



Supporting Information

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Sequential Protein Capture in Multiplex Single Molecule Arrays: A Strategy for Eliminating Assay Cross-Reactivity

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Table S1. Assay Limits of Detection and 4 PL Fitting Parameters. Calibration curves were fit to a 4-parameter logistic (4 PL) regression (Eq. S1) with $1/y^2$ weighting using GraphPad Prism version 8.3.

Assay Format	Marker	A	B	C	D	R ²	LOD (pg/mL)
Standard Multiplex	IL-7	0.00197	1.024	3,065,000	504,000	0.984	0.0022
Standard Multiplex	IL-8	0.00434	0.958	532.7	43.0	0.997	0.0051
Standard Multiplex	IL-10	0.00765	0.998	219.9	98.0	0.998	0.0021
Sequential Multiplex	IL-7	0.00218	1.027	7,102,000	813,000	0.985	0.0148
Sequential Multiplex	IL-8	0.00290	0.969	4,082,000	233,000	0.998	0.0207
Sequential Multiplex	IL-10	0.00628	0.979	274.0	68.0	0.999	0.0035

Equation 1: 4-Parameter Logistic Regression

$$Y(x) = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B}$$

Sequential Multiplex Assay Validation

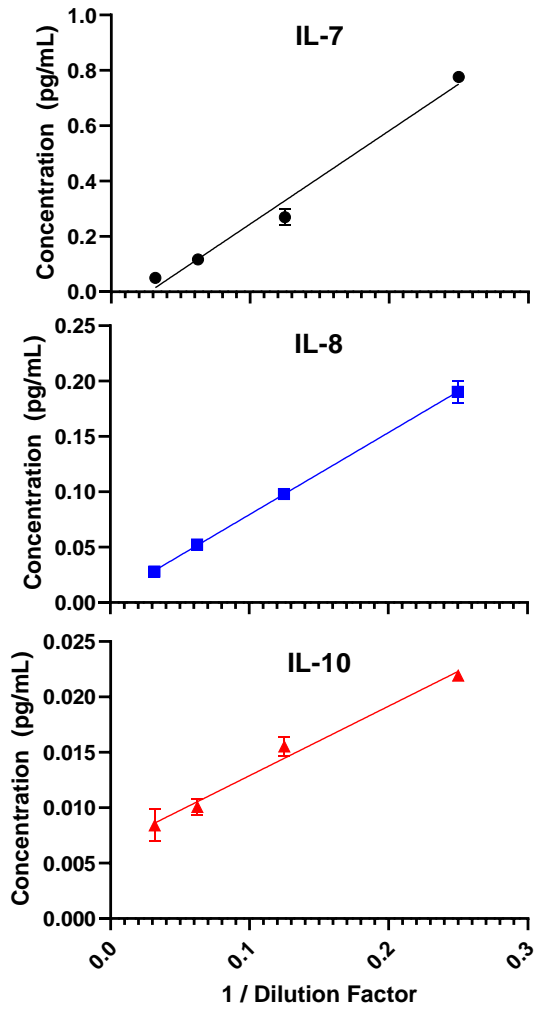


Figure S1. Sequential Multiplex Assay Dilution Linearity. Data for each marker were fit by linear regression. The R^2 value for each fit was 0.9816 for IL-7, 0.9961 for IL-8, and 0.9712 for IL-10. Error bars represent the standard deviations of duplicate measurements. Some error bars are narrow and encompassed within the data point.

Table S2. Sequential Multiplex Assay Spike and Recovery. % Recoveries were calculated by subtracting the measured endogenous protein concentration in a plasma sample with no spiked recombinant protein from the measured protein concentration of plasma samples with spiked recombinant protein, and then dividing by the spiked protein concentration and multiplying by 100. CVs are the coefficients of variation of the measured spiked plasma samples, which were performed in duplicates.

Spiked Protein	IL-7		IL-8		IL-10	
	Average Recovery (%)	CV	Average Recovery (%)	CV	Average Recovery (%)	CV
50 pg/mL	95.1	0.15	100.3	0.053	86.2	0.092
5 pg/mL	78.9	0.006	90.6	0.076	75.9	0.069
0.5 pg/mL	89.2	0.048	88.9	0.041	93.1	0.083

Table S3. Cross-reactivity Analysis of Protein Dropout Experiments. Values displayed are the signal AEB divided by the background AEB (0 pg/mL sample).

Standard Multiplex									
Bead	IL-7	IL-7	IL-7	IL-8	IL-8	IL-8	IL-10	IL-10	IL-10
Protein	IL-7	IL-8	IL-10	IL-7	IL-8	IL-10	IL-7	IL-8	IL-10
	On target	Off target	Off target	Off target	On target	Off target	Off target	Off target	On target
Conc. (pg/mL)	Signal/Background								
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.0244	3.58	2.02	1.78	0.96	1.36	1.01	0.98	1.19	2.18
0.0977	9.13	1.69	1.31	1.00	2.60	1.02	0.86	0.92	5.80
0.391	35.6	2.04	1.83	1.15	8.16	0.89	0.92	0.87	19.6
1.5625	134	2.67	2.73	0.97	29.7	1.32	0.99	1.00	72.7
6.25	525	2.75	3.33	1.83	113	1.97	1.52	1.08	326
25	2560	3.84	8.68	3.06	496	2.95	2.46	1.42	1295
80	10400	11.1		6.47	1497		5.94	2.20	
100			79.9			28.9			4280
200	27500	38.3		52.0	4460		28.1	11.1	
400		59.8			6420			15.6	
Sequential Multiplex									
Bead	IL-7	IL-7	IL-7	IL-8	IL-8	IL-8	IL-10	IL-10	IL-10
Protein	IL-7	IL-8	IL-10	IL-7	IL-8	IL-10	IL-7	IL-8	IL-10
	On target	Off target	Off target	Off target	On target	Off target	Off target	Off target	On target
Conc. (pg/mL)	Signal/Background								
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
0.0244	1.78	0.94	1.06	1.08	1.55	0.88	0.68	0.63	1.37
0.0977	4.04	0.87	1.08	1.32	3.02	0.96	0.70	0.62	3.32
0.391	13.7	1.10	1.08	1.02	9.25	0.85	0.64	0.71	11.5
1.5625	47.8	1.27	0.97	1.02	32.5	0.99	0.68	0.60	39.7
6.25	191	0.83	0.94	1.03	127	0.84	0.67	0.69	158
25	938	0.87	0.89	0.92	529	0.93	0.66	0.69	573
80	3070	0.79	0.67	0.97	1520	0.96	0.69	0.65	1730
200	7950	0.61		0.75	3960		0.65	0.62	
400		0.61			6240			0.71	