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Supplementary Information for

Mobile platform for rapid sub-picogram-per-milliliter, multiplexed, digital droplet detection of proteins

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Table S1. Technologies to perform digital ELISA assays

Ref.	Target	LOD	Volume	Compart-ments	Device material	Multiplex Capability	Readout Equipment	Method of Detection
Single Enzyme Studies								
(1)	β-Gal	0.88 μM	4.2 pL	200k	PDMS	Green only	Fluorescence Microscope	-Single β-Gal in droplets -Measured rate of fluorescence production
(2)	ALP/ β-Gal	7.0 fM	44 fL	7600*120 chamber	CYTOP on glass coverslip	Green Orange	Fluorescence Microscope	-Noted only 2.5% of the substrate was consumed in 20 min
Biological Targets								
(3)	TNF-α and PSA	6 fg/mL (200aM)	50 fL	50k well	Glass wells Silicone gasket/oil	Singleplex	Fluorescence Microscope	Microarray with bead-based dELISA
(4)	P24	100 vir/mL	50 fL	comp	Simoa array	Multiplex Capability	Fluorescence Microscope	Measured number of p24 proteins -Converted to virion count by dividing 2000 p24 proteins/virion Verified with RNA extracted
(5)	PSA	1.2 pg/mL (43 fM)	32 fL	20k drops	PDMS Glass	Demonstrated Singleplex	Fluorescence Microscope	-Static array with around 20k droplets analyzed -Low dynamic range -No false positives reported
(6)	Flu	4 fM in buffer	38 fL	62.5k wells	Patterned Teflon-Af on glass slides	Multiplex Capability	Fluorescence Microscope	-Detected nucleoprotein from flu virus in nasopharyngeal swabs to show dELISA in clinical sample.
(7–9)	Commercial assays	3.8 fg/mL (200 aM) IL-10	40 fL	216k wells 25-50k beads	Cyclic Olefin Polymer Bluray Printing	Multiplex (6-plex 66 samples/h)	Fluorescence Microscope	-Fully automated commercial digital ELISA from Quanterix Simoa HD-1 Analyzer
This	GM-CSF IL6	3.7 fg/mL (300 aM) 7.0 fg/mL (350 aM)	22.5 pL	10M droplets 1M beads	PDMS and Glass 3D stack	Multiplex (Duplex Samples/5 min)	Cell Phone Camera	-Integrated mobile platform with parallelized droplet generation and detection with cloud computing

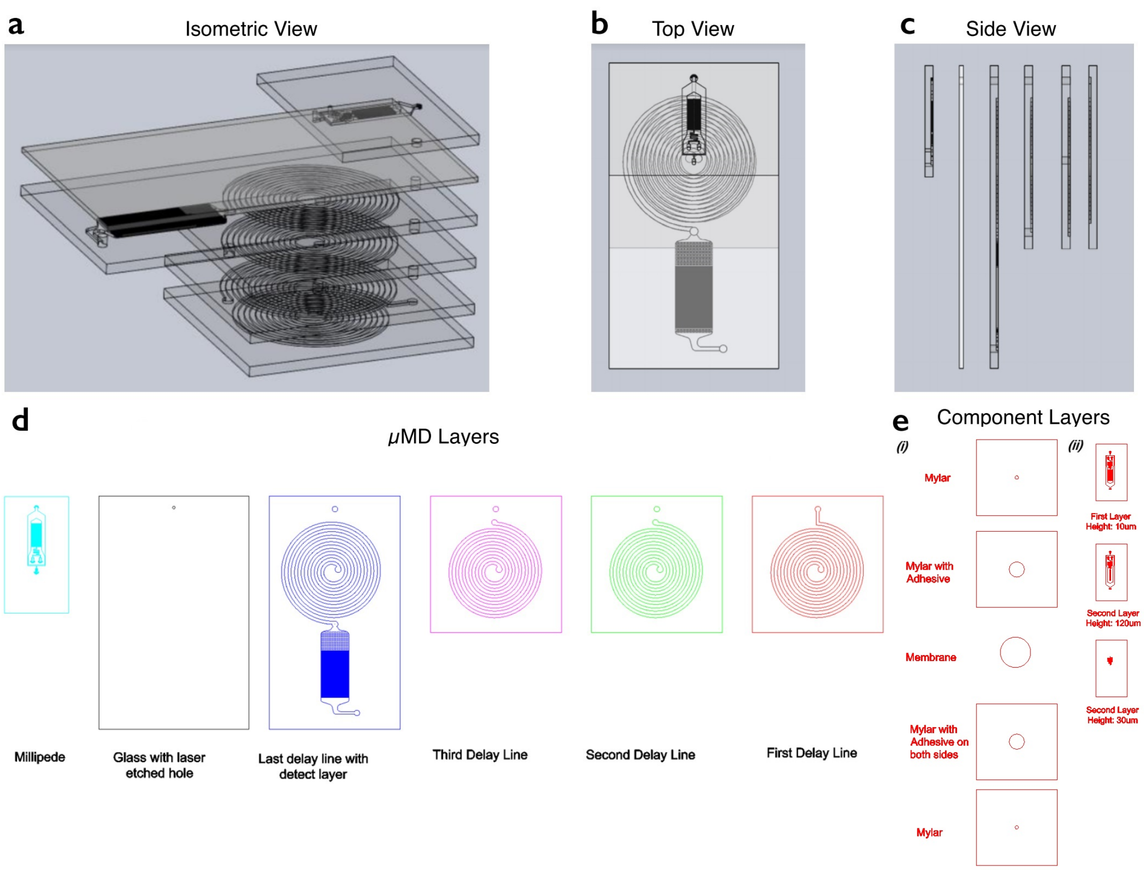

Figure S1. 3D design of chip. a-c. Isometric and side views of the µMD display how the modular components are stacked in a 3d chip. **d.** Draftsight drawings of each layer of the µMD, followed by component layers for the bead processor **(e,i)** and the droplet generator **(e,ii)**.

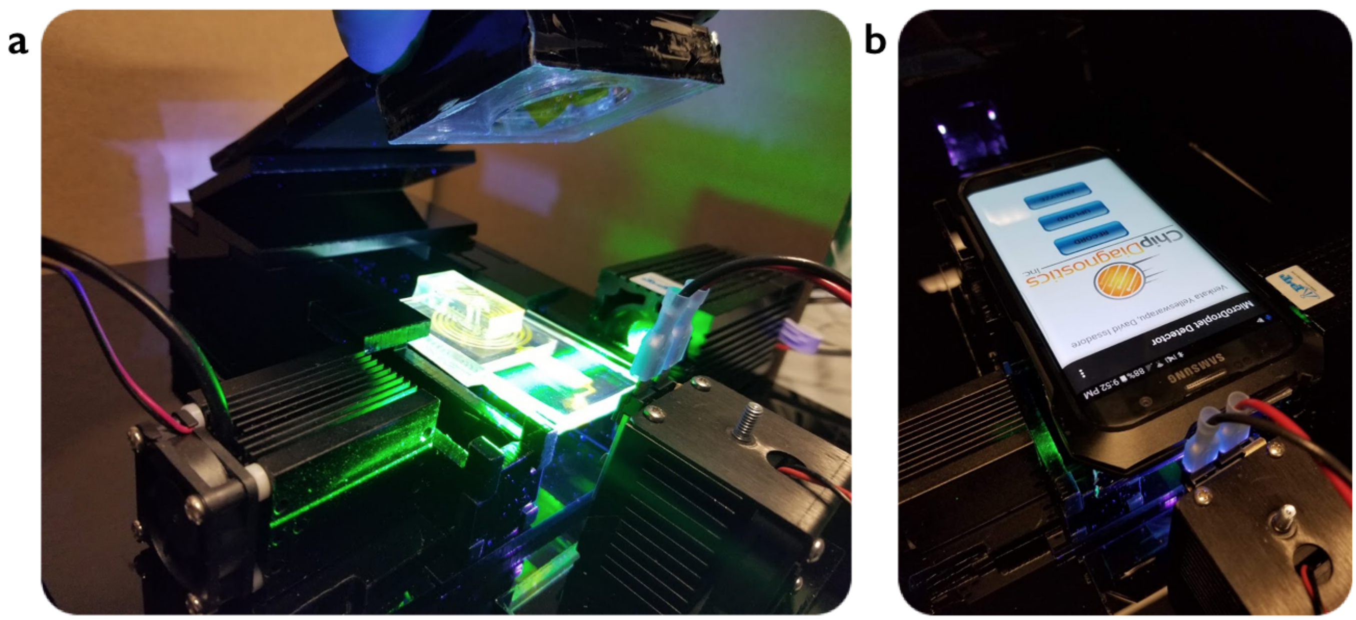

Figure S2. Design of µMD casing. a,b. The µMD consists of a disposable microfluidic chip, a cell phone, and an acrylic casing that we designed. The acrylic casing comes in two parts: one that is attached to the cell phone to fix the distance between the imaging plane and the macro lens, and the second which houses the LEDs and locks the cell phone into position when the disposable chip is inserted.

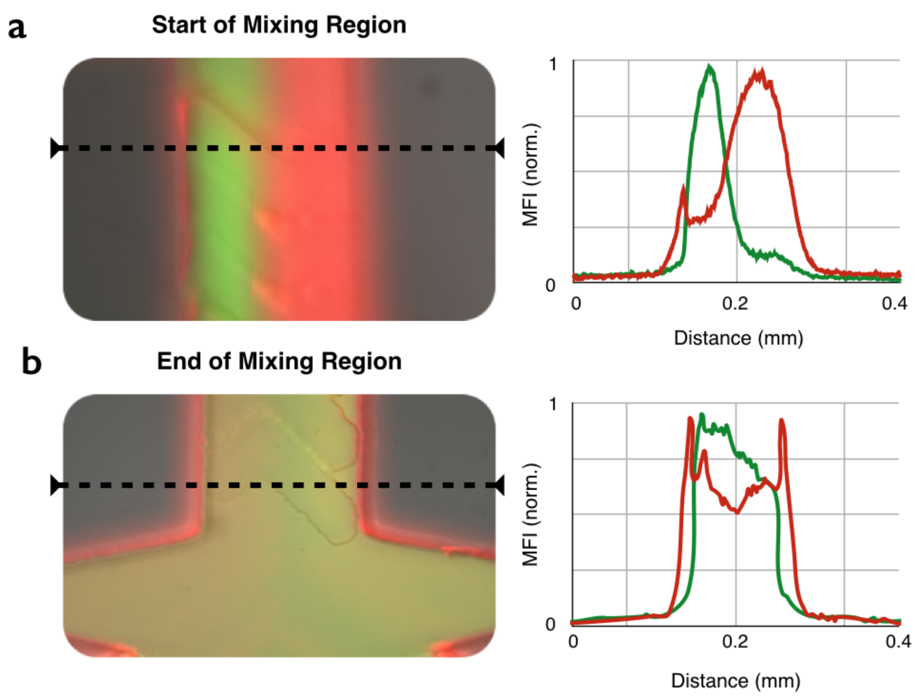

Figure S3. Mixing of aqueous phases. A channel length of 14 mm is used to ensure proper mixing of the beads and the substrate, while minimizing background signal that comes from enzymes generating fluorescence signal before they are encapsulated into droplets.

Movie S1. The video demonstrates the workflow of the droplet digital assay, as well as features of the robust droplet generation and detection.

Supporting Information Text

dELISA Assay. On our chip, the bead processing, droplet generation, droplet incubation, and detection are integrated. By avoiding manual processing steps, loss, contamination, and unreliable reinjection of droplets after incubation, as has been observed in similar systems(10), can be avoided. To functionalize the microbeads used in our assay, we first washed both the 5.4 µm Carboxyl Green [High Intensity Yellow] Particles (CFH-5052-2; Spherotech) and 4.5 µm Carboxyl UV beads (CFP-4041-2; Spherotech) 6 times each, using centrifugation at 15k rcf for 5 min to remove the sodium azide, which inhibits HRP, from the supernatant. Subsequently, we used the PolyLink Protein Coupling Kit (24350-1; PolySciences) to attach anti-human GM-CSF (MAB2172; R&D) and anti-human IL-6 (MAB206; R&D) antibodies onto the beads, respectively. To evaluate our device, we created serial dilutions of the protein targets (#215-GM-010, #206-IL-010; R&D) in low protein binding tubes to reduce protein binding to the surface.

The on-chip bead processing steps were carried out as follows. The input to the device is 1:4 diluted serum, diluted in T20 buffer. Incubation with the beads is performed in a total volume of 100 µL T20 buffer and protein sample. Reagents are stored off-chip in this study, but can be preloaded on-chip in future device generations. The reagents for the HRP substrate are prepared immediately before the assay to reduce the background of the fluorescence substrate. The reagents can be loaded into a pre-loaded tubing and dispensed using a peristaltic pump. On the on-chip membrane, which captures the beads for processing, the following steps are carried out. Following the initial incubation, the beads are washed and then incubated with 0.7 nM concentration of detection antibody in T20 buffer. After an hour of incubation, the sample is washed and replaced with 12.5 pM concentration of HsHRP in T20 Buffer (Life Technologies), and washed again. Subsequently, the flow is reversed so that beads are released and the output is encapsulated into droplets for analysis. For droplet generation, the continuous phase is Biorad Oil. Droplets of diameter d = 40 µm (CV = 5.3%) are generated with the total dispersed phase fixed at ϕ_d = 12 ml/hr with a fixed continuous phase of ϕ_c = 55 ml/hr. QuantaRed™ Enhanced Chemifluorescent HRP Substrate Kit was mixed with the microbeads in the on-chip vortex mixer (**Fig. 2a**, **SI Appendix, Fig. S3**) immediately upstream of the droplet generator. The substrate is introduced at a flow rate of 6 ml/hr. We selected QX200 Droplet Generation Oil for EvaGreen (Biorad) to encapsulate the beads in stable droplets with minimal dye leakage. The µMD uses high fluorescence intensity dye beads, and HRP substrate compared to the low intensity dye beads, the β-gal enzyme, and RGP substrate found in Simoa's technology.(3, 8)

When performing the bead-processing off-chip, we used the following protocol. Capture beads are added into sample in low protein binding tubes, and incubated for an hour. The sample is diluted into 1mL with T20 Buffer and centrifuged at 12k rcf to remove background cell debris and nontarget molecules in the supernatant. The beads are resuspended into 0.7 nM concentration of detection antibody in T20 buffer and incubated for an hour. This solution is diluted into 1mL T20 Buffer and centrifuged at 12k rcf to remove unbound detection antibody, and 12.5 pM concentration of HsHRP in T20 Buffer. This sample is washed 4 times using a centrifuge and resuspended in T20 Buffer, to remove any HsHRP that could result in a false positive. The resulting sample is then introduced as the aqueous inlet for the droplet generating device. For measuring samples spiked into FBS or PBS, we performed the incubation off-chip and all subsequent processing steps on-chip. We first diluted the serum 1:4 in T20 buffer. Incubation with the beads was performed in a total volume of 100 L T20 buffer and protein sample to reduce nonspecific binding, and placed on an end-to-end mixer for an hour to prevent sedimentation.

To calculate the limit of detection (LOD) and limit of quantification (LOQ), we measured the number of false positives in replicate (N = 3) "blank" samples that included FBS but contained no spiked protein. The LOD and LOQ was converted to units pg/mL using the know molecular weights of the target molecules. The LOD was defined as $\text{LOD} = \langle \text{FP} \rangle + 2.5\sigma(\text{FP})$, where $\langle \text{FP} \rangle$ was the mean number of false positives and σ(FP) was the standard deviation of the false positives. The LOQ was defined as $\text{LOQ} = \langle \text{FP} \rangle + 10\sigma(\text{FP})$. We defined these values so that they agree with what is used to describe the gold standard technology, Quanterix's Simoa. (11, 12)

Design of the Non-Disposable Components of the µMD and its Software. The µMD consists of a disposable microfluidic chip, a cell phone, and an acrylic casing that we designed.(**SI Appendix, Fig. S1,S2**) The acrylic casing comes in two parts. The first part is attached to the cell phone and fixes the distance between the imaging plane and the macro lens. The second part houses the LEDs and the cell phone, and sets the position of the disposable chip relative to the excitation sources and the camera (**SI Appendix, Vid S1**). This casing contains a low cost commercial plastic lens (<$4), a bandpass filter ($\lambda_{cwl}$ = 512 ± 11.5 nm, 630 ± 45.5 nm, #87-241; Edmund Optics), and a slot to automatically align the microfluidic chip. The disposable microfluidic chip is constructed of only PDMS, glass and mylar, and is prototyped using soft lithography at The University of Pennsylvania's Singh Center for Nanotechnology. The low cost plastic macro lens (15x magnification, ML-515; Carson HookUpz) is used to image the device Field of View FOV = 7x12 mm^2. (**Fig. 3b**) There are three excitation sources, each mounted in the acrylic casing: an ultra-bright UV LED (λ_{ex} = 400 nm, CBT-90-UV-C31-M400-22; Luminus), a fat beam (laser diameter > 10mm) blue laser diode (450nm, 400mW Laser Diode Module; APT Lighting), and a fat beam green laser (532nm, 300mW Laser Diode Module; APT Lighting). The light sources are driven using external electronics consisting of an LED driver circuit (DK-114N-3; Luminus Development Kit) for the LED, TTL modules for the laser diodes, and a microcontroller (Arduino Mega2560) programmed with unique MLS patterns for each light source. To illuminate the droplets in the microfluidic channels we make use of antiresonant side coupling to achieve uniform illumination(13). The non-disposable cost, excluding the cell phone, of the µMD prototype is < $1000.

The software used in this study implements the data analysis shown in **Fig. 3**. This software detects multiple fluorescent colors in each individual droplet, rather than just one, as was done in previous work. (14, 15) A custom App is written that is installed on a Galaxy S8 phone. This App controls, and coordinates, the multiple components in this experiment, including the cloud computing, and the cell phone camera. A commercially available App Open Camera is used to interface with the cell phone's camera, and allows manual control of the camera's settings. Video collected on the phone is uploaded to a Maltab cloud server (MathWorks Cloud). Optical aberrations in the video are fixed by the software based on a calibration used to calculate the distortion from the macro lens. Small errors in the position and angle of the chip relative to the camera are also corrected. The software then parses the frames into 120 individual channels, and carries out the algorithm described in **Fig. 3**. The data analysis currently takes 10 minutes to analyze 10 million droplets. Data analysis can further be sped up using a GPU or cloud server, but we currently run the process locally on a Using an Ubuntu OS with an Intel Core i7-7700HQ @ 2.80 GHz x 8 and 16 GB RAM. All source code for the software used in this study is included in the **SI Appendix, SI Text**.

Supplementary Code. Source code can be found in the following link to the lab website which contains all of the software with a readme file to explain how to use each of the components. All code is commented thoroughly for ease of use. Source code is shared for: (i) Matlab software for image analysis, (ii) Arduino code that modulates the LED excitation, and (iii) an Android App that connects the software to cloud.

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