The Power of Single Molecule Counting: The Future of Immunoassays

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Introduction

The increasing need for new therapeutics that target different mechanisms of action, and methods for earlier diagnosis require increased understanding of disease processes and progression. Fundamental to this is the ability to distinguish between healthy and disease states. The clinical use of protein biomarkers often necessitates the measurement of small changes at low concentrations, in small volumes, which demands technology that can provide sensitive, precise and accurate quantification. Since the introduction of immunoassay (IA) technology and its application to the measurement of proteins in biological samples, significant improvements have been made in performance, including standardization, precision, and sensitivity. In certain instances, sensitivity levels of 1 – 10 ng/L is adequate for research and diagnostic applications, however, there is an evolving need for sub-picogram/mL quantification to meet the challenges of working with small animals during research, for interrogation of low volume retrospective study samples, and to quantify proteins to evaluate biomarker biology while maintaining a broad dynamic range to accommodate disease progression & therapeutic response. Millipore Sigma's propriety single molecule counting (SMC™) immunoassay technology helps to overcome these challenges in the translation of protein biomarkers to the clinic, due to improved assay precision and sensitivity, which has enabled the measurement of endogenous biomarker concentrations at femtogram levels and the precise monitoring over time.

Figure 1: SMCxPRO™
Fluorescent based platform, enabling the quantification of low abundance biomarkers in different matrices.

Methods

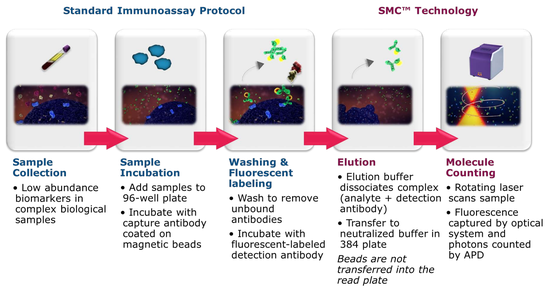


Figure 2: SMC™ Assay Workflow
This figure illustrates the typical bead based assay work flow. A sandwich immunoassay complex is generated on coated magnetic beads. The complex is disassociated from the bead and the eluate is read on the SMCxPRO™ .

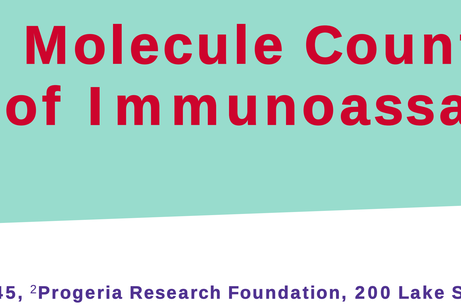


Figure 3: Single Molecule Counting™ Technology
Single molecule counting is performed inside individual wells. A small 5 μm integration space is illuminated by a laser. The laser scans the well, single fluorescently labeled molecules are excited as they pass through the interrogation space. Detected signals with peak intensity above the threshold background of fluorescence are counted as single digital events.

Results (I)

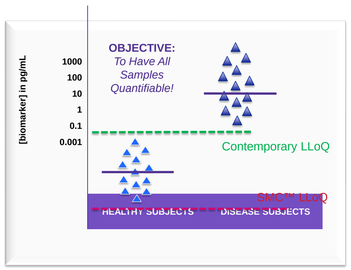


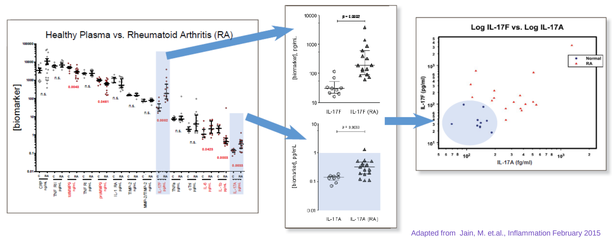
Figure 4: Shifting the Paradigm: Previously undetectable, Quantified
New Biomarker discovery * Monitor small changes in analyte levels * Follow PK studies for longer time courses * Micro-dosing studies for improved tox studies

Results (II)

Immunoassay	Format	Vendor	LLoQ (pg/mL)	Fold Improvement
Original IL-13 ELISA	ELISA	R&D Systems	9.8	–
Erenna Plate assay	Plate	Singulex	1.2	8×
Erenna MP assay	**MP**	**Singulex**	**0.07**	**140×**

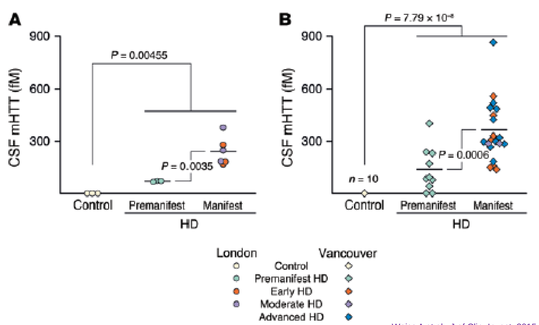
St. Ledger et al., J Immuno Methods, 2009.

Figure 5: IL-13 as Efficacy Marker for Asthma
Accurate measurement of circulating iL-13 levels is needed for PK/PK & MOA studies.
Quantification of baseline levels in clinical study population (n=182, 3 cohorts).
Using 99th% cut-off, quantified all healthy & mild asthmatic subjects.
All < 1 pg/mL – previously not measurable by other methods



Adapted from Jain, M. et.al., Inflammation February 2015

Figure 6: Increase [iL-17A] & [iL-17F] Correlate with RA
Ultra-high sensitivity improves S/N response of samples, thereby improving precision.
Combining the results from the two biomarkers allows for disease identification with improved specificity.



Weiss A et al., J of Clin. Invest. 2015

Figure 7: mHTT is Quantifiable in Mutation Carriers, not Control Participants in 2 Independent Cohorts
CSF mHTT levels are significantly elevated in manifest HD compared to premanifest HD carriers.

Results (III)

Table 1: MS Custom Assays & Sample Testing Group follows Standardized Guidelines
Established in 2005 "Fit for Purpose" Method Development & Validation for Successful Biomarker Measurement

Table II. Fit-for-Purpose Elements of Biomarker Assay Development and Method Validation

Parameters/assay elements	Preanalytical and method development[a]	Exploratory method validation[b]	Advanced method validation[c]
Reagents and reference material	Consistent and accessible source (due diligence)	Initial characterization Stability initiated	Well characterized Inventoried Establish stability Establish change control
Target range	Estimate in biomarker work plan Define expectation of LLOQ and ULOQ	Acquiring data	Establish from incurred samples
Dynamic range (lower and upper quantitation limits)	Determine preliminary assay range with precision profile over target range	Use 3 validation runs	Use at least 6 runs (in-study validation data can be utilized) Establish LLOQ and ULOQ
Sensitivity	Define minimum detectable range Define requirements of sensitivity (LOD) and LLOQ	Estimate sensitivity Consider LOD vs. LLOQ	Establish sensitivity
Curve fitting	Choose appropriate calibration model fitting method and tools	Confirm choice of calibration model from 3 validation runs	Use 6 validation runs to confirm calibration model
Selectivity and specificity	Reagent specificity from supplier or literature Assess matrix effects and minimize if possible. Determine minimum required dilution (MRD) Sample and substitute matrix	Investigate likely sources of interference, including the therapeutic agent	Extensive testing of interference and risk recommendation Assessment of biomarker heterogeneity and isoforms
Parallelism	N/A	Use incurred samples, if available	Investigate in targeted population Determine maximum tolerable dilution
Dilution linearity	Determine if applicable, as defined in the biomarker plan (test range)	Use spiked samples	Use spiked samples and dilution VS if applicable
Precision and accuracy (analytical)	Establish expectations early on in biomarker work plan Consider heterogeneity	Use 3 validation runs	Use of total of at least 6 runs (in study validation data can be utilized)
Relative accuracy/recovery (biological)	Establish expectations early on in biomarker work plan	Use spiked incurred samples at multiple concentrations Addition recovery	Use multiple donors
Robustness (reagent and change control)	Determine need Consider availability of biological matrix	NA	Establish tolerability on crucial elements
Sample handling, collection, processing, and storage	Establish feasible conditions	Establish short-term and bench top stability Optimize conditions and effects on assay	Establish freeze/thaw and long-term sample stability
Documentation	Biomarker work plan Draft procedures Assess outsourcing options	Appropriate documentation to support the intended use of the data	Appropriate documentation to support the intended use of the data

Lee et al., Pharmaceutical Research, Vol. 23, 2006

n	Sample	Mean [Progerin] pg/mL	SD	%CV	[Progerin] Spike pg/mL	Spike Recovery
1	Buffer Control	-	-	-		
2	" rh. Spike	1,048	114.53	11%	1,000	105%
2	" endog Spike	847	2.33	0%	1,019	83%
2	NHP 1	67	16.52	25%		
2	" rh. Spike	1,086	68.72	6%	1,000	102%
2	" endog Spike	940	81.44	9%	1,019	86%
2	NHP 2	-	-	-		
2	" rh. Spike	1,254	1.20	0%	1,000	125%
2	" endog Spike	1,058	6.56	1%	1,019	104%
2	NHP 3	31	0.01	0%		
2	" rh. Spike	1,391	108.44	8%	1,000	136%
2	" endog Spike	1,044	64.55	6%	1,019	99%
2	Sub#110	550	56.56	10%		
2	" rh. Spike	1,920	2.33	0%	1,000	137%
2	" endog Spike	1,745	118.29	7%	1,019	117%
2	Sub#145	559	7.23	1%		
2	" rh. Spike	1,993	93.17	5%	1,000	143%
2	" endog Spike	1,738	51.78	3%	1,019	116%

n	Sample	Dilution Factor	Mean [Progerin] pg/mL	SD	%CV	Dilution Corrected [Progerin] pg/mL	Linearity
2		5	2,261	60.07	13%	11,305	
2	Sub#154	25	509	6.81	7%	12,728	113%
2		125	111	3.79	17%	13,834	109%
2		5	5,613	6.04	1%	28,063	
2	Sub#110	25	985	4.68	2%	24,634	88%
2		125	200	1.99	5%	25,034	102%
2		25	1,075	42.06	0.04	26,865	
2	Sub#145	50	546	12.31	0.02	27,319	102%
2		100	245	6.97	0.03	24,483	90%

Summary

- Using SMC™ technology we have developed a sensitive assay for the specific quantification of Progerin
- The recovery results demonstrate the recombinant standard is appropriate for quantification, whereas the linearity results show accurate measurement at various levels of dilution.
- The sensitivity allows the use of mouse models for low volume testing & the specificity enables testing the efficacy of potential therapeutics.

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