Supplementary Information

Supplementary Text

Determination of reagent cross-reactivity. When adding a new, candidate protein to an existing multiplexed digital ELISA, two experiments were performed to ensure that the new protein and detection reagents did not cross-react with the existing reagents and result in elevated false positive AEB signals for any of the proteins. In experiment #1 (see table below for design), a calibration curve (0 to 100 pg/mL) was generated for the new protein based on its specific capture and detection reagents, with and without: a) the addition of 100 pg/mL of the existing multiplexed proteins to each sample; and b) a mixture of all the biotinylated detection antibodies for the multiplexed proteins used at the detection antibody labeling step. In experiment #2 (see table below for design), 0 and 10 pg/mL of each of the existing multiplexed proteins were spiked into a sample, and detected using the multiplexed digital ELISA, with and without: a) 100 pg/mL of the candidate protein in the sample; and b) the biotinylated detection antibody against the candidate protein at the detection antibody labeling step. If unanticipated increases in false positive signals were observed in either of these experiments then the new protein was not selected to be part of the multiplex. Examples of the data generated in Experiments #1 and #2 are shown in **Supplementary Figure 2**.

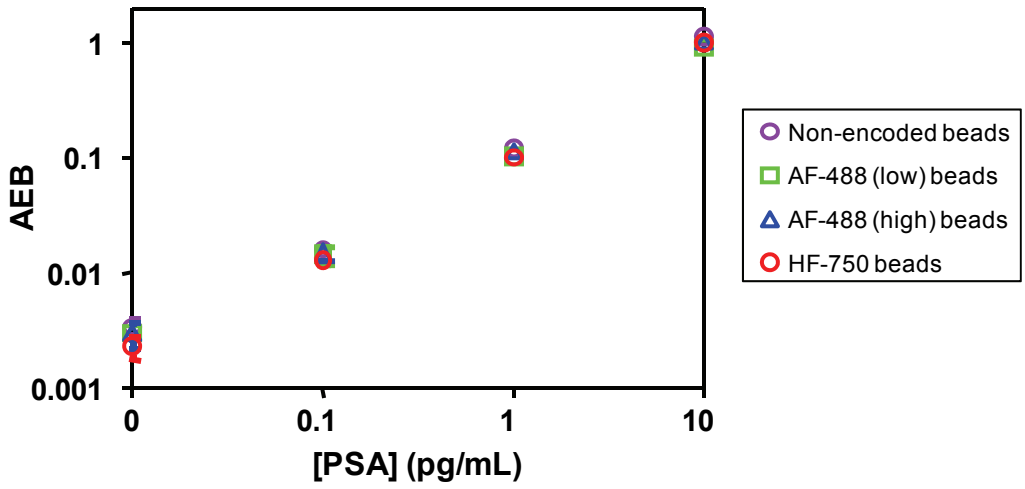
Experiment #1:

	Run 1			**compared to**	**Run 2**	
	Capture	Analyte	Detector	Capture	Analyte	Detector
New protein	X	X	X	X	X	X
Existing protein #1					X	X
Existing protein #2					X	X
…Existing protein #N					X	X

Experiment #2:

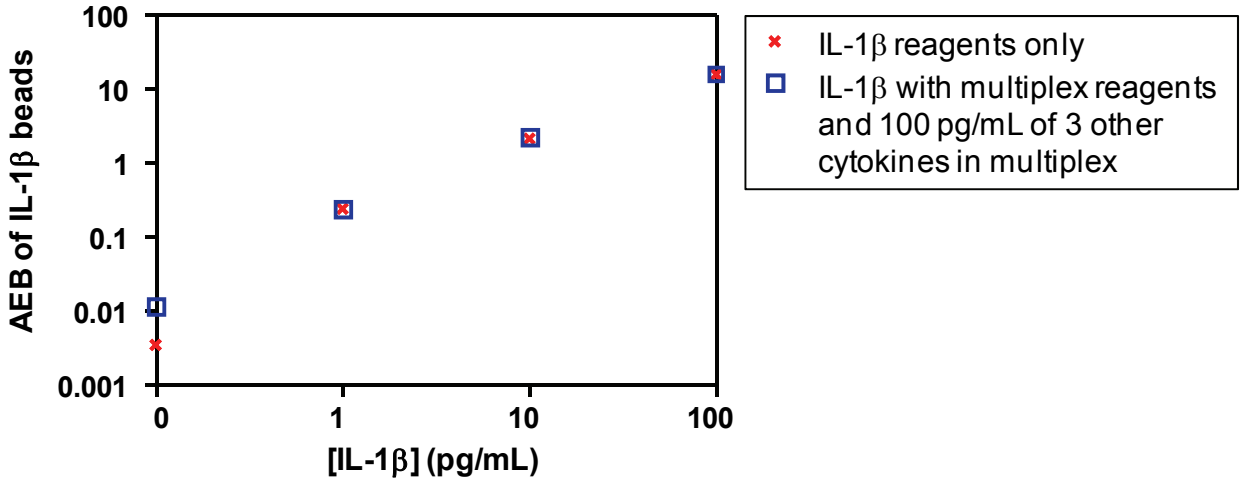
	Run 1			compared to	Run 2	
	Capture	Analyte	Detector	Capture	Analyte	Detector
New protein					X	X
Existing protein #1	X	X	X	X	X	X
Existing protein #2	X	X	X	X	X	X
…Existing protein #N	X	X	X	X	X	X

Supplementary Figures



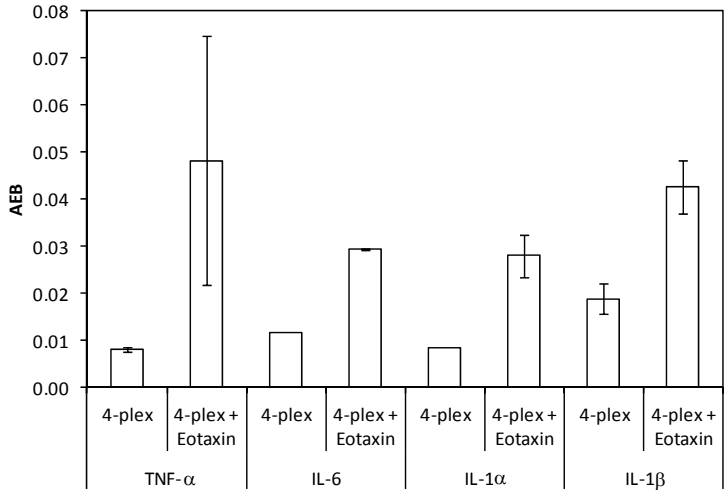
Supplementary Figure 1. Plots of AEB against concentration of PSA for digital ELISAs developed with non-encoded beads and beads labeled with fluorescent dyes to enable decoding.

A



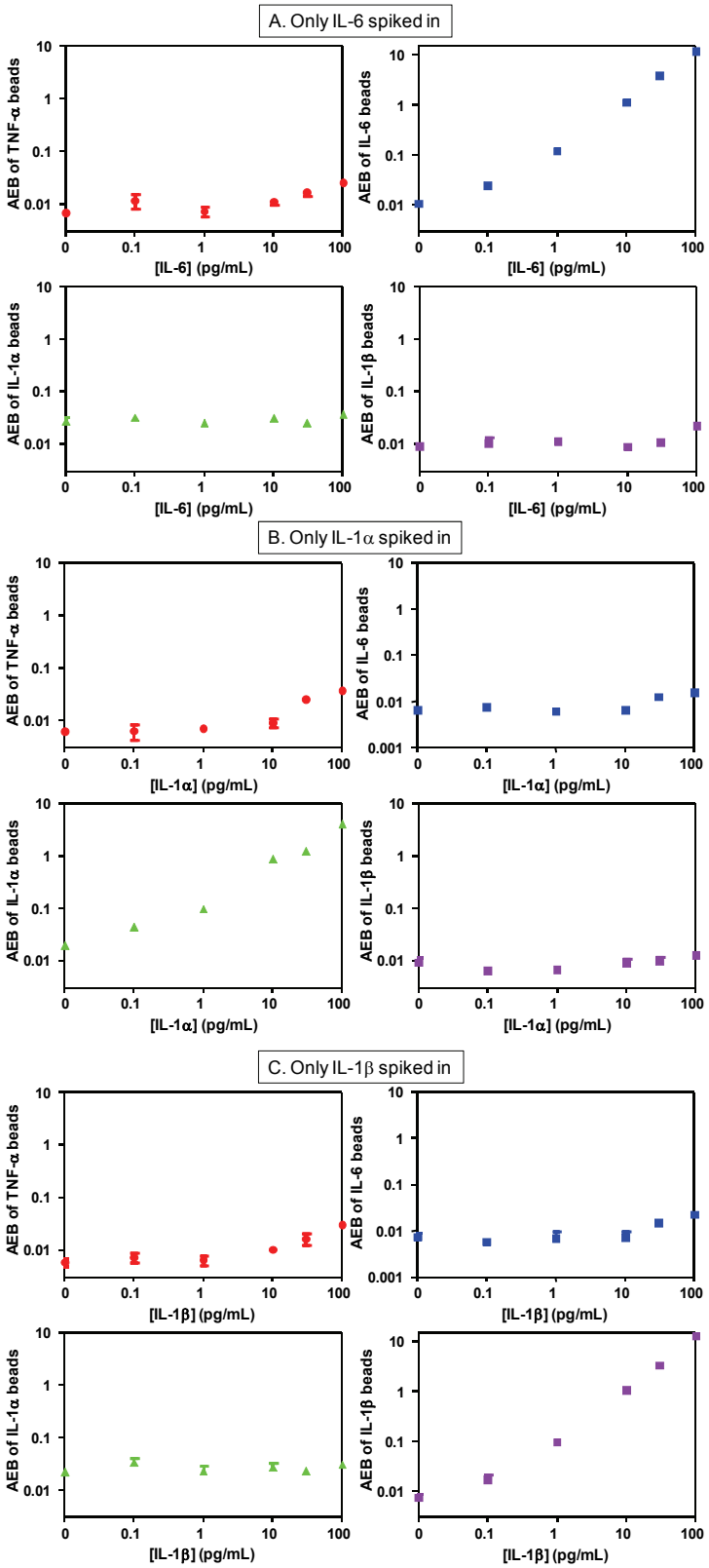
[IL-1β] (pg/mL)	AEB from IL-1b beads IL-1b reagents only	AEB from IL-1b beads IL-1b reagents plus 100 pg/mL of 3 cytokines and their detection antibodies
0	0.0036± 0.0004	0.0118± 0.0007
1	0.2525± 0.0047	0.2355± 0.0319
10	2.159± 0.3658	2.229± 0.3973
100	15.86± 1.295	16.30± 2.243

B



Supplementary Figure 2. Examples of experiments to determine cross-reactivity in multiplexed digital ELISA. A) Example of Experiment #1 described above. IL-1β was being added to an existing 3-plex of TNF-α, IL-6, and GM-CSF. IL-1β beads were run in conventional singleplex mode (red crosses), and also with 100 pg/mL each of TNF-α, IL-6, and GM-CSF, and a mixture of the biotinylated detection antibodies for these 3 cytokines added to the assay (blue squares). The 3-fold increase in background signals for IL-1β beads was expected from the use of four-fold higher concentration of detection

antibodies,[1] but no further increase was observed from the presence of 100 pg/mL of 3 other antigens, so cross-reactivity was acceptable. B) Example of Experiment #2 described above. Eotaxin was being added to an existing 4-plex of TNF-α, IL-6, IL-1α, and IL-1β. The 4-plex was run with all 4 cytokines at 0 pg/mL, with and without 10 pg/mL eotaxin and 0.1 μg/mL of its biotinylated detection antibody to assess the effect on backgrounds. For each of the proteins, the backgrounds increased between 2.3−6.1-fold upon addition of eotaxin, an increase not anticipated by the 20% increase in detection antibody concentration. We inferred significant cross-reactivity with eotaxin reagents giving rise to false positive signals, so eotaxin was not added to this multiplex assay.



Supplementary Figure 3. Plots of AEB against protein concentration for 4 beads specific to 4 cytokines measured in bovine serum samples spiked with: A) only IL-6; B) only IL-1α; and C) only IL-1β.

Supplementary Tables

Supplementary Table 1. Effect of fluorescence of unmodified and encoded beads on the channel used to detect fluorescence (resorufin) from the reaction of single enzymes.

Bead type	Average fluorescence in resorufin detection channel (574 nm/615 nm ex/em)
Unmodified, non-encoded beads	408±14
AF-488 fluorescent beads	390±9
cy5 fluorescent beads (low)	401±12
cy5 fluorescent beads (high)	408±11
HF-750 fluorescent beads	420±12

Supplementary Table 2. AEB values of 4 bead types in a 4-plex measured in samples spiked with IL-6 before and after software correction of crosstalk. Significant crosstalk was observed at 100 pg/mL IL-6 in all three non-IL-6 bead types, and these false positive signals are greatly reduced by correction without affecting the IL-6 bead data.

Beads measured	[IL-6] pg/mL	Before crosstalk correction			After crosstalk correction		
		AEB	s.d.	CV	**AEB**	s.d.	CV
IL-6 beads	0	**0.012**	0.001	8.0%	**0.012**	0.001	8.3%
	1	**0.103**	0.007	6.4%	**0.103**	0.007	6.7%
	10	**0.921**	0.021	2.2%	**0.922**	0.021	2.2%
	100	**6.187**	0.098	1.6%	**6.188**	0.093	1.5%
TNF-α beads	0	**0.019**	0.001	7.5%	**0.019**	0.001	7.5%
	1	**0.020**	0.001	5.1%	**0.021**	0.001	5.5%
	10	**0.021**	0.000	0.9%	**0.021**	0.000	1.5%
	100	**0.060**	0.001	1.9%	**0.031**	0.003	10.0%
IL-1β beads	0	**0.021**	0.001	6.0%	**0.021**	0.001	6.4%
	1	**0.023**	0.001	5.2%	**0.023**	0.001	6.1%
	10	**0.023**	0.004	15.7%	**0.023**	0.004	15.6%
	100	**0.060**	0.002	3.9%	**0.031**	0.000	0.1%
IL-1α beads	0	**0.018**	0.003	16.1%	**0.018**	0.003	17.1%
	1	**0.023**	0.003	12.2%	**0.023**	0.003	13.0%
	10	**0.023**	0.001	3.1%	**0.023**	0.001	3.7%
	100	**0.069**	0.001	0.9%	**0.033**	0.001	1.5%

Supplementary Table 3. AEB as a function of concentration for calibration curves shown in **Figure 5** and **Supplementary Fig. 3**.

Experiment	TNF-α beads				IL-6 beads				IL-1α beads				IL-1β beads			
	[cytokine] pg/mL	AEB	s.d.	CV (%)	[cytokine] pg/mL	AEB	s.d.	CV (%)	[cytokine] pg/mL	AEB	s.d.	CV (%)	[cytokine] pg/mL	AEB	s.d.	CV (%)
TNF-α only spiked in	0	0.0091	0.0011	12%	0	0.0086	0.0016	19%	0	0.0306	0.0029	10%	0	0.0083	0.0038	45%
	0.1	0.0246	0.0059	24%	0.1	0.0127	0.0041	32%	0.1	0.0377	0.0057	15%	0.1	0.0106	0.0009	9%
	1	0.0972	0.0079	8%	1	0.0086	0.0005	6%	1	0.0283	0.0028	10%	1	0.0081	0.0016	19%
	10	0.9197	0.0328	4%	10	0.0074	0.0013	18%	10	0.0411	0.0034	8%	10	0.0107	0.0015	14%
	30	3.0050	0.0799	3%	30	0.0127	0.0032	25%	30	0.0233	0.0035	15%	30	0.0102	0.0022	22%
	100	10.3392	0.4893	5%	100	0.0151	0.0013	9%	100	0.0259	0.0014	5%	100	0.0142	0.0023	16%
IL-6 only spiked in	0	0.0068	0.0008	12%	0	0.0108	0.0001	1%	0	0.0271	0.0058	22%	0	0.0090	0.0008	9%
	0.1	0.0115	0.0034	30%	0.1	0.0245	0.0012	5%	0.1	0.0321	0.0018	6%	0.1	0.0102	0.0034	34%
	1	0.0072	0.0016	23%	1	0.1218	0.0071	6%	1	0.0251	0.0018	7%	1	0.0114	0.0010	8%
	10	0.0110	0.0017	15%	10	1.1289	0.0415	4%	10	0.0309	0.0036	12%	10	0.0089	0.0007	8%
	30	0.0166	0.0026	16%	30	3.8783	0.3436	9%	30	0.0253	0.0034	13%	30	0.0109	0.0018	16%
	100	0.0254	0.0031	12%	100	11.895	0.4263	4%	100	0.0366	0.0044	12%	100	0.0224	0.0019	8%
IL-1α only spiked in	0	0.0062	0.0001	1%	0	0.0067	0.0013	19%	0	0.0195	0.0004	2%	0	0.0093	0.0021	23%
	0.1	0.0063	0.0021	34%	0.1	0.0077	0.0004	6%	0.1	0.0445	0.0045	10%	0.1	0.0064	0.0009	14%
	1	0.0071	0.0009	13%	1	0.0062	0.0011	18%	1	0.0975	0.0052	5%	1	0.0067	0.0005	8%
	10	0.0091	0.0016	17%	10	0.0067	0.0011	17%	10	0.8641	0.0119	1%	10	0.0091	0.0018	19%
	30	0.0255	0.0026	10%	30	0.0126	0.0017	14%	30	1.2379	0.0220	2%	30	0.0098	0.0018	18%
	100	0.0371	0.0003	1%	100	0.0158	0.0008	5%	100	3.9964	0.2728	7%	100	0.0130	0.0021	16%
IL-1β only spiked in	0	0.0058	0.0010	16%	0	0.0075	0.0018	24%	0	0.0221	0.0021	9%	0	0.0075	0.0014	19%
	0.1	0.0072	0.0015	21%	0.1	0.0058	0.0006	11%	0.1	0.0337	0.0068	20%	0.1	0.0173	0.0043	25%
	1	0.0064	0.0014	22%	1	0.0070	0.0026	37%	1	0.0233	0.0060	26%	1	0.0969	0.0128	13%
	10	0.0101	0.0005	5%	10	0.0074	0.0021	29%	10	0.0269	0.0056	21%	10	1.0688	0.0463	4%
	30	0.0163	0.0040	25%	30	0.0152	0.0021	14%	30	0.0235	0.0034	14%	30	3.3097	0.3495	11%
	100	0.0302	0.0033	11%	100	0.0228	0.0040	17%	100	0.0307	0.0030	10%	100	12.6250	1.5968	13%
All 4 cytokines spiked in	0	0.0100	0.0027	27%	0	0.0078	0.0013	16%	0	0.0268	0.0022	8%	0	0.0074	0.0014	19%
	0.1	0.0218	0.0022	10%	0.1	0.0240	0.0026	11%	0.1	0.0515	0.0056	11%	0.1	0.0207	0.0031	15%
	1	0.0949	0.0074	8%	1	0.1248	0.0029	2%	1	0.1085	0.0126	12%	1	0.1045	0.0127	12%
	10	1.0169	0.0112	1%	10	1.3811	0.0416	3%	10	0.8517	0.0502	6%	10	1.0982	0.0146	1%
	30	3.9060	0.3309	8%	30	3.1087	0.2959	10%	30	1.2566	0.0363	3%	30	3.4399	0.2560	7%
	100	12.3860	0.5393	4%	100	9.0958	0.4408	5%	100	5.2287	0.3311	6%	100	12.4415	0.3068	2%

[1] Rissin, D. M.; Kan, C. W.; Campbell, T. G.; Howes, S. C.; Fournier, D. R.; Song, L.; Piech, T.; Patel, P. P.; Chang, L.; Rivnak, A. J.; Ferrell, E. P.; Randall, J. D.; Provuncher, G. K.; Walt, D. R.; Duffy, D. C. *Nat. Biotechnol.* **2010**, *28*, 595-599.