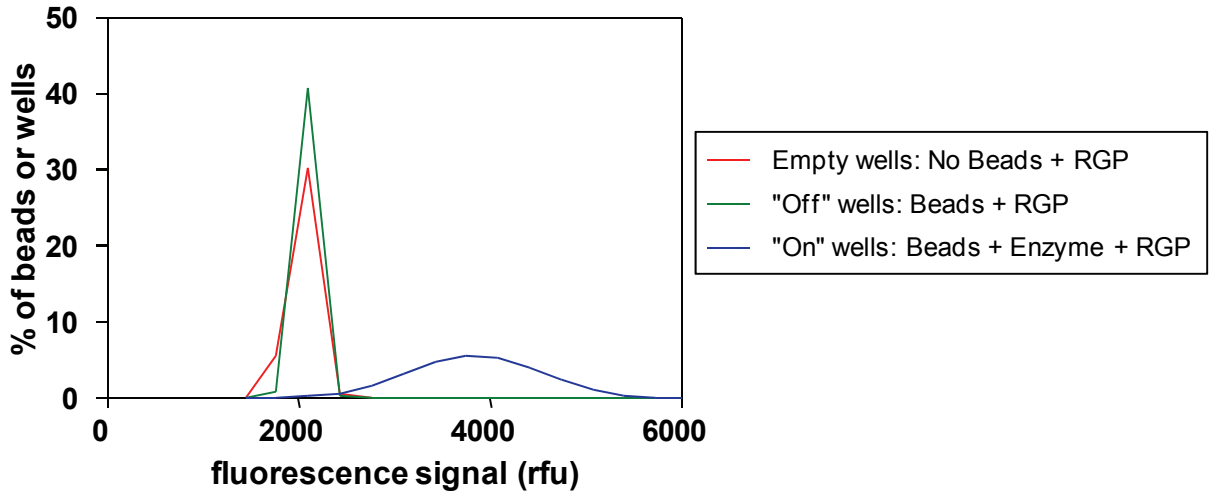
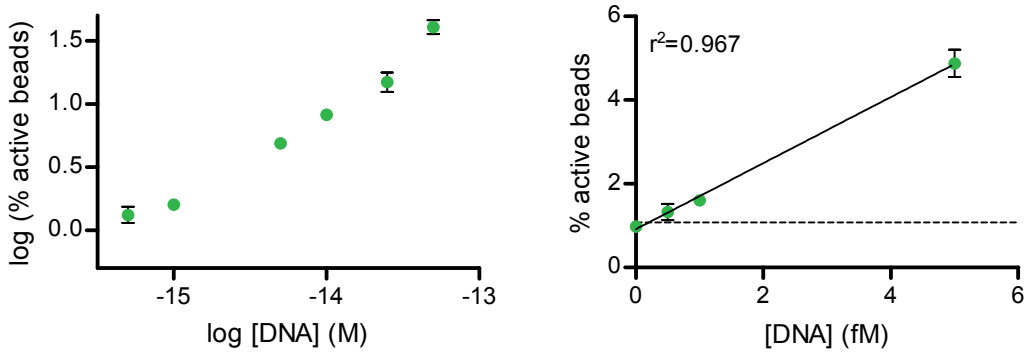
Supplementary Information

1) Supplementary Figures and Legends

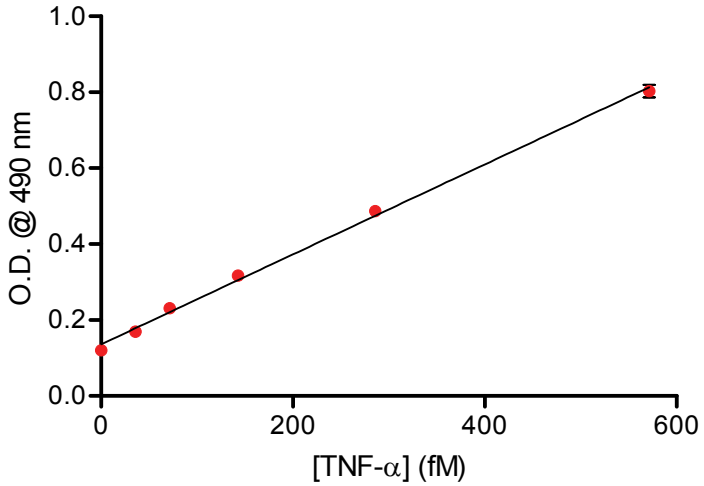


Supplementary Figure 1. Histograms of the average fluorescence intensity of wells in a SiMoA experiment. A representative set of images from a SiMoA experiment (Figure 1) were analyzed to determine populations of wells that: a) contained no beads; b) contained a bead (from white light scatter) but no enzyme (no increase in fluorescence intensity); and, c) contained a bead (from white light scatter) and an enzyme (increasing fluorescence intensity over four consecutive images, and an overall increase in fluorescence of at least 20%). Using this method of calling "on" wells, the occurrence of false positives in the absence of background proteins is very small (<1 in a 50,000-well array, see Figure 2). In the presence of background proteins, e.g., serum samples, there is a significant number of "on" wells from non-specific binding that sets the background signal and limits of detection (see discussion in main text). The fraction of beads within each population is plotted against the average intensity of wells with a bin size of 120 rfu. The red line represents data from empty wells; the green line represents wells with a bead but no enzyme ("off" well); and the blue line represents beads with both a bead and enzymatic activity ("on" well). While a range of enzyme activities are observed as anticipated[21], there is good separation between on and off wells to allow digital quantification.



Supplementary Figure 2. Detection of DNA using digital counting of enzyme labels. Plots of % active beads against different concentrations of DNA spiked into buffer. Experimental details are provided in the Methods section. The plot on the left hand side show the assay response over the

concentration range tested in log-log space. The plot on the right hand side show the assay response in the femtomolar range in linear-linear space. Error bars are plotted as the standard deviation over three replicates. LODs were determined by extrapolating the concentration from the signal equal to background signal plus three standard deviations of the background signal; the dashed lines show the signal at the LOD. The LOD of the digital DNA sandwich assay was ~150 aM, corresponding to about 8000 copies. While these results are preliminary—the DNA was detected in buffer in the absence of non-target DNA—and the sensitivity does not approach that that can be achieved with PCR, they do illustrate that SiMoA can be used to detect nucleic acids without recourse to molecular replication.



Supplementary Figure 3. The R&D Systems HS-ELISA for TNF-α. A TNF-α calibration curve yielded an LOD of 21 fM in whole serum using a Tecan Infinite M200 plate reader. The non-specific binding (NSB) background in this high sensitivity assay was equivalent to about 85 fM of TNF-α.

2) Supplementary Tables

	Column A	Column B	Column C	Column D	Column E	Column F
[SβG] (aM)	Average % active beads observed (Figure 2)	Enzyme/bead ratio from Poisson distribution	Total # of enzymes on 400,000 beads	Background corrected # of enzymes on beads	Calculated # of enzymes in 100-µL sample	Capture Efficiency
0	0.0016%	0.000016	7	---	---	---
0.35	0.0086%	0.000086	34	28	21	132%
0.7	0.0099%	0.000099	40	33	42	79%
3.5	0.0413%	0.000413	165	159	211	75%
7	0.0713%	0.000713	285	279	421	66%
35	0.4461%	0.004471	1789	1782	2107	85%
70	0.8183%	0.008217	3287	3280	4214	78%
350	3.3802%	0.034387	13755	13748	21070	65%
700	7.5865%	0.078897	31559	31552	42140	75%
3500	30.6479%	0.365974	146390	146383	210700	69%
7000	44.5296%	0.589320	235728	235722	421400	56%

Supplementary Table 1. Calculations of the SiMoA capture efficiency of enzyme label from 0.35 aM to 7000 aM. From the fraction of beads that were active in Figure 2 (Column A), the enzyme/bead ratio (Column B) can be determined from the Poisson distribution[23]. In this experiment, ~400,000 beads were incubated with each solution of enzyme (although only ~50,000 beads (~12.5%) on average were interrogated because of the current size of the femtoliter arrays (~50,000 wells)), so the number of enzymes on these beads is determined by multiplying Column B by 400,000 (Column C). After background subtraction, the total number of enzyme molecules captured on the beads can be determined (Column D). The ratio of this number to the total calculated number of enzymes in 100 μL at the start of the experiment (Column E; Volume × Concentration × Avogadro's Number) yields a capture efficiency (Column F). The overall efficiency of capture and detection of enzyme using SiMoA is high (65-85%) in the range where Poisson noise is acceptable (<20%, from 3.5 aM to 3500 aM), and is probably only limited in this experiment by the slow diffusion of the large SβG conjugate (MW ~ 515 kDa). At 7000 aM, where the % active beads > 40%, the % active starts to deviate from linearity (giving rise to an apparent lower capture efficiency) because of difficulties in image analysis at these high fractions (see main text).

NSB Dropout Experiment (SiMoA PSA assay)					
	Average	SD	CV	% NSB	
No dAb; No PSA	0.110%	0.162%	147%	from SbG	20%
No SbG; No PSA	0.000%	0.000%	---	from dAb	80%
NSB	0.541%	0.194%	36%	Total NSB	100%

Supplementary Table 2. A dropout experiment isolating the sources of NSB in the PSA digital ELISA. By comparing 'no detection antibody NSB' (No dAb) and 'no SβG NSB' (No SβG) to total NSB (NSB), the contribution of detection antibody and SβG to the NSB of the assay was determined.

	Centaur ng/mL	**SiMoA ng/mL**
Bio-Rad Control 1	0.838	1.06 ± 0.21
Bio-Rad Control 2	2.47	2.66 ± 0.36
Normals		
ProMedDx S376	2.1	1.60
ProMedDx S378	2.3	1.70
ProMedDx S381	2.9	2.14
ProMedDx S388	4.1	3.95
ProMedDx S395	0.93	0.63
ProMedDx S396	0.9	0.77
ProMedDx S397	1.2	0.66

Supplemental Table 3. Specificity of the PSA digital ELISA. Specificity of the digital ELISA was confirmed using PSA samples from Bio-Rad (controls) and ProMedDx (serum from healthy individuals) that had previously been tested on a commercial immunoanalyzer (ADVIA Centaur, Siemens). While there was good correlation between the two methods (r^2 = 0.96), the PSA concentrations of the healthy

serum samples determined using SiMoA were (24±12)% lower than those originally determined on the ADVIA Centaur. There are two possible explanations for this systematic bias between the two technologies. First, the ADVIA Centaur values were obtained on fresh serum, whereas digital ELISA values were obtained after the sera had been frozen for extended periods of time and had experienced a freeze-thaw cycle. Second, there were likely differences between the PSA calibrators used to generate the calibration curves that would result in differences in PSA concentrations determined. We used Complex PSA from the World Health Organization (WHO) as calibrators; the ADVIA Centaur calibration PSA is unknown.

3) Supplementary Discussion

Sensitivity to Enzyme Label as Sample Volume Increases. For some applications, volumes larger than 100 μL might need to be tested for very low concentrations of analyte. As the volume of sample increases, detecting small numbers of enzyme labels with high efficiency becomes more challenging technically. To achieve the same detection efficiencies in 1 mL and 10 mL of sample that we achieved in 100 μL, would require 10 and 100 times more beads, respectively, both from thermodynamic and kinetic perspectives. These larger numbers of beads (2 million and 20 million) would require much bigger arrays and also larger imaging (CCD) chips or multiple images per array. While these experiments are feasible, they would require a much more complex instrument and longer read times, so might not be justified in a diagnostic setting.

Optimization of Labeling of Captured Proteins in Digital ELISA. The choice of the concentrations of detection antibody and enzyme conjugate (SβG) used to label the captured protein is important for optimizing digital ELISA. The relatively high background signals of ELISAs caused by the non-specific interactions between the capture beads and detection antibody and enzyme conjugate (see Supplementary Table 2), combined with the high sensitivity to label provided by SiMoA, means that often only a fraction of the capture proteins need to be labeled to avoid saturating SiMoA signals. For example, the background levels observed in digital ELISA are equivalent to ~1–2 fM of target protein (see main text), so the ratio of analyte to bead is about 0.3–0.6. At these ratios, the number of active beads would be 25–40% if every protein was labeled with an enzyme, i.e., at the upper end of the digital dynamic range. To ensure that these background signals are closer to the lower end of the digital dynamic range—1% active beads provides a reasonable noise floor for background in digital ELISA—appropriate labeling of the captured protein can be achieved by kinetic control of the labeling steps, either by minimizing the concentrations of both labeling reagents or by using shorter incubation times. We have chosen to minimize label concentrations and use standard ELISA incubation times; as a result, the total assay time is ~6 h. This length of time is acceptable for testing that tolerates a daily turnaround time for samples. For shorter turnaround times of, say, <1 h (e.g., for point-of-care applications), the assay could be performed with short incubations of higher concentrations of labeling reagents.