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Development of an Ultrasensitive Digital Immunoassay on the Single Molecule Array (Simoa™) Platform

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Abstract

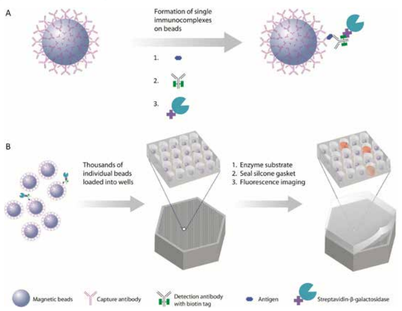
Purpose:
Quanterix's single molecule array Simoa technology is a fully automated immunoassay platform that utilizes arrays of femtoliter sized reaction chambers that can isolate and detect single molecules bound to paramagnetic beads. This method can measure proteins in the femtomolar range, up to 1000-fold improvement in sensitivity over traditional ELISA. Here we describe the development and optimization of a cytokine assay using the Quanterix platform.
Method:
The Quanterix platform is based on the use of capture antibodies coupled to paramagnetic beads and biotinylated detection antibodies, which in turn are detected by the action of a reporter enzyme: streptavidin β-galactosidase (SβG) and resorufin β-D-galactopyranoside as the fluorescence substrate. We executed a 2-variable design of experiments (DOE) strategy experiment in which multiple concentrations of detection antibody and SβG were tested. These are the 2 most critical variables to optimizing Simoa assays.
Results:
Under the optimal conditions identified (0.6 µg/mL detection antibody and 150 pM SβG), we were able to achieve an LOD of 1.4 fg/mL. This represents a 150-fold increase in sensitivity as compared to a current ultrasensitive assay developed on the Mesoscale Discovery platform.
Conclusions:
The Quanterix Simoa platform and technology has been shown to offer increased sensitivities over currently available assays, with homebrew capabilities that allow for the use of proprietary reagents and control of the assay development and optimization process. The fully automated platform allows for rapid development time and efficiencies in sample analysis.

Simoa – Single Molecule Array Technology

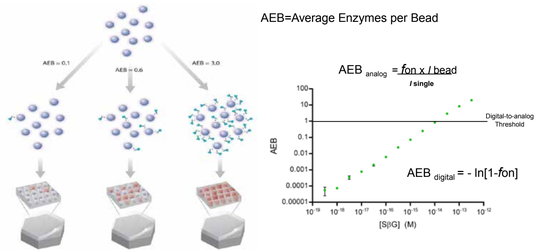
Single molecule array (digital immunoassay) starts with a sandwich antibody complex formed on paramagnetic beads, and bound complexes are labeled with an enzyme, as in conventional bead-based ELISA. When assaying samples containing extremely low concentrations of target proteins, the ratio of protein molecules (and the resulting immunocomplexes) to beads is small (typically < 1:1) and, as such, the percentage of beads that contain a labeled immunocomplex follows a Poisson distribution. At low concentrations of target protein, the Poisson distribution indicates that beads carry either a single immunocomplex or none. It is not possible to detect these low numbers of enzyme labels using standard detection technology, because the fluorophores generated by each enzyme diffuse into a large assay volume (typically greater than 0.1 mL). It takes hundreds of thousands of enzyme labels to generate a fluorescence signal above background.

Simoa technology allows for the detection of very low concentrations of enzyme labels by confining the fluorophores generated by individual enzymes to extremely small volumes (~50 fL) by loading beads into an array of 216,000 femtoliter-sized wells, ensuring a high local concentration of fluorescent signal. Beads possessing a single enzyme-labeled immunocomplex generate a high concentration of fluorescent product in the confined well. By acquiring time-lapsed fluorescence images of the array using standard microscopic optics, it is possible to distinguish beads associated with a single enzyme molecule ("on" well) from those not associated with an enzyme molecule ("off" well). The protein concentration in a test sample is determined by counting the number of wells containing both a bead and fluorescent signal relative to the total number of wells containing beads (AEB – average enzymes per bead). Since the technology enables concentration to be determined digitally rather than by analogue signals, it is termed a digital immunoassay.

Simoa – Single Molecule Array



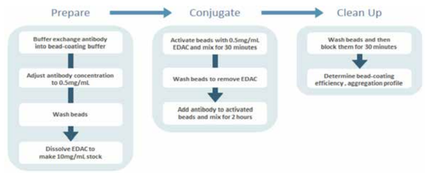
Digital Immunoassay



Assay Development Process on the Simoa Platform

Stage 1: Prepare initial lots of reagents

A. Coupling of capture antibody to paramagnetic beads
(Initial antibody concentration = 0.5 mg/mL)



B. Biotinylation of detection antibody (Initial : 40X molar excess of biotin)

Stage 2: Optimize Coupling

If the capture and/or detector reagents do not meet the criteria for your assay, there is a need to optimize the reagents.

Initial Criterion:

- >80% monomeric beads in the prepared capture bead concentrate
- Calibration curve from initial run suggests that the desired sensitivity level was not met

Optimizing the capture and detector reagents can be done by varying 2 critical parameters:

Factor 1: Antibody concentration in coupling reaction Bracket initial concentration with a high and low concentration 0.3 (low), 0.5 (initial), 0.7 (high) mg/mL

Factor 2: Molar excess of biotin in detector coupling reaction Bracket initial concentration with a high and low concentration 20X (low), 40X (initial), 60X (high) molar excess

Stage 3: Optimize Assay Conditions *Reduce background and increase signal*

With the reagents optimized, now execute a 2-variable design of experiments (DOE).

- Titrate the detector antibody concentrate concentration
  - The recommended starting value is 0.3 µg/mL, to optimize it is recommended to titrate in the range of 0.1–1.2 µg/mL.
- Titrate the SβG concentration
  - The recommended starting value is 150 pM, to optimize it is recommended to titrate in the range of 50–300 pM.
- Evaluate the LOD and signal-to-background ratio to determine the best detector and SβG concentrations. AEB should be less than 0.02 AEB, with ideal background levels of 0.001–0.005 AEB. Signal can reach 15–18 AEB, but levels above 18 AEB should be avoided.

Preparation of Initial Lots of Reagents for Cytokine Assay

- Capture Antibody
  - Conjugated to paramagnetic beads (Agilent LodeStars 2.7 carboxyl)
  - Standard 2-step carbodiimide reaction protocol
  - 0.5 mg/mL capture antibody used in conjugation reaction
- Detection Antibody
  - Biotin labeled detection antibody (NHS-PEG_4)
  - 40:1 challenge of a 1 mg/mL solution
- Assay Buffer
  - Casein in PBS (Thermo)

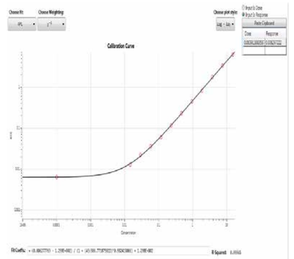
Cytokine Bead Performance

Bead performance is monitored by a Bead Aggregation Assay that is accessed in the Simoa software. The Bead Aggregation Assay is useful for determining actual bead concentration and the aggregation (% monomeric) following bead conjugation reactions. The results below are data from the initial lots of reagents prepared for the development of the cytokine assay. The 94.4% monomeric beads pass the criteria for > 80% monomeric beads in a successful preparation.

	Calculated Bead Concentration [beads/mL]	Calculated Monomeric Bead Percent	Avg beads/mL	Avg beads/mL
Cytokine Beads	2982900.6	95.1	2856325.6	94.4
	2525400.5	91.5		
	2824300.6	95.3		
	3092700.6	95.8		

Cytokine Beads
Multiplication factor = 3330000/2856325.6 = 1.17
Real beads concentration = 1400000/1.17 = **1200857 beads/mL**

Cytokine Callibration Curve Performance



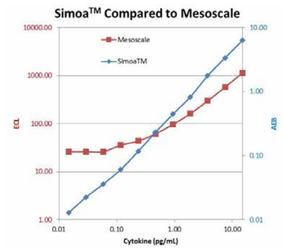
Assay Conditions
- 0.3 µg/mL detection
- 150 pM SβG

Cytokine (pg/mL)	AEB Avg	AEB SD	AEB CV	S:B
0.0	0.0064	0.0011	18%	–
0.0146	0.0128	0.0003	3%	2.01
0.029	0.0224	0.0035	16%	3.50
0.058	0.0361	0.0023	6%	5.66
0.117	0.0607	0.0020	3%	9.51
0.234	0.1168	0.0030	3%	18.29
0.468	0.2315	0.0100	4%	36.27
0.937	0.4444	0.0102	2%	69.63
1.875	0.8248	0.0345	4%	129.22
3.75	1.7717	0.0647	4%	277.58
7.5	3.3641	0.1964	6%	527.09
15.0	6.3087	0.3839	6%	988.44

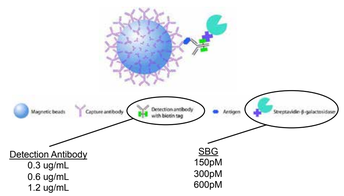
SD = standard deviation; CV = coefficient of variation
S:B = signal to background

Sensitivity Comparison to Current Mesoscale Discovery Method

Shown here is a sensitivity comparison of 2 methods, Simoa and Mesoscale Discovery. Both methods utilized the same capture and detection antibodies, as well as similar assay buffer. Single lots of antibody, assay buffer, and recombinant calibrator were used for both experiments. Represented in the data shown below are calibration curves (0.015–15 pg/mL) generated by each technology.



Optimization of Cytokine Assay Conditions



Cytokine Cal levels pg/mL	0	0.015	0.15	1.5	15.0		0	0.015	0.15	1.5	15.0
0.3 µg/mL Det 150 pM SβG	0.0058	0.0122	0.0712	0.6510	6.3850	**AVG AEB**	0.0056	0.0136	0.0719	0.6257	6.3697
	0.0047	0.0153	0.0704	0.6116	6.3703	**SD AEB**	0.0008	0.0016	0.0020	0.0219	0.0156
	0.0064	0.0133	0.0741	0.6145	6.3539	**CV AEB**	15%	12%	3%	4%	0%
						S:B		2.41	12.75	110.93	1129.38
0.3 µg/mL Det 300 pM SβG	0.0105	0.0194	0.1138	1.0110	9.1893	**AVG AEB**	0.0093	0.0202	0.1129	0.9787	9.0927
	0.0092	0.0201	0.1103	0.9724	9.2593	**SD AEB**	0.0012	0.0009	0.0022	0.0296	0.2307
	0.0082	0.0211	0.1145	0.9528	8.8294	**CV AEB**	13%	4%	2%	3%	3%
						S:B		2.17	12.14	105.29	978.16
0.3 µg/mL Det 600 pM SβG	0.0130	0.0314	0.1677	1.4311	12.6449	**AVG AEB**	0.0139	0.0298	0.1668	1.4308	12.6267
	0.0139	0.0329	0.1721	1.3609	12.1253	**SD AEB**	0.0010	0.0042	0.0058	0.0697	0.4927
	0.0150	0.0250	0.1606	1.5003	13.1101	**CV AEB**	7%	14%	3%	5%	4%
						S:B		2.13	11.96	102.59	905.35
0.6 µg/mL Det 150 pM SβG	0.0089	0.0223	0.1261	1.0952	11.2975	**AVG AEB**	0.0085	0.0223	0.1374	1.1024	11.0839
	0.0085	0.0223	0.1443	1.1562	11.1073	**SD AEB**	0.0005	0.0001	0.0099	0.0506	0.2263
	0.0080	0.0224	0.1419	1.0557	10.8468	**CV AEB**	6%	0%	7%	5%	2%
						S:B		2.63	16.19	129.84	1305.54
0.6 µg/mL Det 300 pM SβG	0.0144	0.0380	0.2110	2.0478	13.9456	**AVG AEB**	0.0151	0.0370	0.2126	2.0807	15.2973
	0.0137	0.0379	0.2147	2.1499	16.3076	**SD AEB**	0.0018	0.0017	0.0019	0.0600	1.2174
	0.0171	0.0351	0.2120	2.0444	15.6386	**CV AEB**	12%	4%	1%	3%	8%
						S:B		2.45	14.10	138.06	1015.00
0.6 µg/mL Det 300 pM SβG	0.0230	0.0576	0.3010	2.7644	16.0141	**AVG AEB**	0.0242	0.0560	0.2964	2.7921	18.2676
	0.0259	0.0558	0.2881	2.8705	18.0147	**SD AEB**	0.0015	0.0015	0.0072	0.0688	2.3900
	0.0238	0.0545	0.3000	2.7415	20.7740	**CV AEB**	6%	3%	2%	2%	13%
						S:B		2.31	12.22	115.15	753.37

Minimal Required Dilution (MRD)

To test the linearity of dilution and to establish the MRD, four (4) EDTA plasma samples were diluted between 1:2 and 1:8 in assay buffer. Based on the results shown below, there is no clear evidence of matrix effects so it was determined that samples would be run at 1:2 dilution in assay buffer.

	Dilution	Cytokine (pg/mL)	% Change From Neat
Sample A	Neat	0.213	–
	1:2	0.199	-6.5
	1:4	0.224	5.3
	1:8	0.255	20.1
Sample B	Neat	0.064	–
	1:2	0.074	15.1
	1:4	0.074	16.1
	1:8	0.071	11.2
Sample C	Neat	1.866	–
	1:2	1.933	3.6
	1:4	2.252	20.7
	1:8	2.019	8.2
Sample D	Neat	0.171	–
	1:2	0.271	58.2
	1:4	0.194	13.2
	1:8	0.178	4.3

Recovery of Recombinant Cytokine Spike

Four (4) EDTA plasma samples were spiked with recombinant cytokine calibrator at a concentration of 2.0 pg/mL. Control samples were prepared in assay buffer and analyzed along with the spiked and unspiked plasma samples. Mean results across all four (4) samples were 75.4%, ranging from 66.5%–85.8% recovery.

% Recovery was calculated based on the following formula: ([Observed spiked plasma – Observed unspiked plasma] / Observed spike in assay buffer) x100

	Endogenous Cytokine (pg/mL)	Observed Cytokine (pg/mL)	% Recovery
Assay Buffer	0	2.090	–
Sample A	0.255	1.97	81.9
Sample B	0.074	1.479	67.2
Sample C	0.171	1.561	66.5
Sample D	1.933	3.726	85.8

Conclusions

- The Quanterix Simoa platform and technology has been shown to offer increased sensitivities over currently available assays. We have shown >100-fold increases in sensitivity in the presented cytokine assay.
- Homebrew capabilities allow for the use of proprietary reagents and control of the assay development process.
- Fully automated platform allows for rapid development and efficiencies in sample analysis.

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