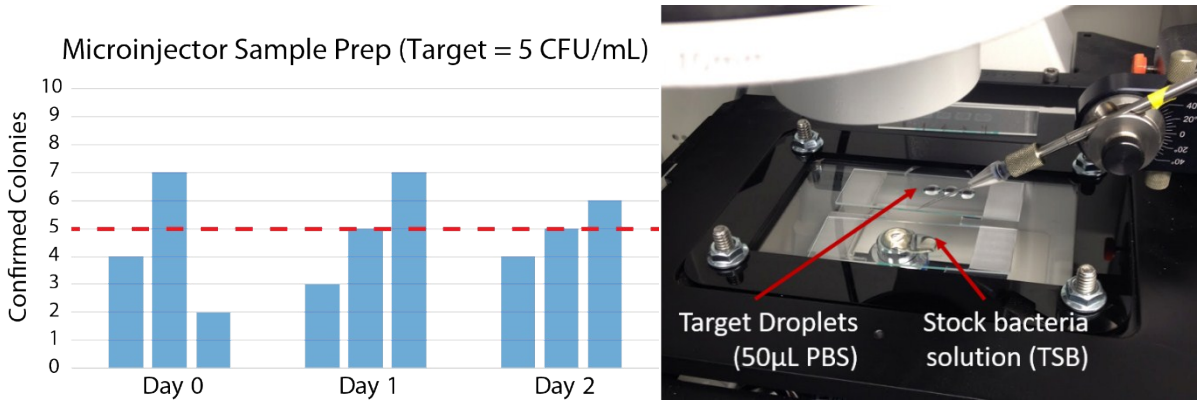


Figure S8. Microinjector sample preparation validation. Left panel) Growth study demonstrating consistent colony count for ultra-low concentrations. Right panel) Photograph of microinjector setup and positioning of stock bacteria and target droplet for transfer.

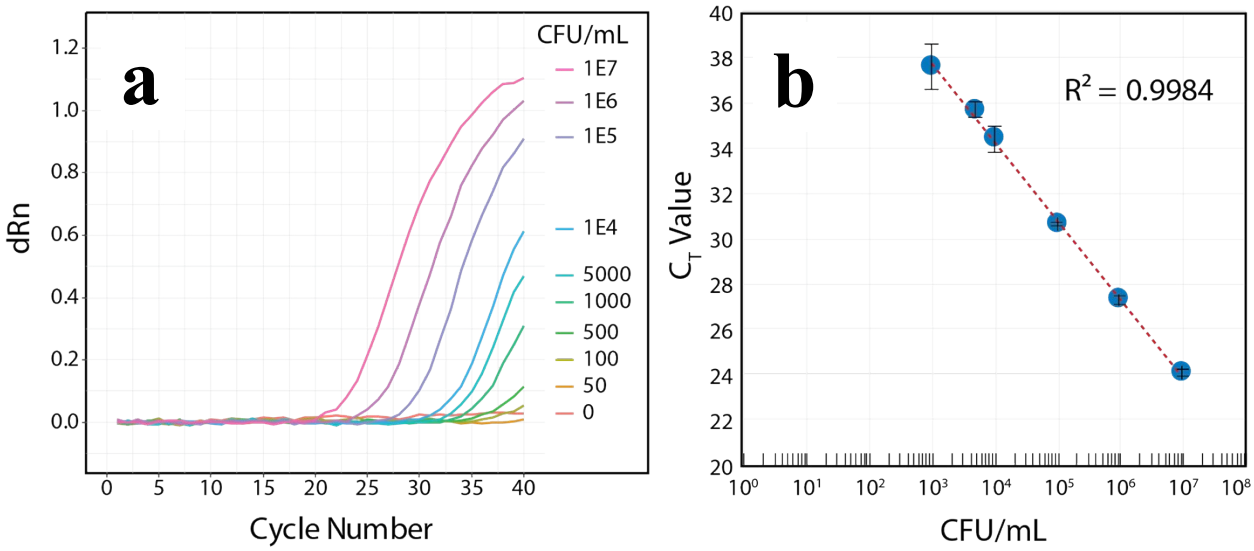
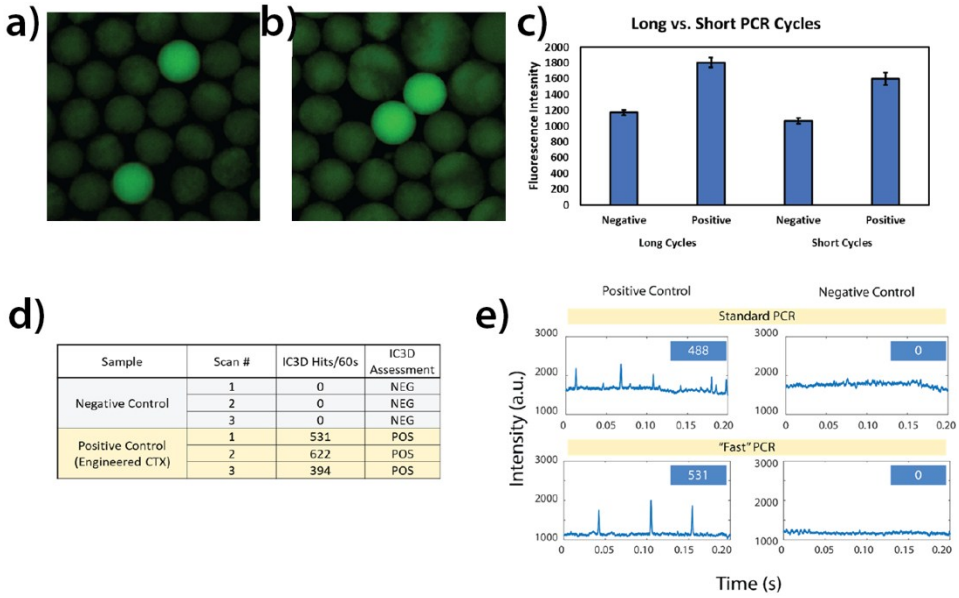


Figure S9. Bulk PCR results (single-digit sensitivity study). **a)** Amplification curves from negative control and 9 different final concentrations of 50, 100, 500, 1,000, 5,000, 10^4, 10^5, 10^6, and 10^7 CFU/ml per PCR mixture. **b)** Quantitation cycle threshold values (n=4) for negative control and serial dilutions of bacteria per PCR reaction. The linear regression line (R^2 = 0.9984) was generated by plotting the mean Ct values versus the serial dilutions of bacteria over a range of concentrations from 10^7 LogCFU/ml to 10^3 LogCFU/ml and demonstrated a limit of detection of 1000 CFU/ml with bulk PCR.



Sample	Scan #	IC3D Hits/60s	IC3D Assessment
Negative Control	1	0	NEG
	2	0	NEG
	3	0	NEG
Positive Control (Engineered CTX)	1	531	POS
	2	622	POS
	3	394	POS

Figure S10. Fast IC3D blood ddPCR proof-of-concept study. Demonstration of sample to result in less than 1 hour. **a)** Representative droplets image of short PCR cycles compared to **b)** standard PCR cycles. **c)** Droplets thermocycled using short cycles show a minor drop in relative fluorescent intensity compared to standard droplets, for both positive and negative droplets. **d)** (Raw IC 3D hits/60s for negative vs. "Fast PCR"). **e)** Representative time trace for standard PCR cycles vs. short PCR cycles ("Fast" PCR). (Bacteria used is *E. coli* with truncated $bla_{\text{CTX-M-9}}$ engineered plasmid. 10% blood, double-quenched FAM probe)

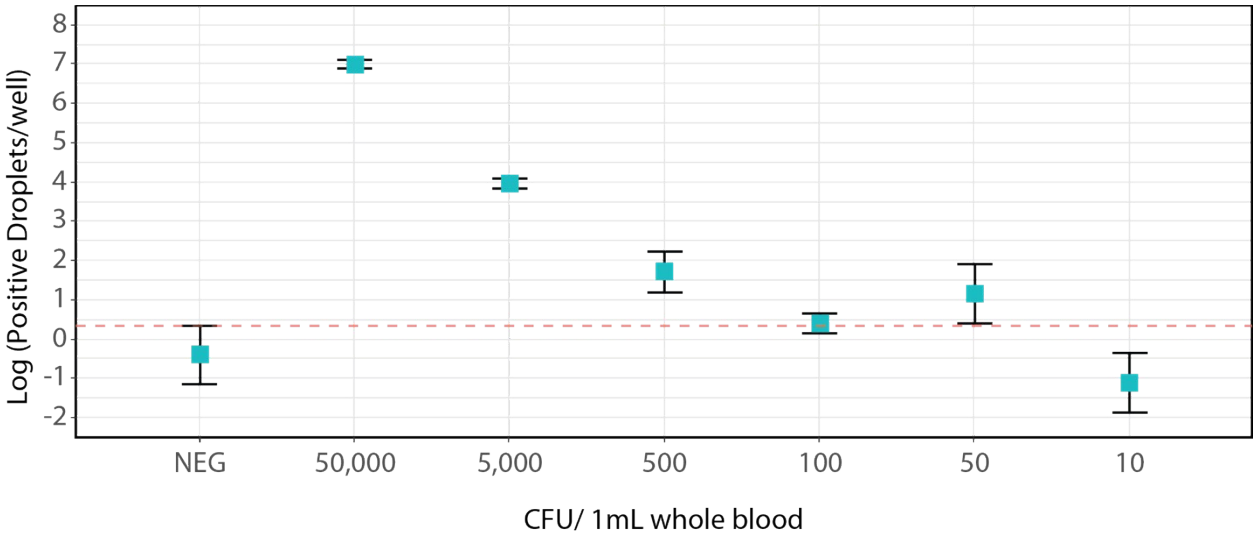


Figure S11. Equivalent performance of BioRad ddPCR system in detecting model *E. coli* with synthetic $bla_{\text{CTX-M-9}}$ target gene used in previous IC3D experiments. Error bars represent standard deviation among measurements from 3 independent extractions from samples with the same initial starting concentrations of bacteria in whole blood. Using a common definition for limit of detection as the lowest detectable concentration at which the measurement is statistically distinct from the negative control (higher than the upper error bar), the LOD for the BioRad ddPCR system is between 50 – 100 CFU/ml compared to 10 CFU/ml for the IC3D system.

Table S1. Complete list of all primer/probe sequences tested in paper studies.

Target	Target Gene	Ref	Forward primer sequence	Reverse primer sequence	Probe Sequence
16s - pan-probe (v.1)	16s rDNA		ACAACCCGAAGGCCTTCTTC	CTAGCTGGTCTGAGAGGATGAC	/5'Alex488n/CGGTCCAGACTCCTACGGGAGGCAGCAG/3'BHQ1/
16s - pan-probe (v.2)	16s rDNA	[(5)]	GAGGCAGCAGTRGGGAAT	CGCGCTTTACGCCCA	/5'Quasar670/CGTATTACCGCGGCTGCTGGCAC/3'BHQ2/
Methicillin resistance	*mecA*		CCAATTTGTCTGCCAGTTTCT	GGTATGCAACAAGTCGTAAATAAAAC	/5'Quasar670/TTGAGTTCTGCAGTACCGGATTTGCCAATT/3'BHQ2/
Staph aureus	*nuc*		GGTGTAGAGAAATATGGTCCTGAA	CCAAGCCTTGACGAACTAAAAGC	/5'6-FAM/TGGACGTGG/ZEN/CTTAGCGTATATTTATGCTG/3'IABkFQ/
ESBL	$bla_{\text{CTX-M-1}}$		TTCTTCAGCACCGCG	CGAATTAGAGCGGCAGTC	/5'Quasar670/CATCGCAAAGCGCTCATCAGCACGATAAAGT/3'BHQ2/
ESBL	$bla_{\text{CTX-M-2}}$		GGATTGTAGTTAACCAGGTCG	ATGTGCAGTACCAGTAAGGTGAT	/5'Quasar670/TTATCGCTCTCGCTCTGTTTAAGCACCGC/3'BHQ2/
ESBL	$bla_{\text{CTX-M-9}}$		CCATAACTTTACTGGTACTGCAC	GTCGCGCTCATCGATAC	/5'Quasar670/TGGAAAGCGTTCATCACCGCGATAAAGCA/3'BHQ2/
CRE	bla_{KPC}		ATAGTCATTTGCCGTGCCATAC	TGATTGGCTAAAGGGAAACACG	/5'Quasar670/TTCCGGTTTTGTCTCCGACTGCCCA/3'BHQ2/
CRE	$bla_{\text{OXA-48}}$		AAGACTTGGTGTTCATCCTTAACC	GAATGAGAATAAGCAGCAAGGA	/5'Quasar670/CCAAATCGAGGGCGATCAAGCTATTGGGA/3'BHQ2/
CRE	$bla_{\text{NDM-1}}$		CCATCCCTGACGATCAAAC	GACCAACGGTTTGGCGATCT	/5'Quasar670/AAACCCGGCATGTCGAGATAGGAAGTGTG/3'BHQ2/
VRE	*vanA*		CCATGTTGATGTAGCATTTTCAGC	CAAGGTCTGTTTGAATTGTCCG	/5'Quasar670/TGCATGGCAAGTCAGGTGAAGATGGATCC/3'BHQ2/
E. coli	*uidA*	[(6)]	CGGAAGCAACGCGTAAACTC	TGAGCGTCGCAGAACATTACA	/5'Quasar670/CGCGTCCGATCACCTGCGTC/3'BHQ2/
Klebsiella spp.	*khe*	[(7)]	GATGAAACGACCTGATTGCATTC	CCGGGCTGTCGGGATAAG	/5'Quasar670/CGCGAACTGGAAGGGCCCG/3'BHQ2/

Table S2. Experimental Design for single-digit sensitivity study

Microorganism used	*E. coli* pBR322 *ctx-m-9* kan^r
Sample matrix	Whole blood diluted to 10% in PCR reagent master mix
Bacteria concentration in final reaction	0 (negative control), 1, 5, 10, 100, 1x10^4 CFU/ml; prepared by serial dilutions of overnight stock
Measurement timing	End point (i.e. 3 hr after droplet formation)
Number of preparations	2 overnight stocks
Number of replicates per preparation	3
Total number of runs	36

Table S3. IC3D blood ddPCR results for "single-digit sensitivity study"

		IC3D Hits/60s for Different Concentrations (CFU/ml)					
Day	Replicate	0	1	5	10	100	10,000
1	A	0.00	4.00	20.00	56.00	82.67	163.33
1	B	0.00	5.33	0.00	26.00	28.67	146.00
1	C	0.00	0.00	0.00	0.00	74.00	213.33
2	A	0.00	4.00	26.00	44.00	91.33	207.33
2	B	0.00	0.00	0.00	2.67	0.00	205.33
2	C	0.00	0.00	1.33	13.33	0.00	78.00

Table S4. qPCR C_t values for "single-digit sensitivity study"

CFU/ml	C_T (Rep1)	C_T (Rep2)	C_T (Rep3)	C_T (Rep4)	C_T (Avg)	SD
0	N.A.	N.A.	N.A.	N.A.	-	-
50	N.A.	N.A.	N.A.	N.A.	-	-
100	N.A.	N.A.	N.A.	39.62	39.62	-
500	N.A.	N.A.	N.A.	38.59	38.59	-
1,000	37.63	39.00	37.57	36.56	37.7	1.0
5,000	36.14	35.68	35.33	35.91	35.8	0.3
10^4	35.19	34.67	33.84	34.27	34.5	0.6
10^5	30.68	30.67	30.80	30.73	30.7	0.1
10^6	27.62	27.45	27.40	27.10	27.4	0.2
10^7	24.19	24.07	24.07	24.37	24.2	0.1

Table S5. Blinded samples agreed 100% with clinical isolate preparations in identifying 4 positive-CTX-M14 samples and 6 negative samples. Raw fluorescence data was translated into "hits/60s" after applying the same shape fitting algorithm to all data.

Sample	IC3D Hits/60s	IC3D Assessment	Clinical Assessment	Beta-Lactamases Genes Present
Negative Control	0	NEG	-	-
Positive Control (Engineered)	488	POS	-	**CTX-M-14**
Sample 1	397	POS	POS	TEM-1B, CMY-2, **CTX-M-14**
Sample 2	281	POS	POS	TEM-1B, **CTX-M-14**
Sample 3	0	NEG	NEG	NDM-1, OXA-9, TEM-1A, CTX-M-15, OXA-1
Sample 4	0	NEG	NEG	OXA-9, TEM-1A, SHV-83, CTX-M-2, OXA-10
Sample 5	402	POS	POS	TEM-1B, **CTX-M-14**, SHV-11, DHA-1
Sample 6	0	NEG	NEG	KPC-3, OXA-1
Sample 7	0	NEG	NEG	CMY-94, SHV-1
Sample 8	515	POS	POS	TEM-1B, **CTX-M-14**
Sample 9	0	NEG	NEG	CMY-80
Sample 10	0	NEG	NEG	KPC-4, TEM-1A, ACT-5

Table S6. IC 3D blood ddPCR results from Fast-PCR proof-of-concept study

Sample	Scan #	IC3D Hits/60s	IC3D Assessment	Lab Assessment
Negative Control	1	0	NEG	NEG
	2	0	NEG	NEG
	3	0	NEG	NEG
Positive Control (Engineered CTX-M-9)	1	531	POS	POS
	2	622	POS	POS
	3	394	POS	POS

Table S7. Performance comparison of droplet detection technologies

Reference	Method of Detection	Detection Throughput (droplets per sec)	Analytical Sensitivity (CFU/mL)	Estimated Sample-to-result Time	Sample Matrix	Sample processing required?	Suitable for BSI bacterial ID/AST?
IC3D	High volume 3D fluorescent droplet scanning	25 - 350 kHz*	10	1 hr	Blood	No	Yes
BioRad	1D Fluorescent Droplet scanning	1 kHz	50 – 100	6 hrs	Purified gDNA	Yes	No
Raindance	1D Fluorescent Droplet scanning	~ 1 kHz	NA	NA	Purified gDNA	Yes	No
Stilla Technologies	2D droplet array imaging	600 Hz	NA	NA	Purified gDNA	Yes	No
V. Yelleswarapu (8)	Time-domain encoded optofluidics with cellphone camera	1 MHz	NA	NA	Buffer	Yes	No
M. Kim (9)	CMOS sensor with PDMS channels mounted on top	254 kHz	NA	NA	Buffer	Yes	No
S. Lim (10) S. Terekhov (11)	Flow Cytometry**	10 kHz	NA	NA	Buffer	Yes	No

* Since IC3D is not "flow-based", droplet size directly factors into "droplet throughput" calculations. As a volumetric detection methodology, IC3D interrogates a volume of droplets at a rate of approximately 470 µl/min. For droplets of approximately 84 µm diameter (present study), this equates to a droplet throughput of approximately 25 kHz. For 35 µm droplets, this would equate to 350 kHz.

** Typically requires double-emulsions for compatibility with conventional flow cytometry instrumentation

NA: Data not available because method is not suitable for direct blood testing

References

1. Pekin D, Skhiri Y, Baret J-C, Le Corre D, Mazutis L, Salem CB, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip. 2011 Jul 7;11(13):2156–66.

2. Ou C-Y, Vu T, Grunwald JT, Toledano M, Zimak J, Toosky M, et al. An ultrasensitive test for profiling circulating tumor DNA using integrated comprehensive droplet digital detection. Lab Chip. 2019;19:993–1005.

3. Kreader CA. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and environmental microbiology. 1996 Mar;62(3):1102–6.

4. BioRad Laboratories, Inc. ddPCR Supermix for Probes: "Recommendations for Optimal Results."

5. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology. 2002;148(1):257–66.

6. Silkie SS, Tolcher MP, Nelson KL. Reagent decontamination to eliminate false-positives in Escherichia coli qPCR. J Microbiol Methods. 2008 Mar;72(3):275–82.

7. Hartman LJ, Selby EB, Whitehouse CA, Coyne SR, Jaissle JG, Twenhafel NA, et al. Rapid real-time PCR assays for detection of Klebsiella pneumoniae with the rmpA or magA genes associated with the hypermucoviscosity phenotype: screening of nonhuman primates. J Mol Diagn. 2009 Sep;11(5):464–71.

8. Yelleswarapu VR, Jeong H-H, Yadavali S, Issadore D. Ultra-high throughput detection (1 million droplets per second) of fluorescent droplets using a cell phone camera and time domain encoded optofluidics. Lab Chip. 2017;17(6):1083–94.

9. Kim M, Pan M, Gai Y, Pang S, Han C, Yang C, et al. Optofluidic ultrahigh-throughput detection of fluorescent drops. Lab Chip. 2015;15(6):1417–23.

10. Lim SW, Abate AR. Ultrahigh-throughput sorting of microfluidic drops with flow cytometry. Lab Chip. 2013 Dec 7;13(23):4563–72.

11. Terekhov SS, Smirnov IV, Stepanova AV, Bobik TV, Mokrushina YA, Ponomarenko NA, et al. Microfluidic droplet platform for ultrahigh-throughput single-cell screening of biodiversity. Proc Natl Acad Sci U S A. 2017 Mar 7;114(10):2550–5.