

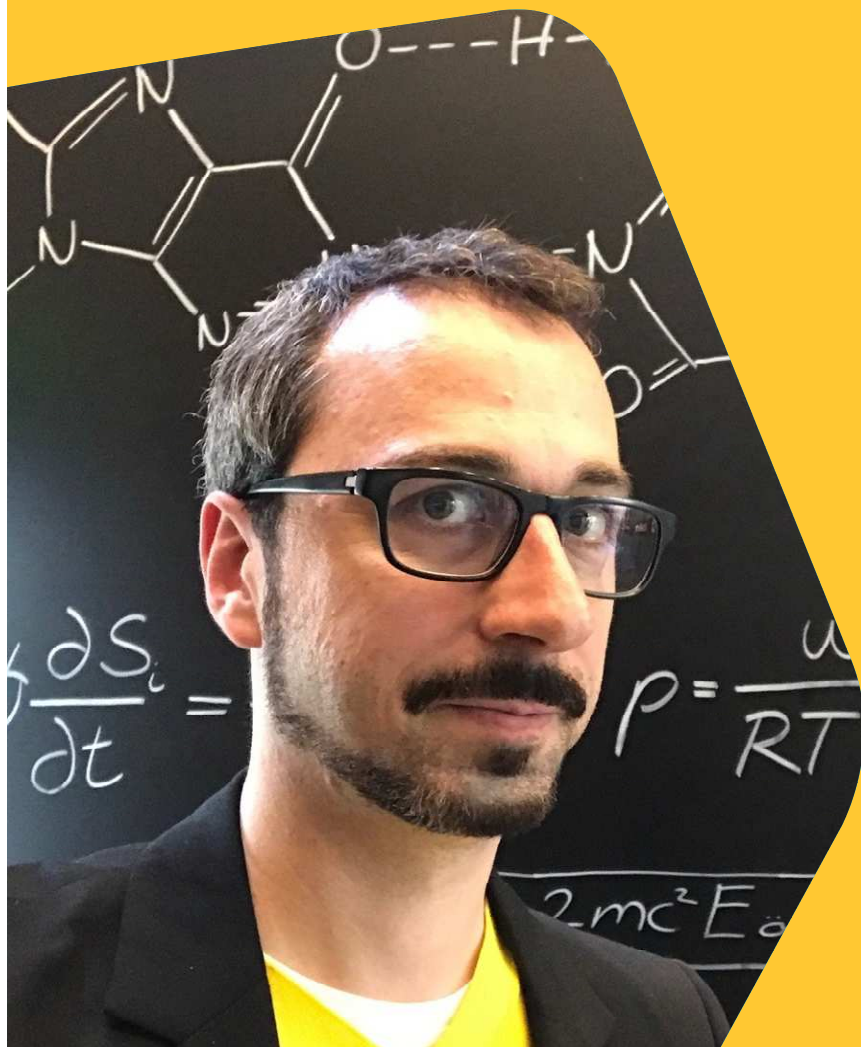
current trends in immunoassays

**Introduction to the
state-of-the-art xMAP®
and SMC™ technologies
for quantification of
protein biomarkers**

Stanislav KUKLA, MSc.
Senior Field Marketing Manager
Life Science division of Merck CZE

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stanislav kukla

- Background in physical, macromolecular, organic chemistry and biochemistry
- Several years at the Institute of Macromolecular Chemistry and Charles University in Prague
- **Senior Field Marketing Manager at the Life Science division of Merck, EE&R+ region, protein workflow specialist**
- Working closely with internal & external partners (R&D, production, customers, KOLs)
- Promoting new innovative technologies and services that we believe will allow for faster, safer and more productive Life Science research and will help researchers discover new therapies for the most challenging diseases

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agenda



Protein biomarkers basics, introduction to immunoassays



Fit-for-purpose approach, current trends in immunoassays



Multiplexing using xMAP® technology



A few comments on validation of multiplexing assays



Introduction to SMC™ technology for high sensitivity detection

„A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention.“

WHOLE ORGANISM



MOLECULAR MARKERS



PROTEINS IN PARTICULAR

bioMarker



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protein biomarkers



Protein biomarkers offer a great degree of differentiated information and nicely describe patient and disease heterogeneity. **They provide a critical link between basic biomedical research and personalised healthcare concept (translational approach).** They also have the potential to integrate and reflect environmental and lifestyle co-factors, such as stress and many more...

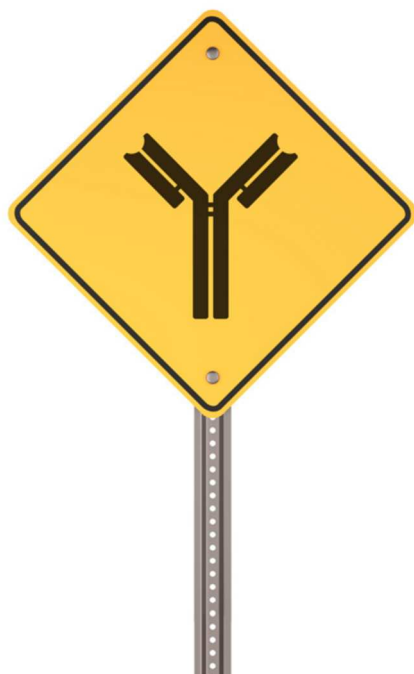
INTRACELLULAR:

up- or down regulation of a particular protein, activation of a signalling cascade, PTMs, formation of protein-protein complexes

EXTRACELLULAR (SECRETED):

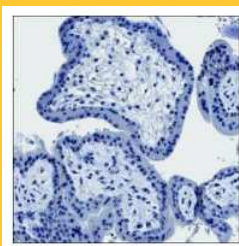
Proinflammatory cytokines, growth factors, protein/peptide hormones

currently used techniques

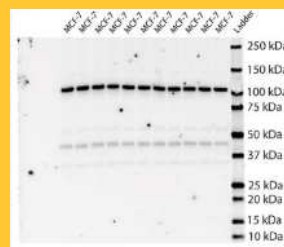


Antibody-based techniques have provided understanding of both normal and pathological processes for many decades and still play the key role in protein biomarker profiling both for intra- and extracellular proteins.

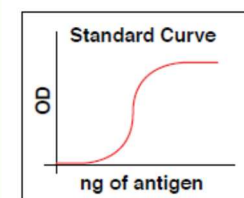
IHC/IF



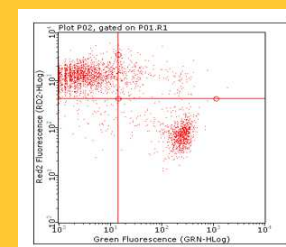
WB



ELISA



FC



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translational approach



Quantitation of biomarkers in biofluids plays a central role in basic research to management of patient care and is **routinely used in clinical laboratories and academic institutions („bench-to-bedside“)**. Standard immunoassays, such as ELISA, have provided understanding of both normal and pathological processes for many decades.

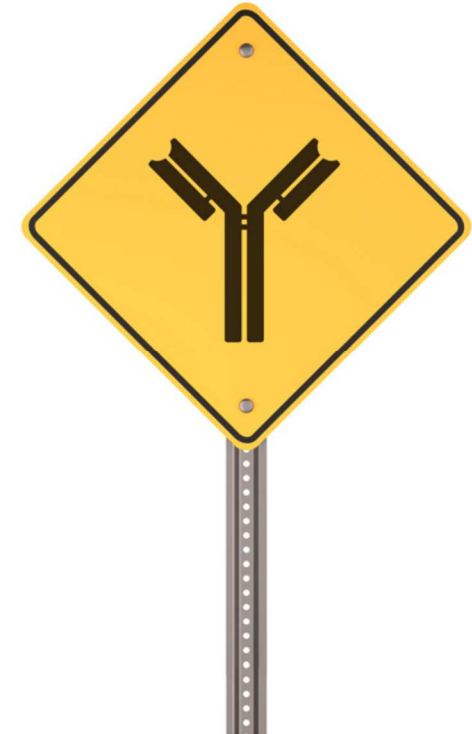


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Basic principles of immunoanalytical techniques

Immunoanalytics makes use of antibody properties:

- Antibodies can be prepared against and are able to **bind various types of antigens** – proteins, viruses, low-molecular weight compounds etc.
- Antibody-antigen interaction is **extremely specific** – allows for detection in the presence of other (similar) compounds and in complex matrices without demanding sample preparation steps
- Antibody-antigen binding is **very strong** – in a combination with sufficiently sensitive detection system, very low detection limits can be achieved (radioisotopes, enzyme systems, fluorescence probes, digital single molecule counting etc.)



Immunoanalytical techniques are used in clinical as well as basic research and translational areas, incl. testing of new potential drugs and biomarkers.

Historical intermezzo

1959 – Rosalyn Yalow and Solomon Berson describe the principle of competitive RIA method and by that they **lay the the foundation of immunoanalytical techniques**



Immunochemistry. Pergamon Press 1971. Vol. 8, pp. 871–874. Printed in Great Britain

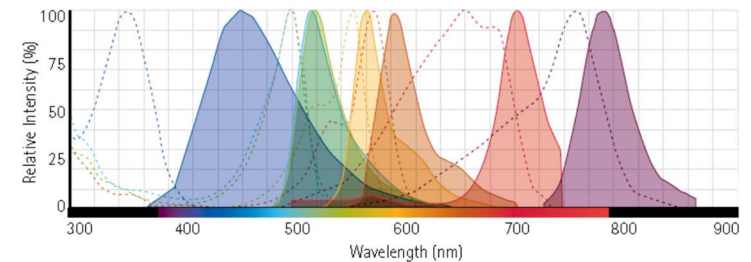
1971 – Eva Engvall and Peter Perlman publish the first article in which they describe and set the term **ELISA**

Enzyme-linked immunosorbent assay (ELISA) **Quantitative assay of immunoglobulin G**

(First received 3 December 1970; in revised form 15 February 1971)

1980's – boom of immunoassays as a result of development of a method of monoclonal antibody production (Georges Köhler and César Milstein, 1975)

■ Alexa Fluor® 488
■ Alexa Fluor® 750
■ Cy3
■ Cy5.5-Allophycocyanin
■ Rhodamine Red
■ Fluorescein (FITC)
■ Hoechst 33258



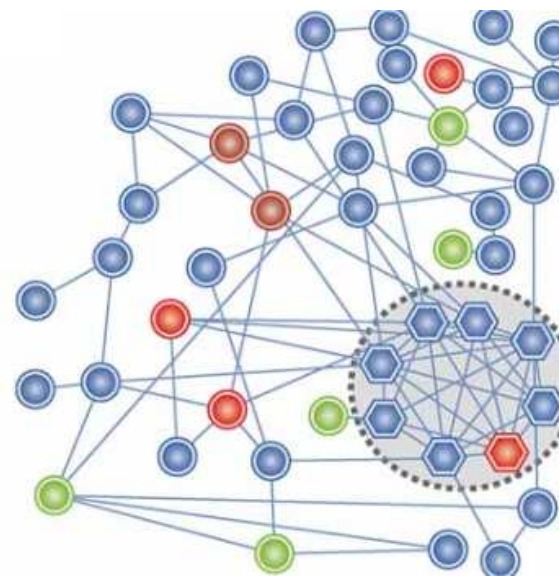
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Current needs and trends in biomarker research – science perspective

Individualization

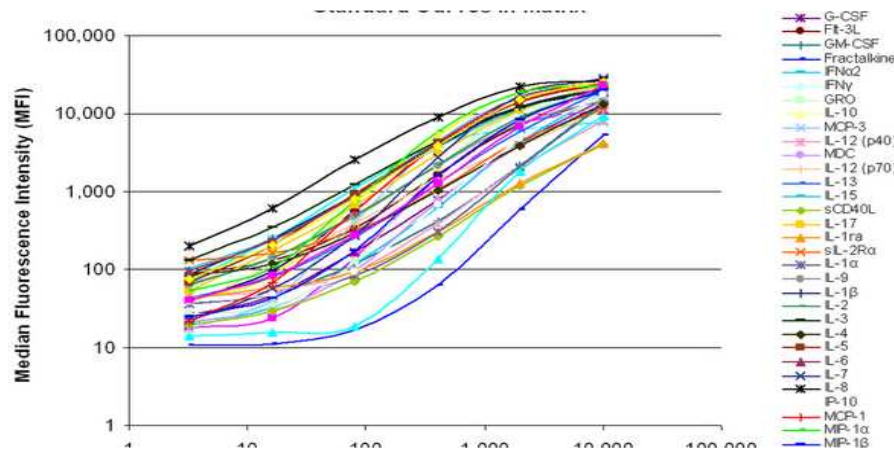


Systemalization (systems biology)

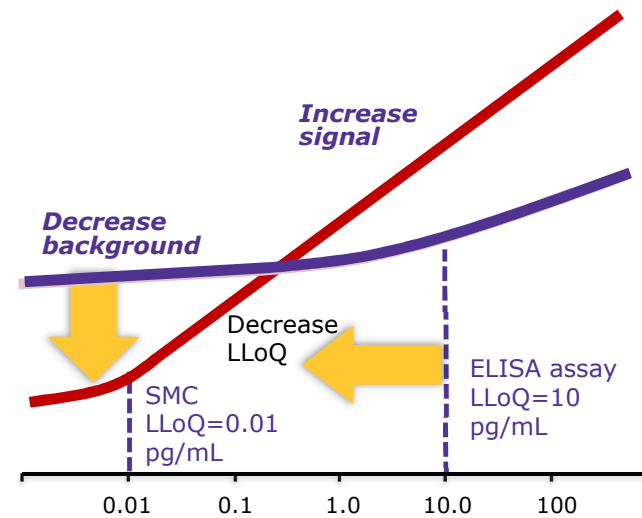


Current needs and trends in biomarker research – practical perspective

Multiplexing



Increasing sensitivity



„Fit-for-purpose“ immunoassays approach @ Merck



Platform	ELISA	xMAP® Luminex	SMC™
Quantitative	Yes	Yes	Yes
Multiplex capability	No	Yes	No
Sensitivity	picogram/ml	picogram/ml	femtogram/ml
Sample volume	50 – 100 µl	~25 µl	5 – 100 µl
Dynamic range	2 – 3 log	3 – 4 log	4 – 5 log
Fit-for-purpose	Plate reader compatible Most widely used	Multiplex detection Flexible platform	Ultrasensitivity High performance

Biomarker research may feel like ...



Looking for the needle in a haystack



-> mulch or



-> animal feed

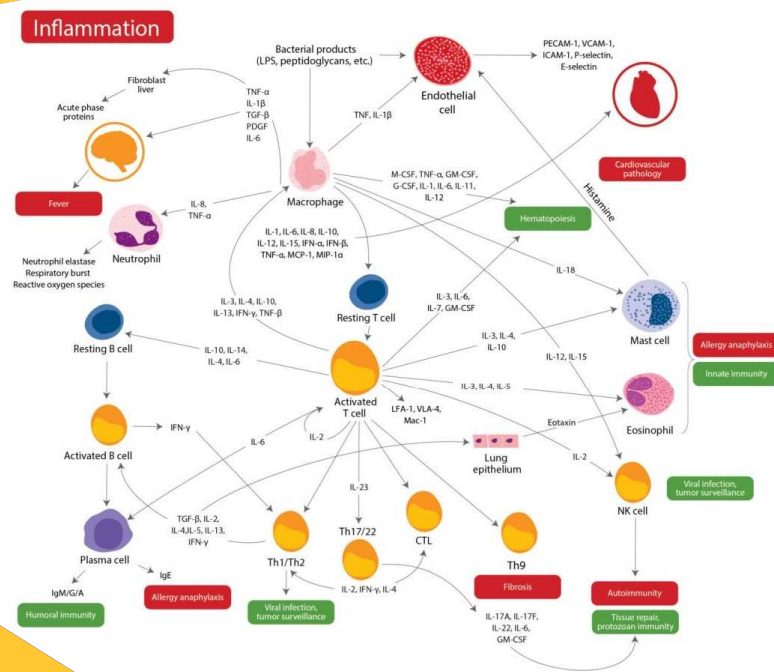
Critical to find the technology (or technologies) that fit your application and expectations... „fit-for-purpose“

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Multiplexing of protein biomarkers

Multiplexed measurement is logical for biological discovery with proteins because they function within networks, pathways, complexes and families. The activity of an individual protein is dependent not only on its abundance, but also on the effects on interacting, modifying, antagonistic and synergistic proteins.

Cytokine biology provides a nice example of the complexity of protein networks.



Summary of benefits of multiplexing approach

Budget

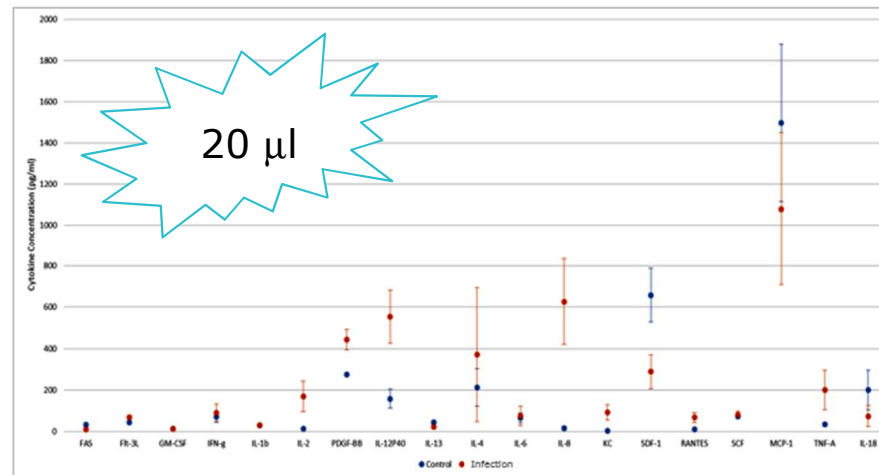
Time

Data hygiene

Systems biology

Sample volume

- Generates **more data with less sample**
- **Reduces costs and labor** per data point
- Deeper insight into **complex biology mechanisms**
- **Less laboratory animals** – financial/ethical considerations
- **Decreased work load** gives less confounding variables in your data – organization, pipetting, sample storage etc.

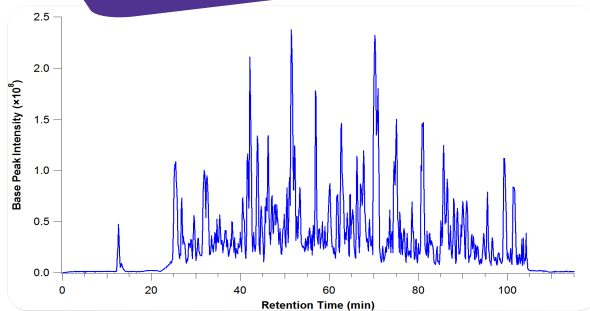


Proof of concept data courtesy of Professor Danielle Gunn-Moore¹, Dr Janet Alexander², Dr Connor O'Halloran¹, Dr Laura McCulloch³ and Lawrence Rentoul⁴ at the (1)University of Edinburgh (Dick School of Veterinary Medicine), (2) WALTHAM Centre for Pet Nutrition, (3) The Roslin Institute and (4) Merck KGaA.

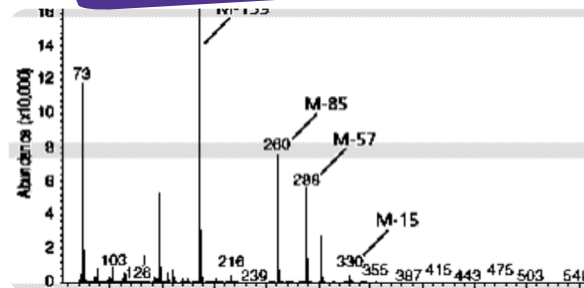
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Overview of multiplex analytical techniques used in proteomics

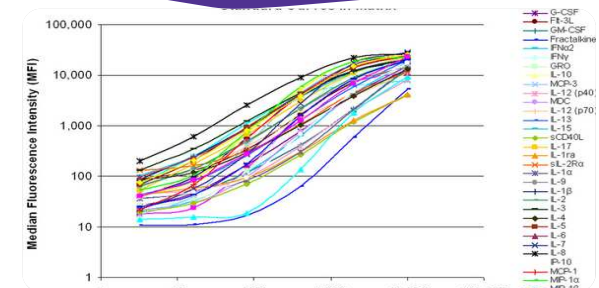
Separations



Mass spectrometry



Immunoanalytics



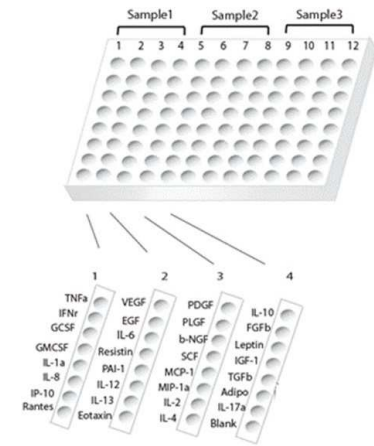
Separation in time of detection based on physico-chemical properties of the analytes and the extent of their interactions with a separation medium (stationary phase in HPLC) or when external fields are applied (magnetic field in TOF-MS)

Based on antibody-antigen binding principle. The only possibility for multiplexing is to have a **spatial separation of different antibody-antigen complexes for detection.**

Spatial separation for multiplex immunoassays

In parallel wells

- Not a real multiplexing
- In this example 32 analytes are measured in a sample but each separately in one of individual 32 wells -> 32x sample volume needed



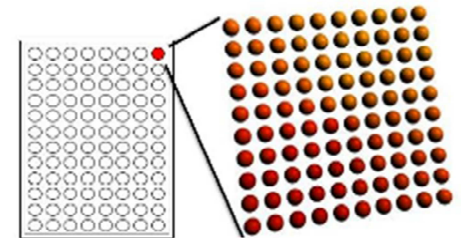
Planar arrays (chips)

- Sample exposed to multiple capture antibodies immobilized in separated spots in one well
- High-throughput, but poor reproducibility



In-solution bead-based

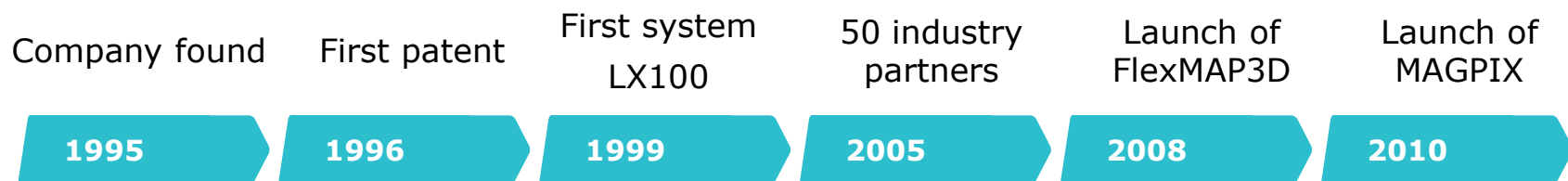
- Antibodies bound to **sets of multiple distinguishable beads that are added to the sample in solution**
- Large batches of beads can be made and flexibility in custom coating
- In-solution kinetics is faster and brings broader detection ranges



xMAP® technology – invented and marketed by Luminex®

- Leader in microsphere-based multiplexing
- Over 14 000 system installations
- Production of the machines and beads
- **cooperation with strategic partners that bring an added value to the technology** by manufacturing reagent kits

Luminex
complexity simplified.



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xMAP® technology – respected and highly cited

PROTEIN EXPRESSION PROFILING

- Inflammation/cytokines
- Cancer markers
- Cardiac markers
- Cellular signaling
- Endocrine
- Metabolic markers
- Neurobiology

GENOMIC RESEARCH

HLA typing

BIODEFENSE/ENVIRONMENTAL

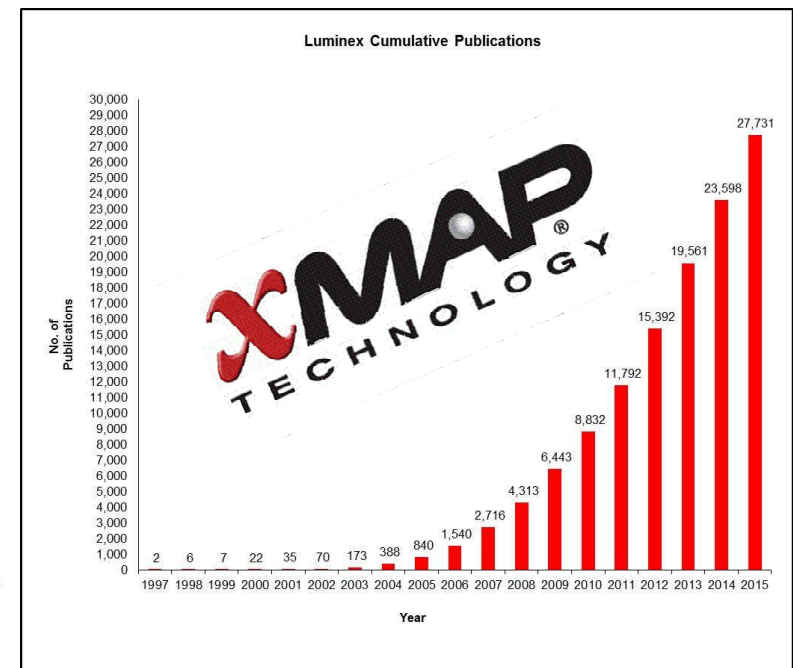


xMAP Technology name origin

x = biomarker or disease panel
to be tested

MAP = Multi-Analyte Profiling

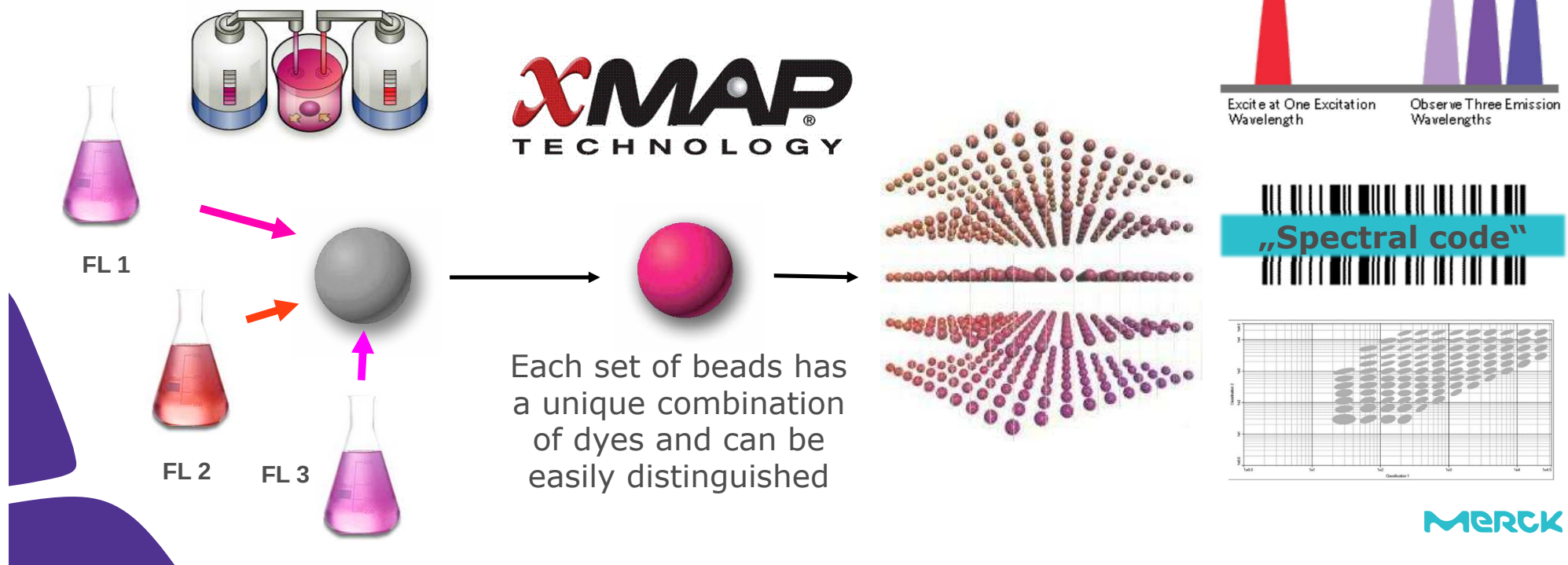
*x*MAP = multiplex biological testing of
up to 500 analytes in a single
sample volume



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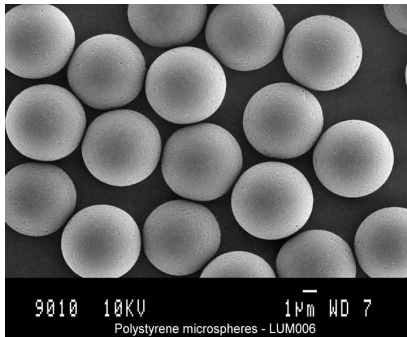
xMAP® technology – basic principle

Bead-based array that uses **spectrally coded microspheres** with chemically modifiable surface.

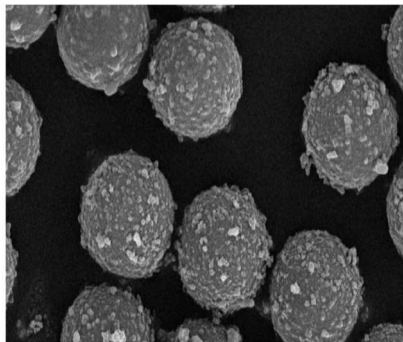
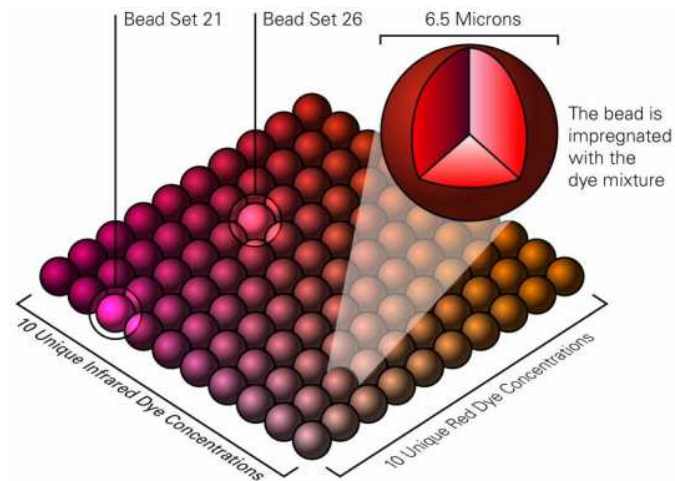


xMAP® technology – microspheres & coupling possibilities

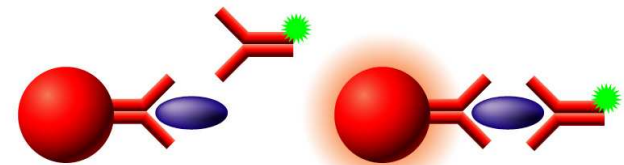
Polystyrene 5,6 um



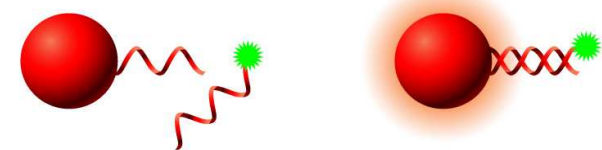
Magnetic 6,5 um



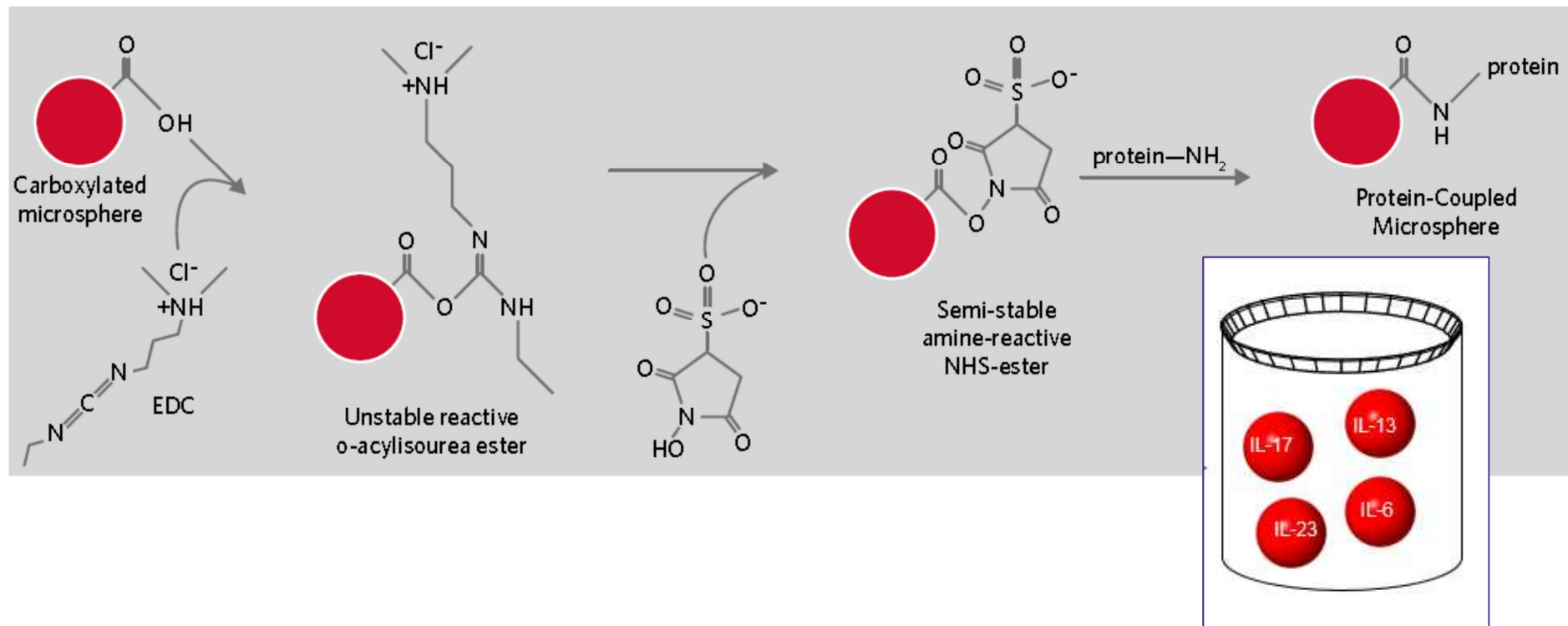
Immunoassay



Nucleic Acid Assay

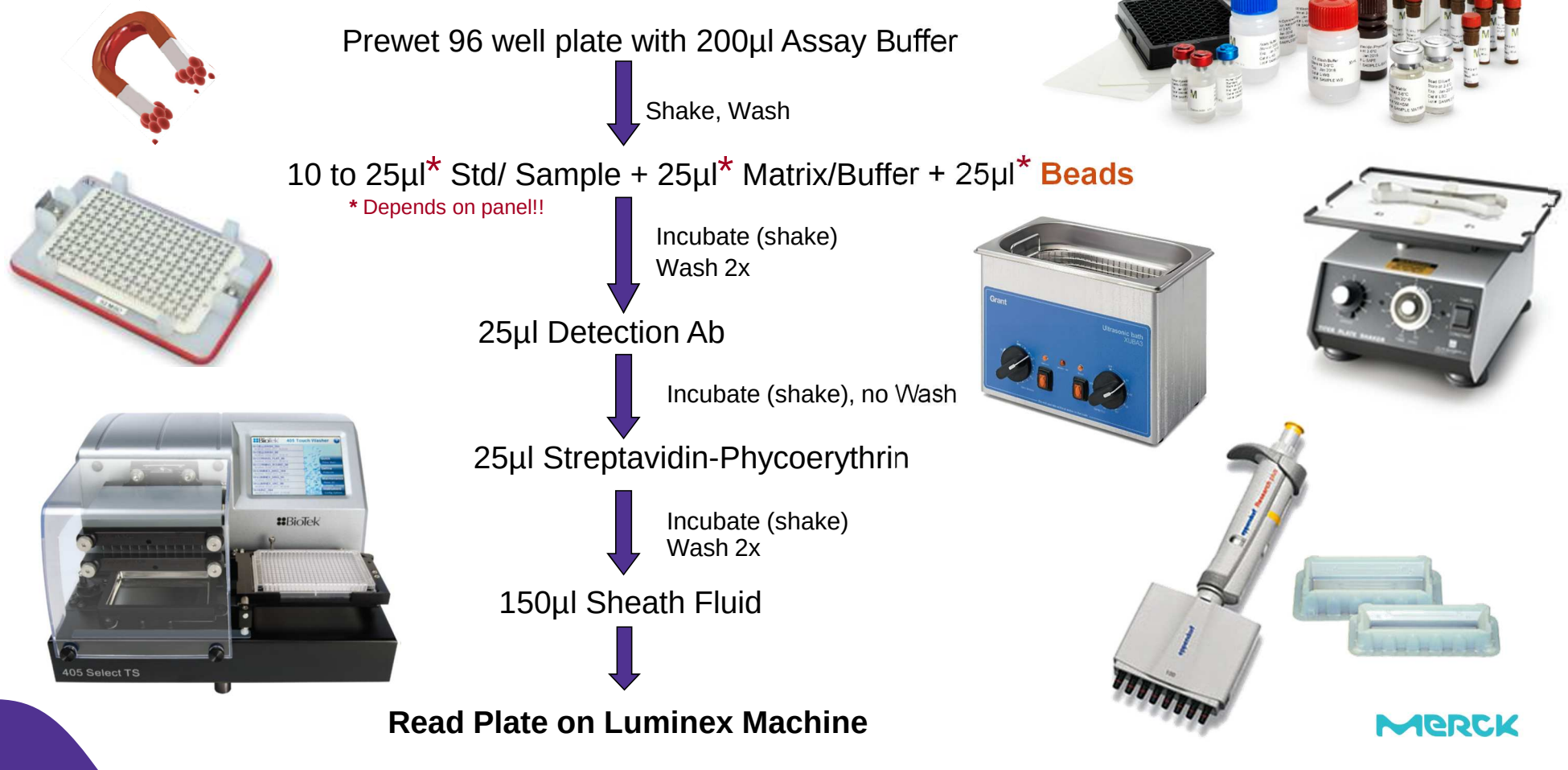


xMAP® technology – surface coupling chemistry

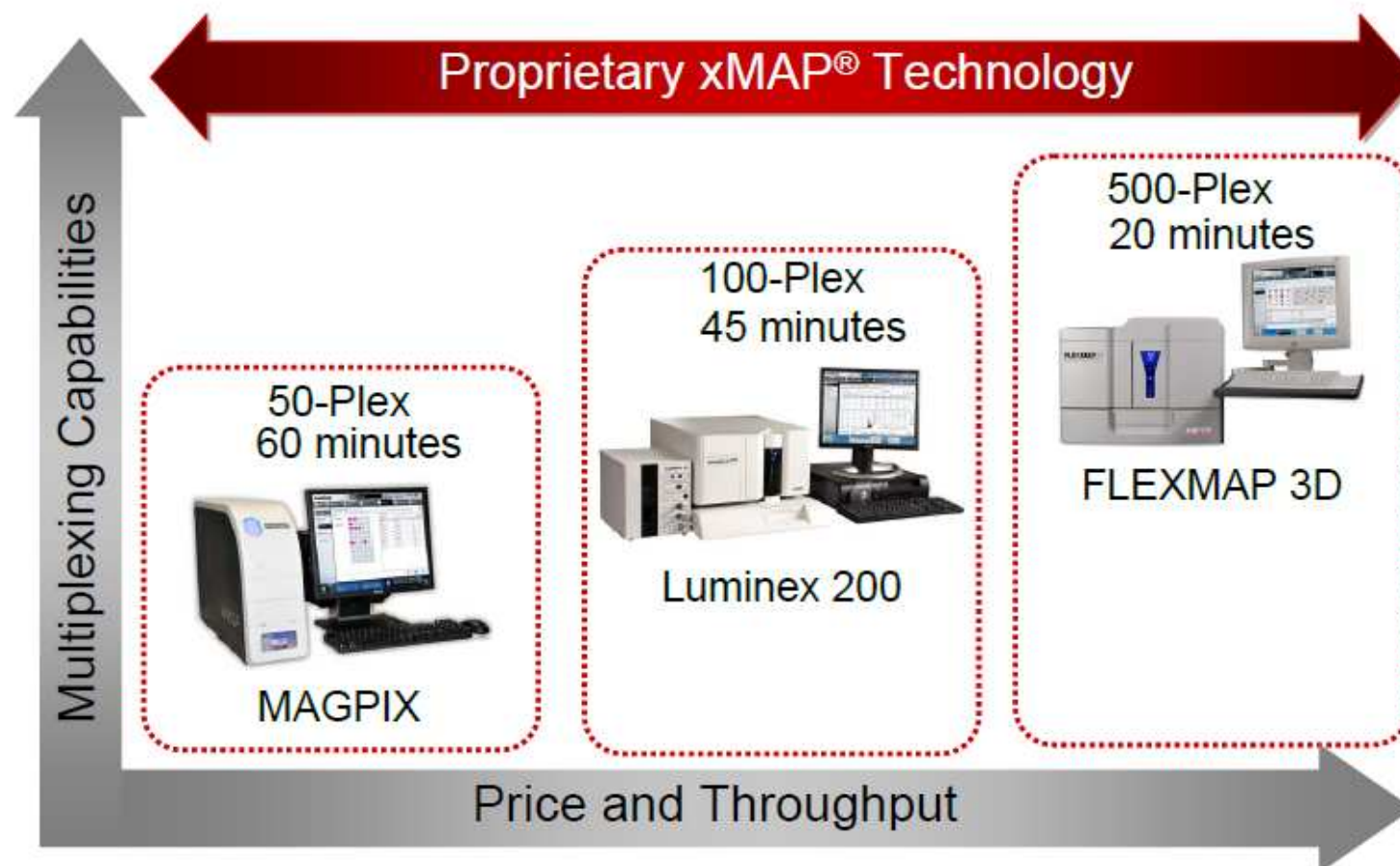




xMAP® technology – general assay protocol

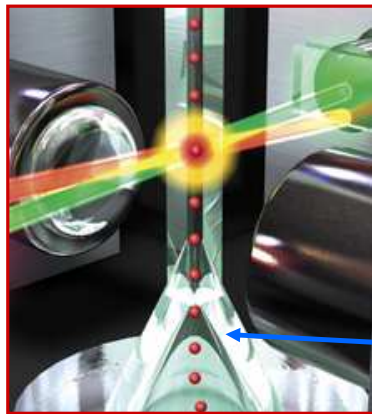


xMAP® technology - instrumentation



xMAP® technology – two types of systems

Flow Cytometry-Based Analysis LMX200 & FM3D



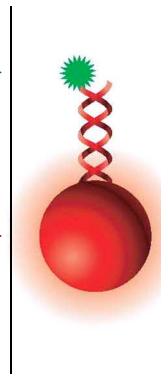
Interrogate
label with green
laser (525 nm)



Interrogate
bead with red
laser (635 nm)



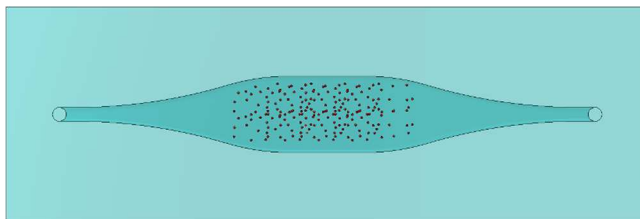
Sheath Fluid



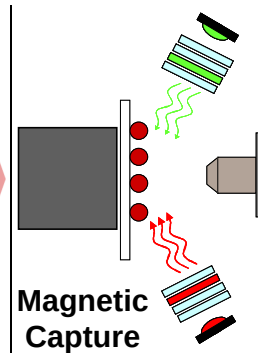
Quantify binding
events

Identify bead region
based on internal
dye concentrations

LED/Image-Based Analysis- MagPix



Beads in Chamber



Interrogate label
with green LEDs
(525 nm)

Identify and quantify
with CCD imager

Interrogate bead
with red LEDs
(635 nm)

xMAP® technology – what is needed to perform the multiplex assay

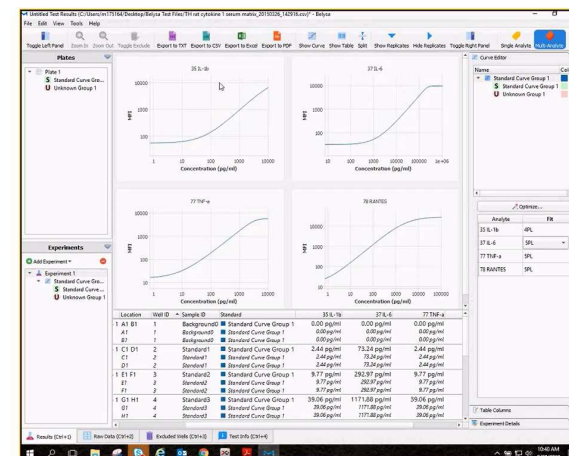
Set of reagents



Instrumentation



Analysis SW



MILLIPLEX® MAP

xMAP
TECHNOLOGY

Belysa™
Immunoassay Curve
Fitting Software

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Milliplex® MAP kits – our ongoing dedication to multiplexing



Antibody
production

1978



RIA kits

1988



ELISA kits

1997

First xMAP kit
on the market

2000



Acquired by
Millipore

2006



Acquired by
Merck

2010

- Milliplex® MAP assays offer the **broadest selection of analytes** across a wide range of research areas and species.
- **Industry-leading expertise backed by decades of experience in assay design, validation and quality assurance**



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Milliplex® MAP kits - overview

- Reagents are offered as panels that contain biomarkers configured with respect to known or expected connections in their function
- Final user will choose **any number of analytes from one panel**
- These are **RUO** kits

Human Metabolic Hormone

(Cat. No. HMHEMAG-34K)

Amylin (active) + ▮	IL-6
Amylin (total) + ▮	Insulin
C-Peptide	Leptin
Ghrelin (active) ▮	MCP-1/CCL2
GIP (total)	Pancreatic
GLP-1 (active) + ▮	Polypeptide (PP)
GLP-1 (total) + ▮	PYY (total)
Glucagon ▮	TNFα

384-well Human High Sensitivity T Cell NEW

(Cat. No. HSTC384-28K)

21 (Cat No. HSTCMAG384-PX21)

21 (Bulk Cat. No. HSTCMAG384PX21BK)

Fractalkine/ CX3CL1	IL-12 (p70)
GM-CSF	IL-13
IFNγ	IL-17A/CTLA8
IL-1β	IL-21
IL-2	IL-23
IL-4	I-TAC/CXCL11
IL-5	MIP-1α/CCL3
IL-6	MIP-1β/CCL4
IL-7	MIP-3α/CCL20
IL-8/CXCL8	TNFα
IL-10	

„MILLIPLEXABILITY“

- 1 menu
- 1 assay
- A multitude of options
- Pre-mixes available
- **Biggest panel has 48 analytes (NEW human cytokine panel A)**

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The science of biomarker validation

- is increasing its importance as assay technology develops, especially in **regulated labs**
- RUO assays are coming under increasing scrutiny by Pharmaceutical and Contract Research Companies.
- guidance and consensus publications are written by those in industry who form forums which discuss and advise up on the validation which should be applied to assays for use in regulated environments.

LOOK closer

All biomarker kits are not
created equal

With assay performance data included in every
protocol, you can discover with confidence.



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Recommendations for biomarker multiplex assays validation

Multiplex assays present **unique challenges** not encountered with single-plexed assays. Unique challenges such as different detection ranges, cross-reactivity with multiplex assay reagents, increased specificity issues and matrix interference, cross-talk across assays, and changes in sensitivity, **if not carefully addressed, can generate misleading results.**



EBF



The AAPS Journal, Vol. 18, No. 1, January 2016 (© 2015)
DOI: 10.1208/s12248-015-9820-y

White Paper

Recommendations for Use and Fit-for-Purpose Validation of Biomarker Multiplex Ligand Binding Assays in Drug Development

Darshana Jani,^{1,13} John Allinson,² Flora Berisha,³ Kyra J. Cowan,⁴ Viswanath Devanarayan,⁵ Carol Gleason,⁶ Andreas Jeromin,⁷ Steve Keller,⁸ Masood U. Khan,⁹ Bill Nowatzke,¹⁰ Paul Rhyne,¹¹ and Laurie Stephen¹²

Guidance for Industry

Bioanalytical Method Validation

DRAFT GUIDANCE

This guidance document is being distributed for comment purposes only.

Comments and suggestions regarding this draft document should be submitted within 90 days of publication in the *Federal Register* of the notice announcing the availability of the draft guidance. Submit electronic comments to <http://www.regulations.gov>. Submit written comments to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. All comments should be identified with the docket number listed in the notice of availability that publishes in the *Federal Register*.

For questions regarding this draft document contact (CDER) Brian Booth, 301-796-1508 or (CVM) John Kadevil, John.Kadevil@FDA.HHS.gov.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Veterinary Medicine (CVM)

September 2013
Biopharmaceutics

Revision 1

MERCK

Parameter	Definition
Robustness	The ability of a method to remain unaffected by small variations in method parameters
Precision	The closeness of agreement between independent test results obtained under stipulated conditions
Trueness	The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value
Uncertainty	A parameter associated with the result of a measurement, that characterizes the dispersion of the values could reasonably be attributed to the measurand
Limits of quantification	Highest and lowest concentrations of analyte that have been demonstrated to be measurable with acceptable levels of precision and accuracy
Dilutional linearity	Dilutional linearity is performed to demonstrate that a sample with a spiked concentration above the ULOQ can be diluted to a concentration within the working range and still give a reliable result
Parallelism	Relative accuracy from recovery tests on the biological matrix or diluted matrix against the calibrators in a substitute matrix
Recovery	The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in the solvent
Selectivity	The ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present
Sample stability	The chemical stability of an analyte in a given matrix under specific conditions for given time intervals

Milliplex® MAP kits – validation steps for new kit development

- Specificity testing: cross-reactivity
- Stringent criteria for precision (Inter- and Intra-assay CVs)
- Sensitivity: LLOD and LLOQ, DCs
- Matrix effect: Spike and recovery analysis
- Quantification: precision, linearity and/or parallelism, dynamic range, hook effect
- Accuracy: standard calibration, benchmark to gold standard, sample correlation
- Studies for stability including heat-stress and real time
- Raw material supply chain research
- Sample testing for biological relevance and normal range
- Relative Potency values monitored for each standard cocktail and compared to WHO where available
- QC Batch Records maintained to monitor performance over time and between lots

Challenges with Cross-Reactivity (related to assay specificity)

Cross-reactivity occurs when the capture or detection reagents in a multiplex assay recognize similar epitopes on other analytes present in the sample. **Multiplex assays are in general more susceptible to cross reactivity issues than single-plexed assays.**

Approaches to testing

- Using single bead and multiplexed detection Ab cocktail
- compare multiplex to well-characterized single-plexed assays
- “missing man” technique where all analytes except one are added to the assay

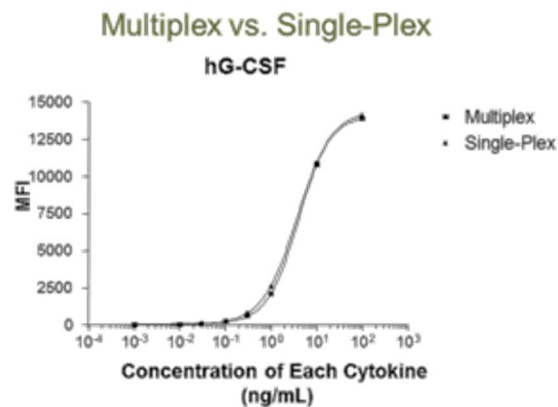
Our cross-reactivity limit is 5%, average is <1%.

	NCAM1	CA9	Mesothelin	Midkine	Hepsin	KLK6	TGM2	ALDH1A1	EpCAM	CD44
Blank	26	34	26	23	20	24	19	20	23	27
S1	50	44	29	46	39	72	32	58	58	36
S2	83	74	45	112	80	186	51	139	130	65
S3	188	150	113	408	193	451	110	327	339	240
S4	515	390	607	1829	498	1234	262	919	919	1032
S5	1517	1160	2861	7913	1136	3393	700	2366	2463	4763
S6	3490	3078	6558	11335	1978	7226	1377	4892	5073	6784
S7	7007	7273	9461	13266	2990	12159	2399	7724	8538	7323
NCAM1	7113	32	23	22	20	25	20	22	22	25
	6989	36	24	25	19	25	21	21	19	27
CA9	33	7100	29	26	18	27	23	23	21	27
	26	6018	27	21	18	23	22	19	19	26
Mesothelin	26	34	9064	26	22	25	22	21	23	27
	26	32	10463	25	19	26	19	19	26	28
Midkine	120	227	109	13128	63	58	43	129	29	57
	122	199	94	13470	66	60	43	119	30	60
Hepsin	25	28	26	20	2521	24	20	16	21	27
	26	33	29	24	2680	24	24	21	23	28
KLK6	31	33	28	23	20	12428	19	22	22	27
	31	35	31	24	18	12511	20	21	22	26
TGM2	31	38	29	27	20	54	2434	24	21	29
	28	40	24	29	19	49	2363	23	21	28
ALDH1A1	25	38	26	23	21	28	21	6429	22	25
	23	31	23	23	20	27	21	6087	21	28
EpCAM	30	38	28	24	19	29	23	19	8622	27
	30	33	22	20	18	25	21	22	7484	28
CD44	31	37	27	24	22	29	20	21	24	7481
	30	31	24	27	20	27	18	24	24	7315

Human Circulating Cancer Biomarker Panel 4

Specificity and consistency of assay profiles

Performance evaluated in both a single plex and multiplex format. Ensured that the reported signal does not fluctuate between the two. Can be challenging for the scientist to perform due to a lack of availability of single-plexed reagents. Data from the vendor can give confidence in the specificity.



Validated panels yield consistent performance irrespective of plex size or other analytes in the panel

CSF Samples 1:2 Dilution	4-plex				1-plex			
	Concentration (pg/ml)							
	Aβ 40	Aβ 42	tTau	pTau 181	Aβ 40	Aβ 42	tTau	pTau 181
1	1329	471	3162	103	1374	431	2963	99
1	1425	481	3252	105	1396	476	3251	110
2	2033	1001	3432	141	2060	979	3291	145
2	1934	1030	3213	144	2046	953	3291	123
3	773	237	2051	144	786	232	1921	142
3	827	256	2038	151	794	250	2148	154
4	1449	649	3446	137	1472	630	3155	137
4	1447	636	3482	139	1523	622	3226	139

4-plex vs. Single Plex A β / Tau Kit

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Performance in sample matrix

Blood is a complex matrix and various proteins may interfere with accurate measurement. Standard curve in buffer does not simulate the conditions in which the native protein is analyzed and this can lead to inaccurate analyte quantification.

Commercially Available Assays are tested using purified proteins/protein mixtures in assay buffer and occasionally tested in matrix like serum or plasma.

Milliplex assays are always validated in the expected matrix noted in the protocol.

We are the only company using a serum matrix reagent to compensate for matrix effect.

SERUM MATRIX = Lyophilized serum, stripped of endogenous proteins, from the native model required by the assay

Rat Bone Panel 1 (Serum/Plasma samples)

(Cat. No. RBN1MAG-31K)

ACTH	Leptin
DKK1	Osteoprotegerin (OPG)
FGF-23	PTH
Insulin	Sclerostin (SOST)

Rat Bone Panel 2 (Cell culture samples)

(Cat. No. RBN2MAG-31K)

ACTH	Osteocalcin (OC)
DKK1	Osteopontin (OPN)
FGF-23	Osteoprotegerin (OPG)
Insulin	PTH
Leptin	Sclerostin (SOST)

How to deal with matrix effects in biological samples

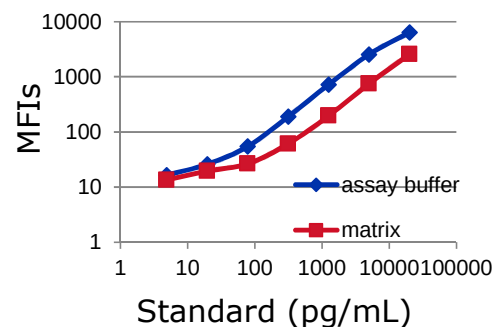
If spike **recovery is outside acceptance criteria (80-120%)**, it shows non-specific matrix effect from samples. Therefore requirement to normalize against this non-specific effect, shifting the actual curve so it matches the recovery in the samples.

Sample dilution

Sample Dilution	Eotaxin	IFN-g	IL-1b
Neat	71%	34%	40%
Diln 1:4	69%	49%	63%
Diln 1:10	72%	61%	74%
Diln 1:20	77%	69%	81%

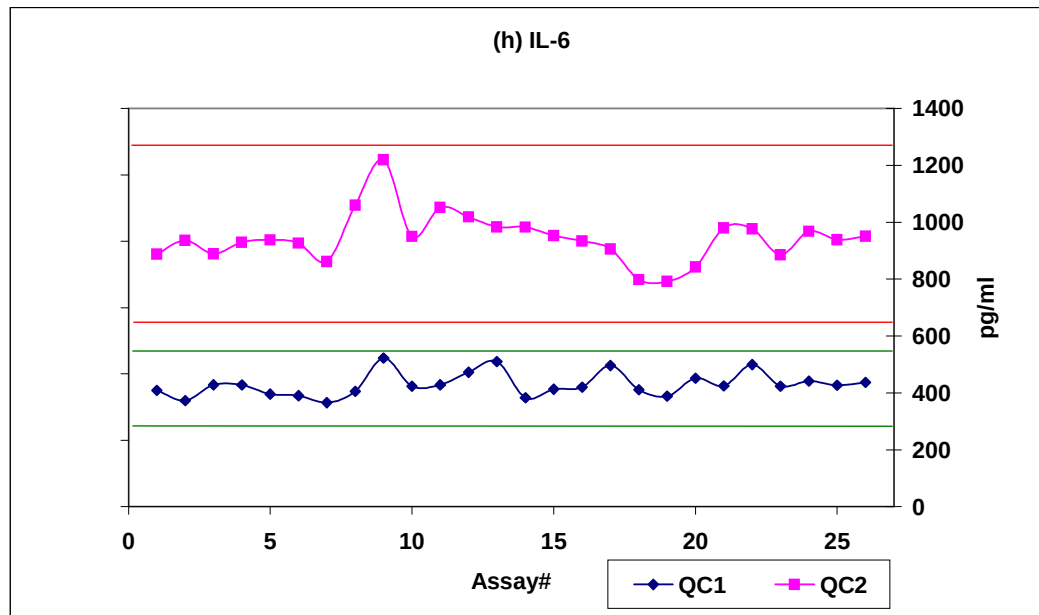
Addition of matrix to standards

Sample Dilution	Eotaxin	IFN-g	IL-1b
Neat	89%	83%	117%



The importance of using quality controls (QC1 & QC2)

Two vials containing a mixture of analytes with **expected concentration ranges** in the lower and higher part of the calibration curve are included in all standard Milliplex® MAP kits.



QUALITY
CONTROLS

MERCK

Intra-assay precision: within run repeatability

Intra-Assay precision is determined by running QC1 and QC2 samples at least 6 times.

Human IGF Panel Validation Data

QC1 Sample	IGF-I	IGF-II
	pg/ml	
1	916	7282
2	924	7046
3	920	7107
4	949	7472
5	898	7369
6	894	7183
7	910	7090
8	907	7052
Average	915	7200
Standard Dev.	17	159
CV (%)	2	2

QC2 Sample	IGF-I	IGF-II
	pg/ml	
1	3336	50602
2	3196	49047
3	3217	48391
4	3415	48021
5	3256	50741
6	3308	49323
7	3178	48367
8	3190	47867
Average	3262	49045
Standard Dev.	84	1115
CV (%)	3	2

	IGF-I	IGF-II
QC1 & QC2 CV (%)	2	2

Inter-assay precision: plate-to-plate consistency

Inter-Assay precision is determined by running QC1 and QC2 as samples at least 6 times.

Human IGF Panel Validation Data

QC1 Sample	IGF-I	IGF-II
	pg/ml	
1	915	7200
2	967	5977
3	797	5254
4	891	7596
5	796	6820
6	811	6266
7	799	6496
8	957	7026
Average	867	6579
Standard Dev.	74	746
CV (%)	9	11

QC2 Sample	IGF-I	IGF-II
	pg/ml	
1	3262	49045
2	3185	47531
3	2859	43125
4	3342	56982
5	2977	47263
6	2896	48900
7	2716	46030
8	3371	48909
Average	3076	48473
Standard Dev.	246	3965
CV (%)	8	8

	IGF-1	IGF-II
QC1 & QC2 CV (%)	8	10

Assay detectability: range

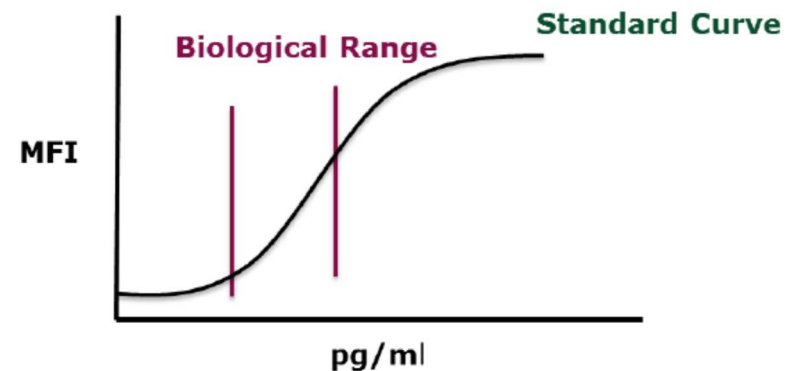
Considerations

Biological Relevance: Does the curve reflect endogenous protein values?

Sensitivity: Does the curve get close to min DC with a low %CV?

Linear Range: Where does the curve linearize, allowing for data resolution?

Example: determining biological sample ranges – consult literature and run a full plate with samples in duplicates (approx 38 samples) using the appropriate sample types (controls vs. diseased – e.g. for cytokines normal vs. sepsis samples)

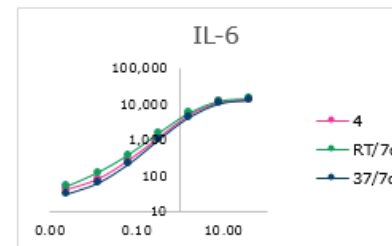
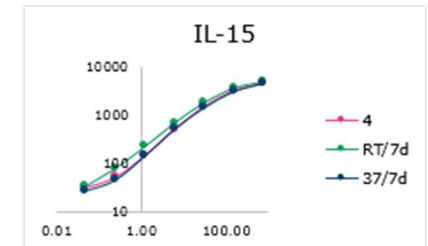


Assesment of stability

Same for multiplex assays as for single-plexed assays. Challenge is determining the conditions that are amenable for all the analytes. **Stability will be limited by the least stable analyte.**

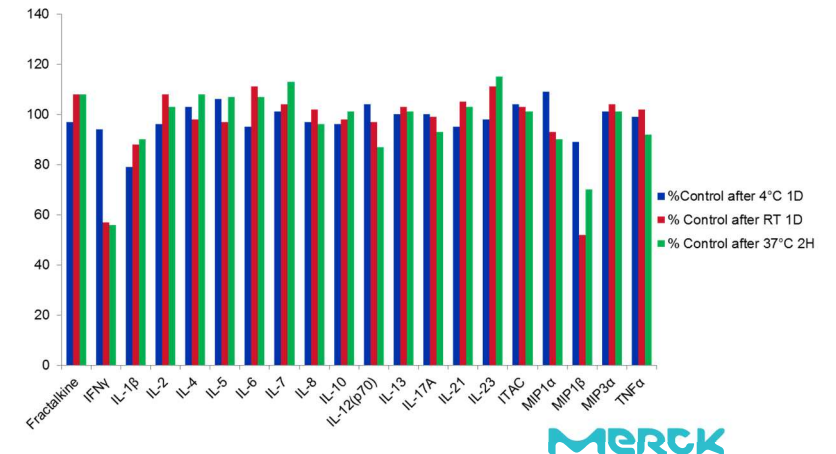
Three metrics are defined for MILLIPLEX MAP

1. Sample stability at a range of temperatures.
2. Freeze/Thaw impact to three cycles
3. Kit Shipping Stability



Sample Stability: Temperature

	Diluted Sample: % of Control Sample (Freeze/Thaw Assay)												
Neat	BDNF	EPO	IL6	IL15	FGF21	FKN	FSTL1	MSTN	Irisin	LIF	OSM	OSTN	SPARC
0 cycles		100%		100%	100%	100%			100%	100%	100%	100%	100%
1 cycle		108%		100%	112%	89%			104%	100%	100%	90%	103%
2 cycles		100%		106%	109%	100%			58%	100%	100%	95%	106%
3 cycles		108%		100%	114%	100%			59%	101%	100%	80%	108%



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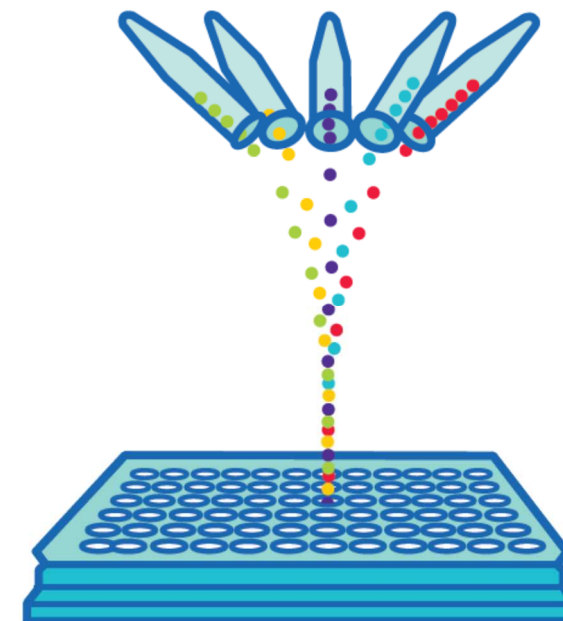
Summary of xMAP® technology & Milliplex® MAP kits

- 1 Multiplexing:** Data generated simultaneously is less prone to user error, enabling to construct cleaner data sets to explore multiple parameters.
- 2 Low sample volume:** With minimal hands-on time, screen multiple analytes in a single sample using a maximum of 25 µL per well.
- 3 Magnetic bead-based format:** Responds rapidly and efficiently to a magnetic field, enabling better and faster washing and detection techniques
- 4 Reproducibility:** High-volume production of xMAP® microspheres allows assay standardization that solid-phased flat arrays cannot provide.
- 5 Economical sense:** Time and resources savings.
- 6 Broadest portfolio:** > 1 000 analytes, custom services possible
Industry leading validation



Multiplexing
gives you the
gift of time
and sample ...

objective:
MORE from less
MORE for less
MORE with less



XMAP
TECHNOLOGY

MERCK



plenty of protein biology „under the surface“

The limitation really might be the platforms that researchers are using at the current time



missing a lot of biology that is occurring under the surface, with a lot of important data still existing underneath (current limits of detection)



Detection of the previously undetectable

SCIENTIFIC BENEFITS:

- Gaining completely new insights into biological mechanisms
- New biomarker discovery

PRACTICAL BENEFITS:

- Reduce research program costs and improve productivity
- Elimination of „non-detectable“ results
- Sample effectiveness & data quality
- Getting more publication quality data



ACCELERATING DRUG DISCOVERY

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What purposes does SMC™ fit and what are the benefits?

SCIENTIFIC

- New biomarker discovery
- Comparisons between Endogenous and Disease Analyte Levels
- Monitoring changes in biomarker levels
- Pharmacokinetics/pharmacodynamics
- Toxicology/drug safety
- Accelerate drug discovery and development
- **Gain insights into novel biological mechanisms**

PRACTICAL

- Eliminate „non-detectable“ results obtained by other immunoanalytical methods
- Sample effectiveness
- Data quality and assay robustness
- Flexibility to design own assays
- Require fewer data points for critical decision making
- **Reduce research program costs and improve productivity**



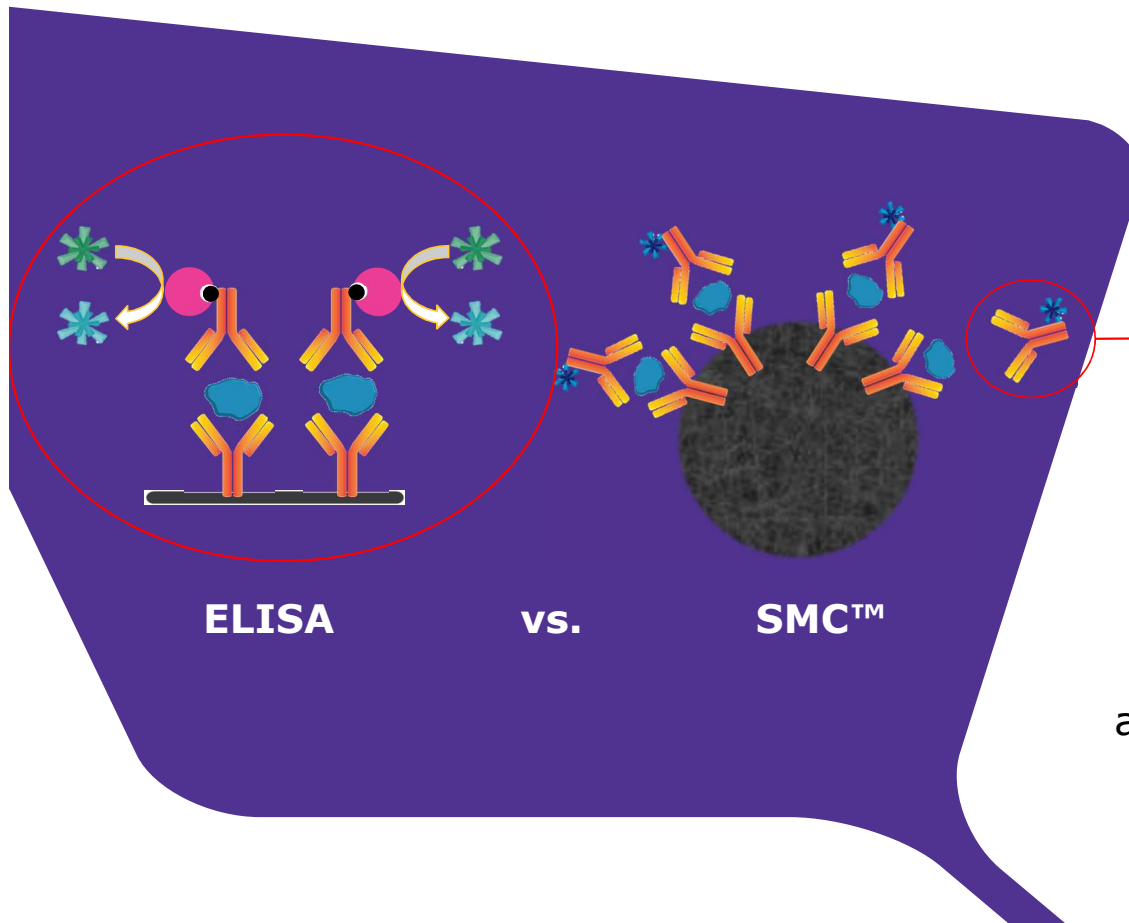
Quantify previously undetectable analytes

discovery depends on detection



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SMC™ = Single Molecule Counting at a glance

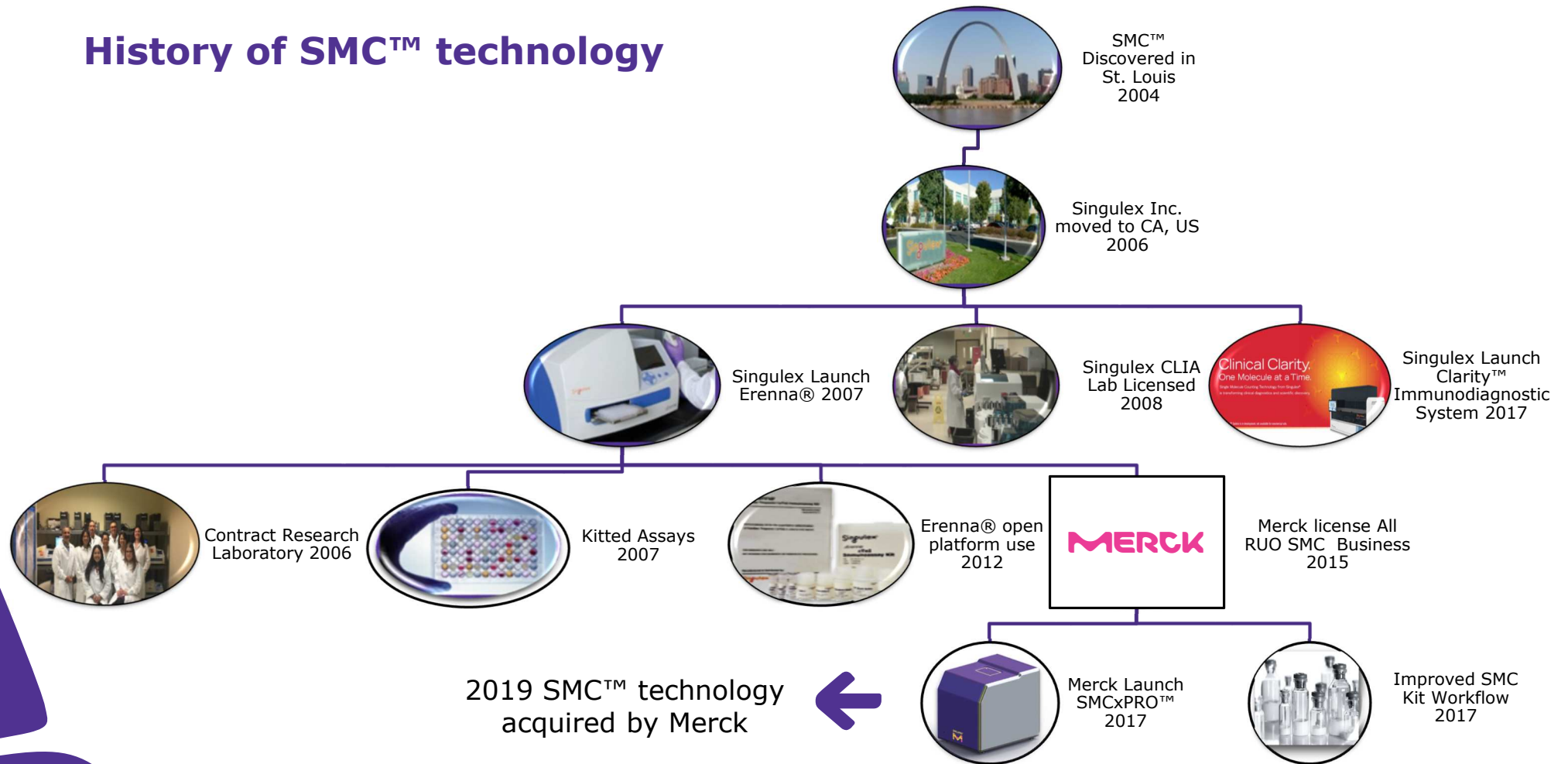


SMCXPRO®

Ultrasensitive immunoassay detection system for digital quantification of low-abundant proteins that you were not able to detect before e.g. with your classical ELISA

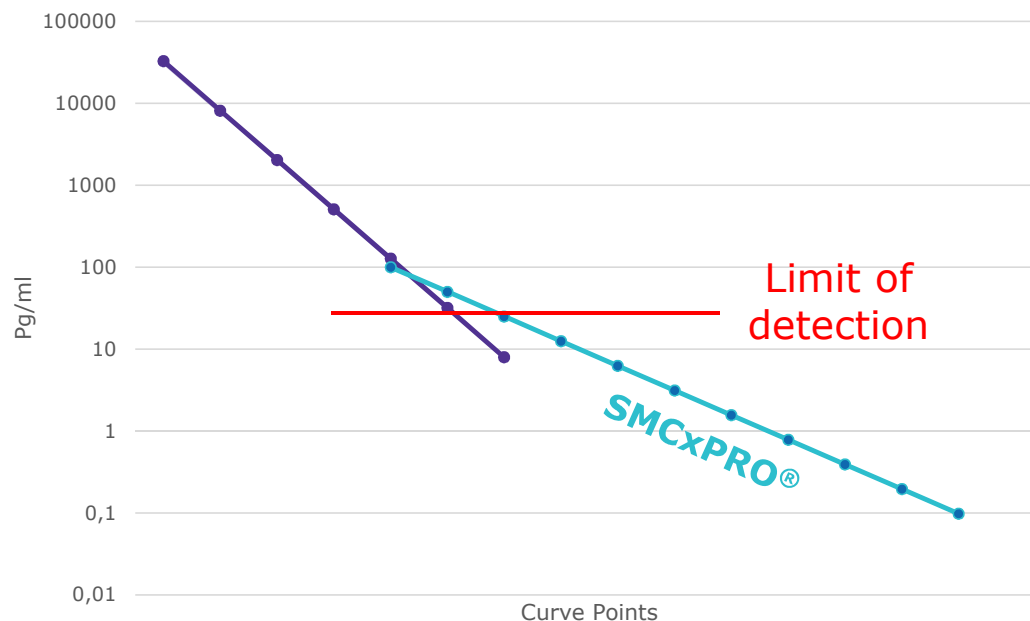
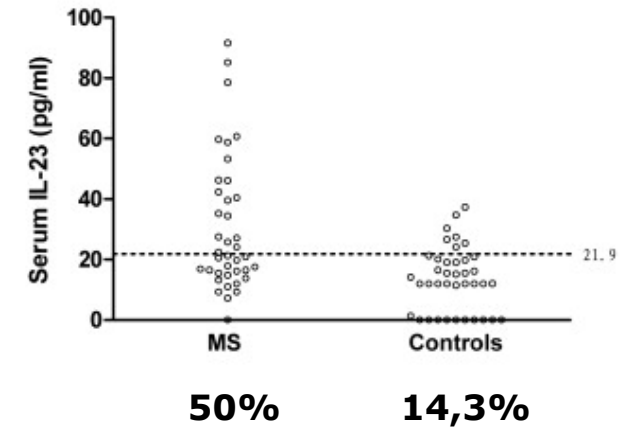
MERCK

History of SMC™ technology



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SMC™ - shifting the detection paradigm

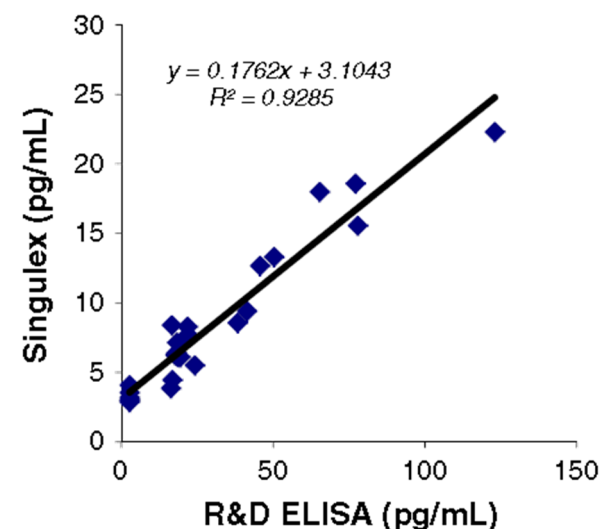


Chen et al looked at the relative values of IL-23 in Chinese patients with Multiple Sclerosis. Using Cytometric Bead Array kits they were looking to establish median population values, however they were not able to achieve detection in all of the patients.

Chen et al, Serum levels of interleukin (IL)-18, IL-23 and IL-17 in Chinese patients with multiple sclerosis; Journal of Neuroimmunology, 243 pp 56-60, 2012

Case study 1 – quantification of IL-22 in human serum

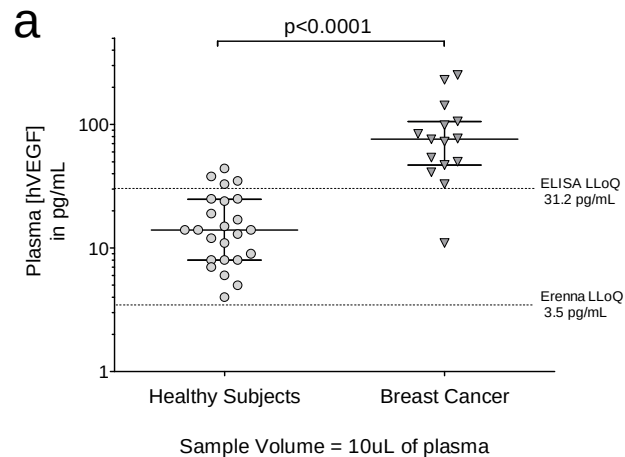
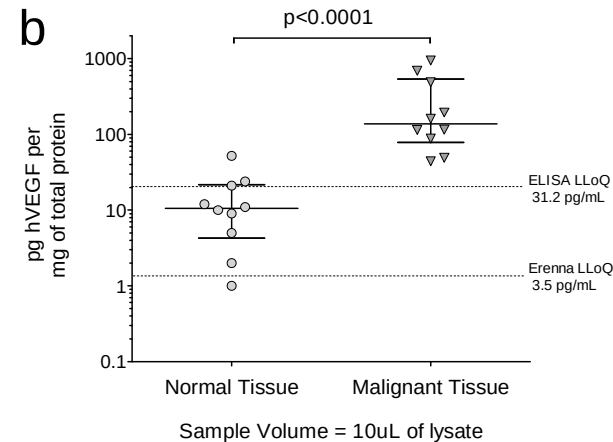
Standard	Theoretical concentration	N ^a	Mean	SD	%CV	% Difference	
	pg/mL		pg/mL				
STD 0	0.0	15	0.0	0.00	0.0	0.0	
STD 11	0.1	15	0.1	0.05	43.6	14.6	
STD 10	0.2	15	0.2	0.03	16.2	-4.0	LLOQ
STD 9	0.4	15	0.4	0.04	9.7	3.7	
STD 8	0.8	15	0.7	0.05	6.4	-4.5	
STD 7	1.6	15	1.6	0.07	4.4	4.2	
STD 6	3.1	15	3.2	0.15	4.6	1.6	
STD 5	6.2	15	6.2	0.62	9.9	-0.7	
STD 4	12.5	15	12.2	0.83	6.7	-2.0	
STD 3	25.0	15	26.5	0.95	3.5	6.0	
STD 2	50.0	15	49.2	2.05	4.1	-1.6	
STD 1	100.0	15	99.5	0.6	0.6	-0.4	ULOQ



Problem: Using ELISA 67% of samples were below LLoQ

Solution: IL-22 SMC™ assay was developed which had 400-fold increased sensitivity. Using SMC it was possible to quantify all 1300 samples in a subsequent clinical study. This indicates that the SMC™ IL-22 assay is a better platform to measure baseline levels of IL-22 and to assess this cytokine as a pharmacodynamic biomarker.

Case study 2 – VEGF in different vascular compartments

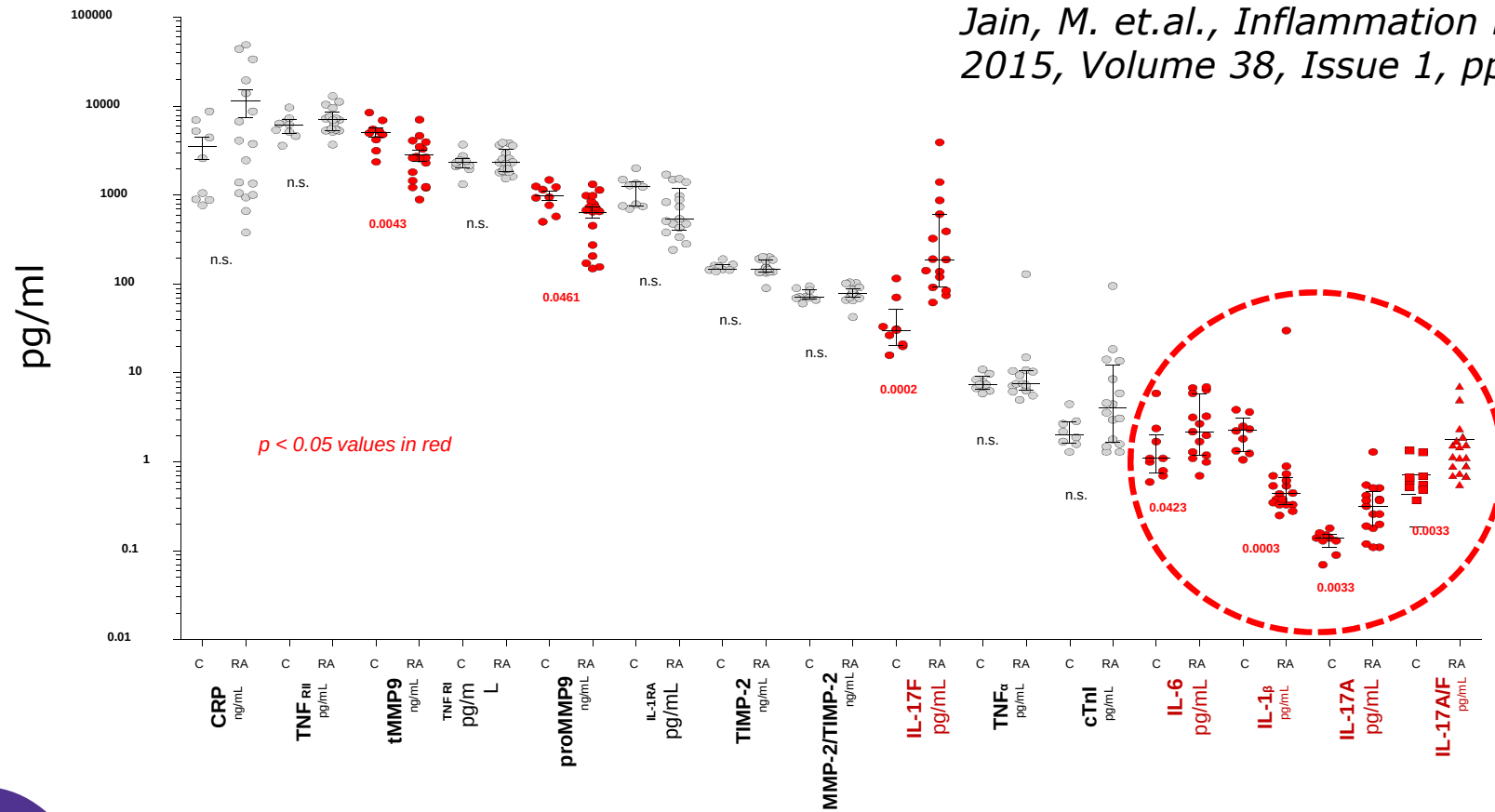


Problem: Unable to quantify the baseline levels using existing ELISA and also limited sample volume available (tissue biopsy lysate).

Solution: SMC™ assay developed with „only“ 8-fold increase in sensitivity but consuming „only“ 10 µL of plasma or tissue biopsy. Similar increases in VEGF found in both vascular compartments (tissue and plasma), enabling them to utilize a less invasive method of measurement and use VEGF as a potential early predictor biomarker.

Case study 3 – rheumatoid arthritis (standard and novel biomarkers)

Jain, M. et.al., Inflammation February 2015, Volume 38, Issue 1, pp 180-186



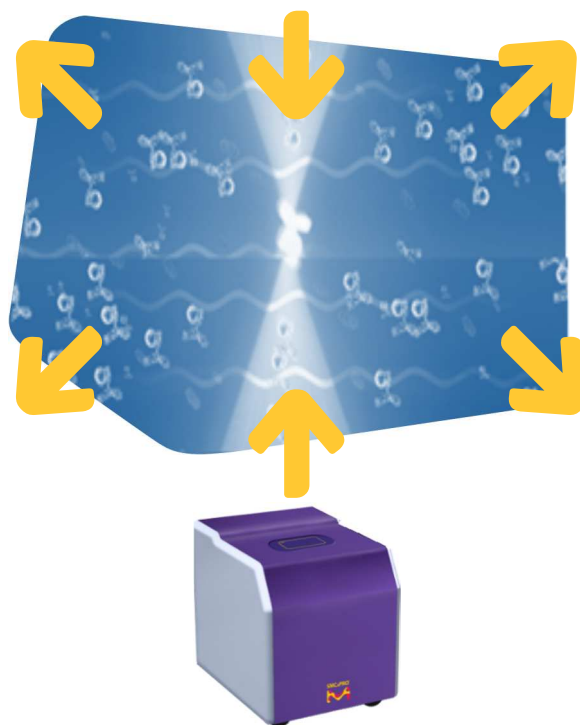
How is high sensitivity achieved?

Use of SMC™ reagents

Proprietary, optimized assay reagents and blocking agents

Concentrating the detection area by workflow

True signal concentration with typical assay input volume of 100 µl and elution read-out volume of 20 µl



Use of magnetic particles

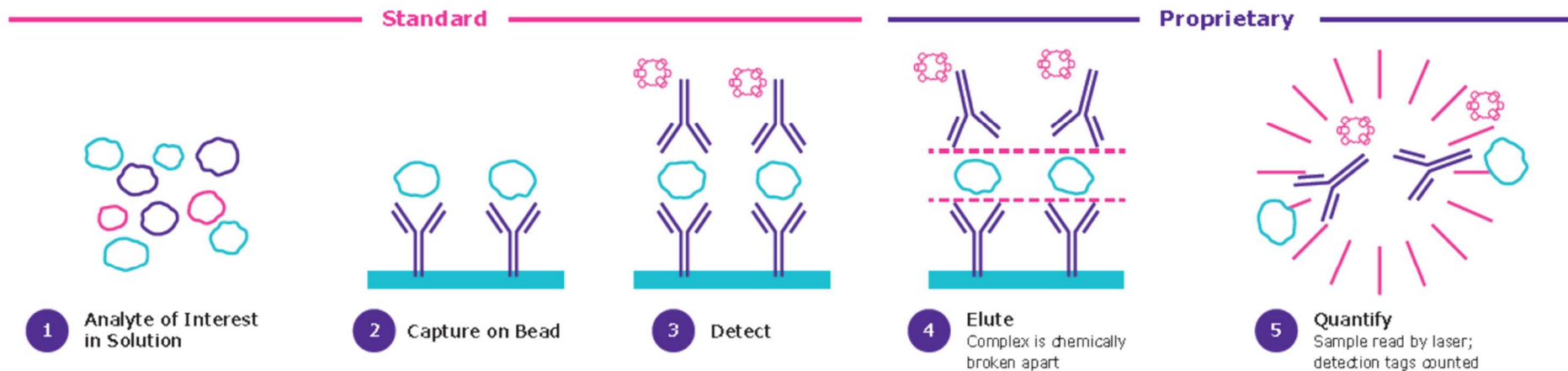
Increased surface area, more capture antibodies, enhanced binding and improved solution kinetics

SMC™ technology (HW/SW)

Background noise reduction by looking at individual detection molecules in a very small interrogation space and removing the detector from the assay plate

SMC™ – the power of single molecule counting technology

Single molecule counting (SMC™) technology provides maximum immunoassay performance while following a workflow very similar to traditional ELISA technology. **By combining a unique assay elution step and robust digital counting, SMC™ technology achieves improved signal-to-noise ratios over traditional immunoassay technologies**, thus providing quantification at both low and high levels of expression on one complete system.



SMC™ – assay workflow

Sample

Plate map

96-well

384-well



Sample collection,
typical volumes
5 – 100 µl of
serum/plasma

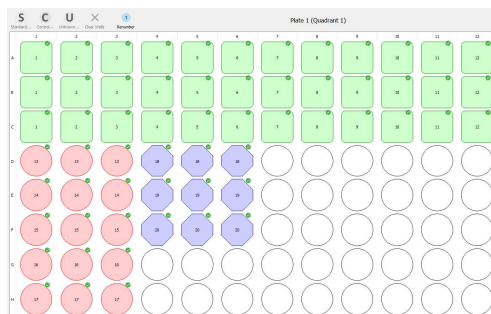


Plate set up **similar to**
classical immunoassay,
includes standards,
internal controls and
samples



Convenient plate
preparation using
magnetic
microparticles
separation



Aurora
Microplates

Transfer of final
eluate into a 384-
well Aurora plate
which is used for
measurement

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SMCxPRO® – extract from instrument specification



Performance:

Slope: >1.2 Response/fM¹

Background: <3 Response¹

Limit of Detection (LoD): < 1 fM

Precision: $<10\%$ CV¹

¹Measurements based on 30fM calibrator (150 kDa antibody labeled with fluorophore), $n = 20$

Dimensions:

Height: 17.8" (452 mm)

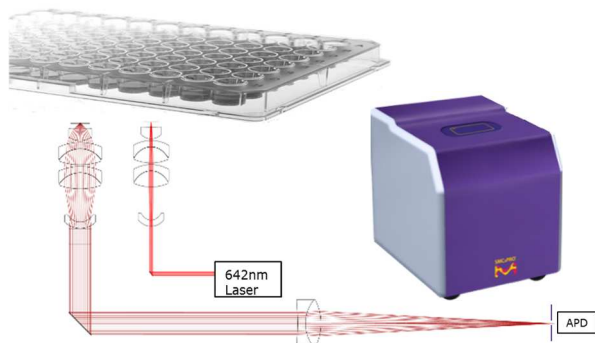
Width: 14.0" (353 mm)

Depth: 15.3" (387 mm)

Fluidics: No

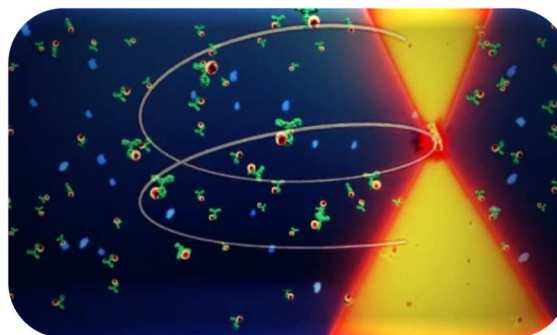
SMCxPRO® – technology principle of single molecule counting

Laser excitation



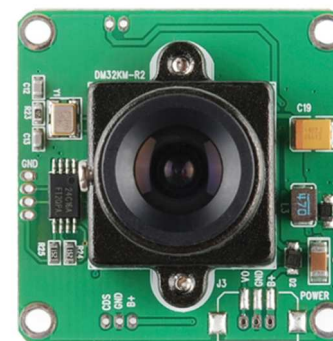
642nm-11mW laser focused 276µm above plate bottom to **single-point-in-time interrogation zone** of the order of 25 femtoliters

Sample scanning



The laser spot is then **scanned through the sample** in a rotating manner at 55 mm/sec, exciting any fluorescent material it passes through.

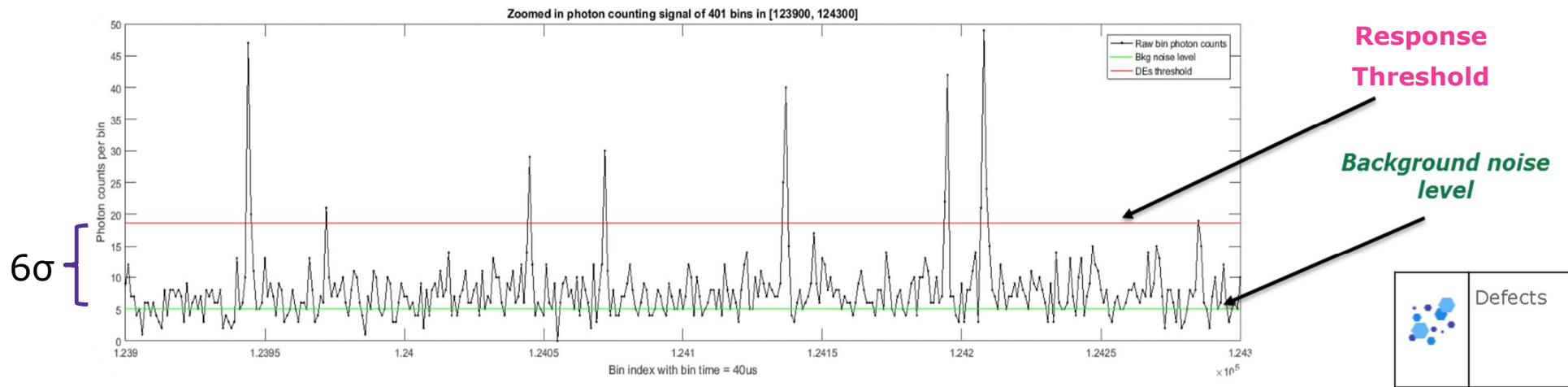
APD detection



The Fluorescent Response passes through a 690/50nm bandpass filter and a Confocal Stop to reduce noise, and then onto a **Single Photon Counting Detector**

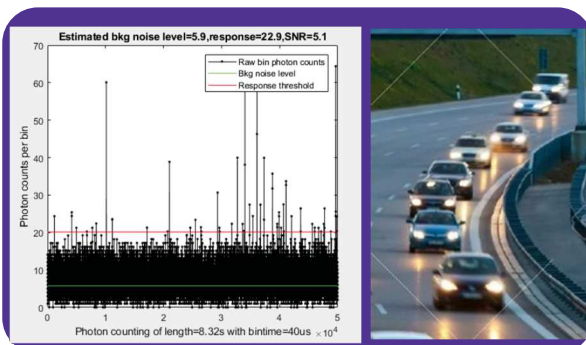
MERCK

SMCxPRO® – analysis of detected signals

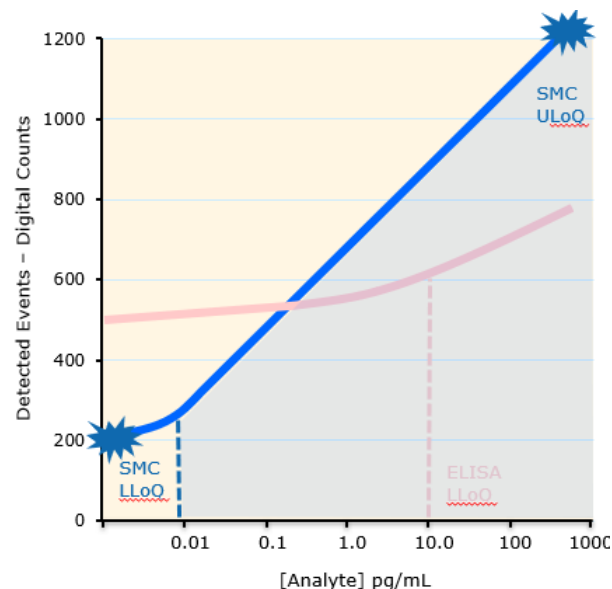


The SMCxPRO® instrument and SW then digitize the detector signal into discrete time series called bins. If, for example, the laser passes through a fluorescent antibody, the instrument will measure a much higher photon count during that bin than when the laser is scanning the non-fluorescent suspension fluid. **Finally, using a proprietary algorithm, data are converted into a single response number which is a measurement of how many detection molecules were seen on a per-second basis for each well (so called response events)**

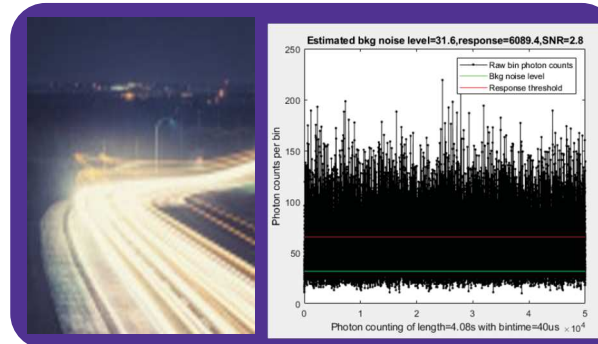
SMCxPRO® – broad dynamic range based on signal processing



Lower end quantification as individual molecules are counted with reduced background



Upper end quantification with proprietary algorithm as signal is increasing



Data are converted into a single response number which is a measurement of **how many detection molecules were seen on a per-second basis for each well** (so called response events)

SMC™ data analysis (example IL-22 assay, lung lysate)

Plate	Group	Expected (pg/mL)	Interp. (pg/mL)	Flag	Interp. CV	Response	Recovery
▶ Plate 1	S Q1 Standard Curve Group	100,000	101,530		13,8%	3993,662	101,5%
▶ Plate 1	S Q1 Standard Curve Group	50,000	47,984		1,8%	3262,508	96,0%
▶ Plate 1	S Q1 Standard Curve Group	25,000	26,275		2,4%	2348,025	105,1%
▶ Plate 1	S Q1 Standard Curve Group	12,500	12,152		7,9%	1261,877	97,2%
▶ Plate 1	S Q1 Standard Curve Group	6,250	6,148		9,5%	660,449	98,4%
▶ Plate 1	S Q1 Standard Curve Group	3,125	3,126		4,2%	336,399	100,0%
▶ Plate 1	S Q1 Standard Curve Group	1,563	1,681		1,8%	179,945	107,6%
▶ Plate 1	S Q1 Standard Curve Group	0,781	0,747		3,8%	79,824	95,6%
▶ Plate 1	S Q1 Standard Curve Group	0,391	0,379		14,6%	41,137	97,1%
▶ Plate 1	S Q1 Standard Curve Group	0,195	0,223		8,5%	24,942	114,2%
▶ Plate 1	S Q1 Standard Curve Group	0,098	0,090		14,1%	11,388	92,0%
▶ Plate 1	S Q1 Standard Curve Group	0,000	0,003	✓ EXT	86,4%	2,921	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,049	✓ EXT	141,4%	6,073	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,740		9,2%	79,124	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,415		1,7%	44,828	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,078	▲ BLOQ	5,4%	10,190	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,175	✖ Defects	1,8%	19,986	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,067	▲ BLOQ	2,1%	9,091	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,525		25,2%	56,408	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	1,419		0,8%	151,714	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,142		10,6%	16,651	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,314		1,1%	34,314	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,628		1,9%	67,197	0,0%
▶ Plate 1	U Q1 Unknown Group	0,000	0,035	▲ BLOQ	20,9%	5,922	0,0%

Read-out parameters:

- ELISA – absorbance
- xMAP – MFI
- SMC – RE

Q1 Standard Curve Group	5PL
LoD : 0,005	
LLoQ: 0,098	
Slope: 96,226	
RSquared: 0,999	
S Q1 Standard Curve Group	
U Q1 Unknown Group	

xPRO SW features

- Familiar „windows“ interface
- Modern user friendly elements (drag and drop, undo/redo)
- Flexible workflow – pre and post-run modification (e.g. Plate maps)
- Reporting to .txt .scv .pdf
- LIMS compatible
- Enables 21 CFR Part 11 compliance
- Automated System-wide Suite of Instrument Self Tests(ASSIST) - A suite of automated calibrations, utilities and tests used to ascertain the operational state of the instrument

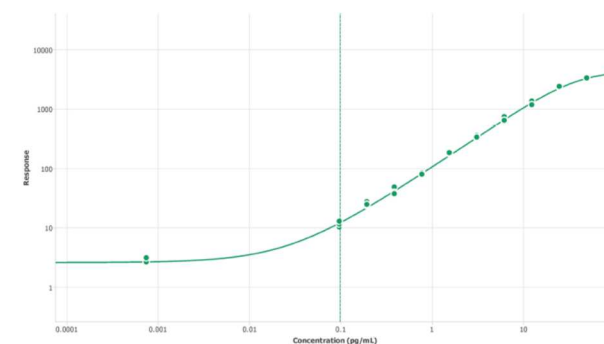
xPRO Final Report



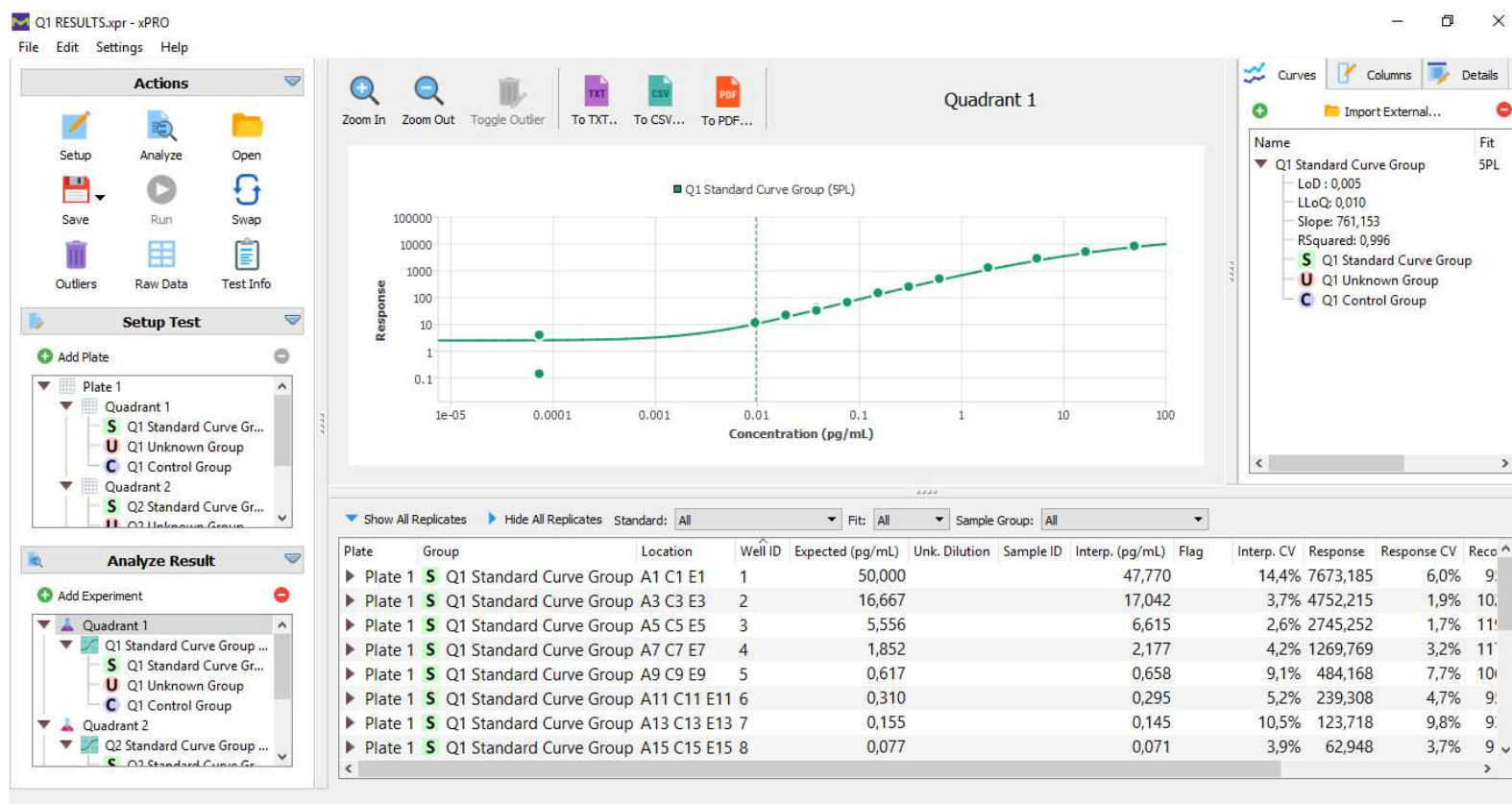
File Name:	2018-09-30 IL-22 READ1.xpr	Printed By:	M140572
Experiment Created By:	S230558	Printed On:	po srp 27 2018 10:26:26 Central Europe Daylight Time
Created On:	so ble 31 2018 1:17:32 Central Europe Daylight Time	Last Modified On:	po srp 27 2018 10:12:50 Central Europe Daylight Time
Last Modified By:	M140572	Executed By:	S230558
Experiment Name:	Quadrant 1	Instrument:	SMC001b
Executed On:	so ble 31 2018 1:57:29 Central Europe Daylight Time		

Standard Curves

Group	Fit	LoQ (pg/mL)	R Squared	Slope	LoD (pg/mL)
Q1 Standard Curve Group	SPL	0,098	0,999	96,226	0,005



xPRO SW features



SMC™ – off the shelf kits @ www.merckmillipore.com/SMCxPRO

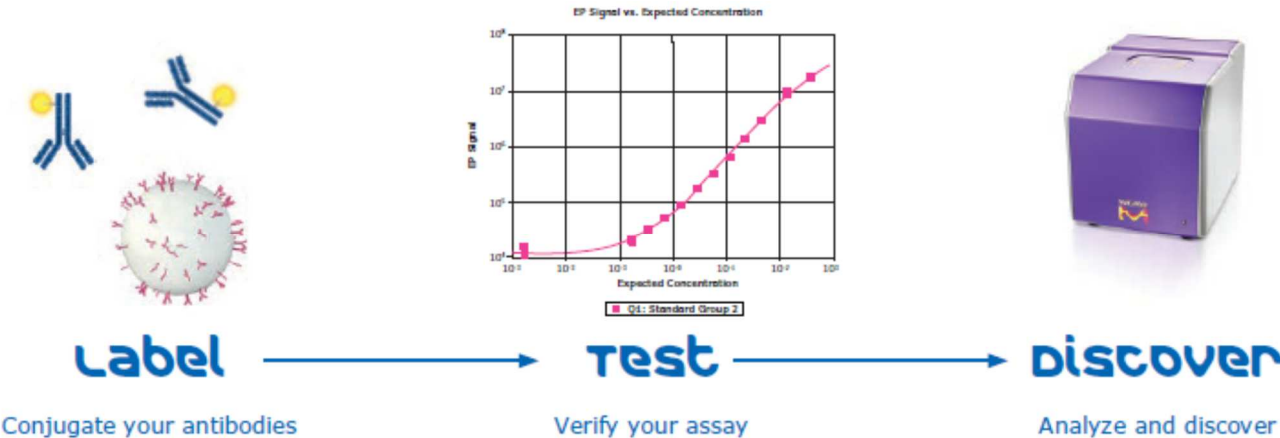
- SMC™ Human **IL-1 β** Immunoassay kit
- SMC™ Human **IL-4** Immunoassay kit
- SMC™ Human **IL-6** Immunoassay kit
- SMC™ Human **IL-13** Immunoassay kit
- SMC™ Human **IL-23** Immunoassay kit
- SMC™ Human **IL-22** Immunoassay kit
- SMC™ Human **TNF α** Immunoassay kit
- SMC™ Human **cTnI** Immunoassay kit
- SMC™ Human **Amyloid Beta 1-40** Immunoassay Kit
- SMC™ Human **Amyloid Beta 1-42** Immunoassay Kit
- SMC™ Human **Glucagon** Immunoassay kit



Growing menu of kits available to address areas of Cardiovascular & Neuro Research, Inflammation, Metabolism & Kidney Tox

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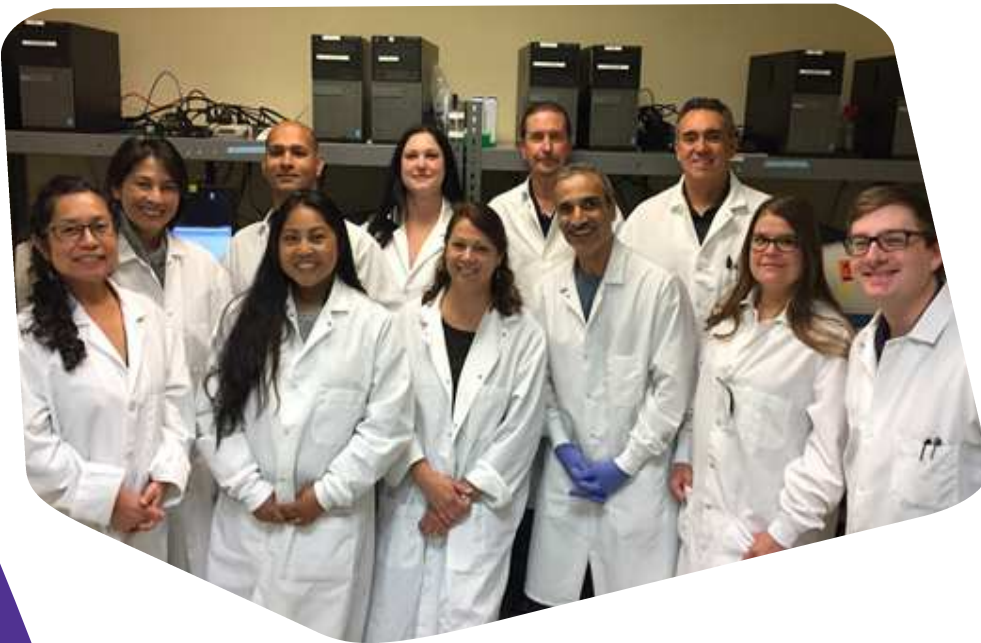
SMC™ – custom assay development @ your lab



	1	2	3	4	5	6	7	8	9	10	11	12		
	Analyte pg/mL												ug IgG per mg MP	ug MP per well
A	5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		12.5	5
B	0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL			10
C	5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL			5
D	0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL			10
E	5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		25	5
F	0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL			10
G	5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL		5 pg/mL			5
H	0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL		0 pg/mL			10
	50		100		500		1000						Det Ab	



SMC™ – custom assay development @ Merck



Prototype Assay Development

- Using proprietary or commercial reagents to evaluate likelihood of assay sensitivity & ability to quantify samples of interest

Full Assay Development

- Includes optimization with verification that the assay is specific, precise and identifies the appropriate dilution levels for operation

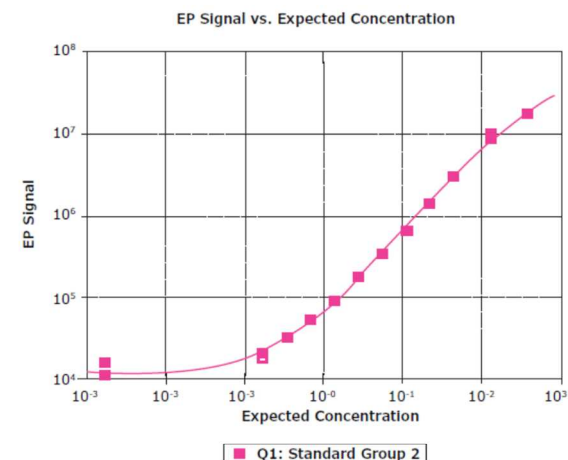
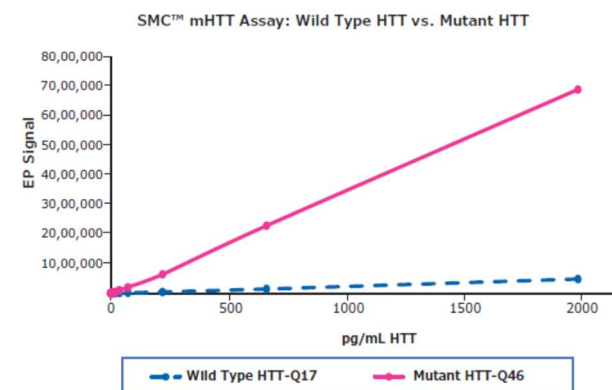
Conversion of a Milliplex Kit or ELISA to the SMC™ platform for improved sensitivity

Sample testing

Example: Sample Testing Services for Measuring Mutant Huntingtin Protein in Cerebrospinal Fluid (CSF) and Brain Homogenates/Lysates

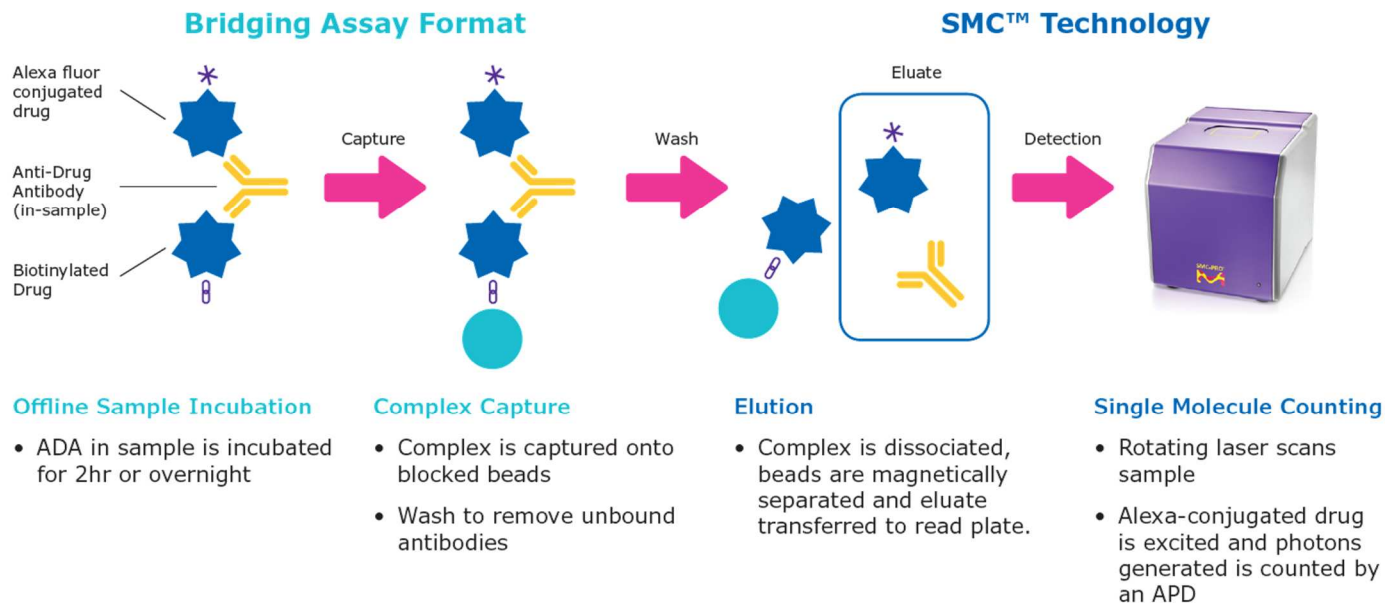
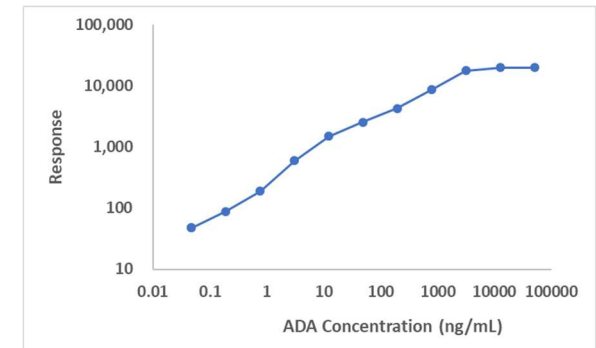
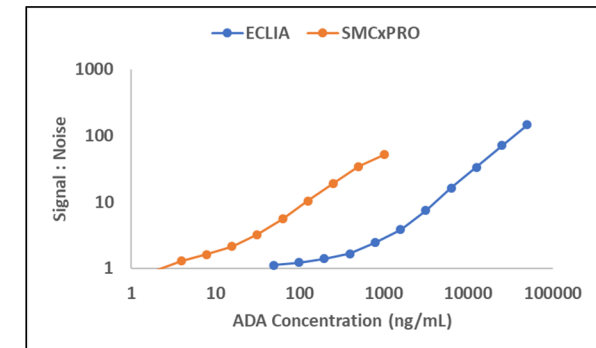
- Measurement down to 0.35 pg/ml for mHTT-Q45 (1-573)
- Measurement in Rat and Human Cerebrospinal Fluid (CSF) and cell lysates.
- Small sample volumes required (10 µL of CSF per well)
- **Assay is specific to Mutant Huntingtin (HTT) Protein (>36 poly-glutamine sequence repeats). Assay does not detect Wild type HTT (<23 poly-glutamine sequence repeats).**

HTT	Description	LLOQ (pg/mL)	MW (g/mol)	M	LLOQ (fM)	Binding Affinity
Q17	(1-3144) full length	> 2000	352,178	NA	NA	Very Low
Q46	(1-3144) full length	2.31	355,894	6.49.E-15	6.49	High



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Immunogenicity testing and assay development



The Federal Drug Administration (FDA) and pharmaceutical experts in the area of immunogenicity testing have recently published guidelines for the design and optimization of immunoassays used in the detection of antibodies against biopharmaceutical drug products in patient samples in the absence of drug and more importantly, when drug is present. **FDA recommends that screening and confirmatory IgG and IgM assays achieve a sensitivity of 100–500 ng/mL (FDA Guidance 2016).**

What is in single molecule counting for you?

Improved sample preparation and workflow

Use of magnetic particles, plate transfer and small elution volume

SMC™ technology (HW/SW)

Background noise reduction by looking at individual detection molecules in a very small interrogation space and removing the detector from the assay plate



Large dynamic range

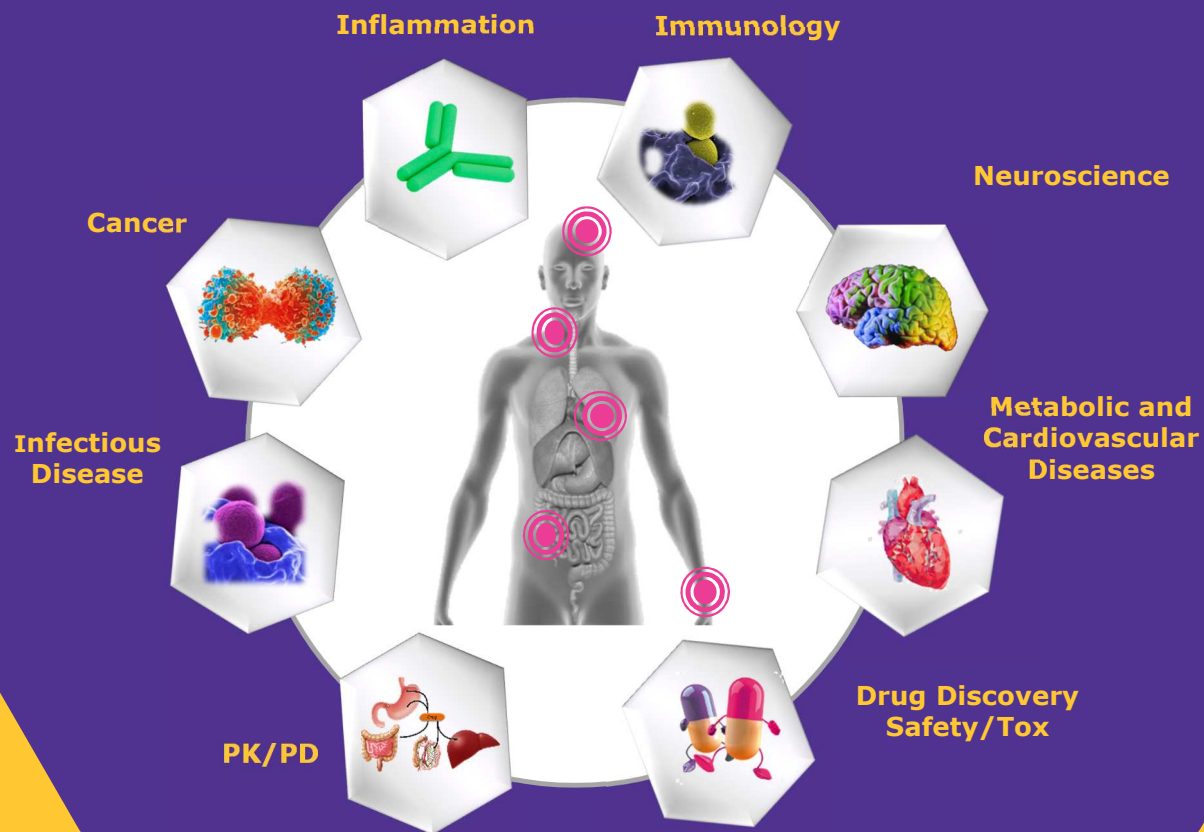
Robust assays with excellent precision with 5log dynamic range capturing protein biomarker concentrations down to fg/ml

Flexibility of assay development

Possibility to develop custom assays using kits specifically developed for that (including „bridging“ assays for immunogenicity profiling)

**Every
single
Molecule
counts ...**

**objective:
to have all samples quantifiable**



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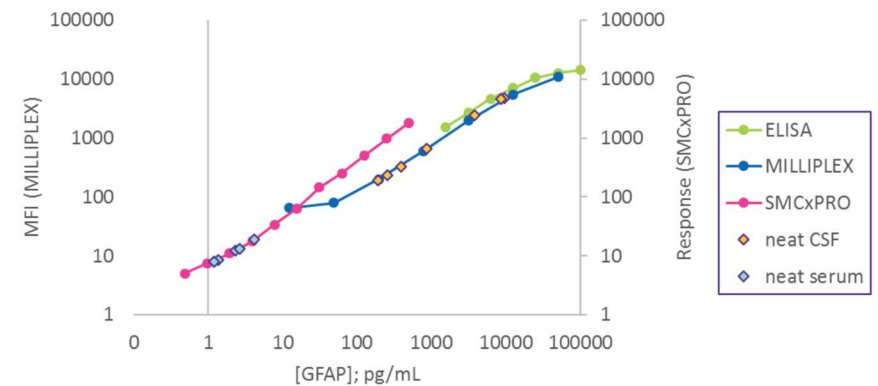
comparative sensitivity

of ELISA, MILLIPLEX, and SMC for measurement of GFAP in biological samples from TBI

Most neat CSF samples fall on the lower end of the MILLIPLEX GFAP assay curve and are undetectable by ELISA. While the MILLIPLEX GFAP assay is proficient at measuring CSF samples, plasma and serum samples, where GFAP is much less abundant, were largely undetectable.

By transitioning our GFAP assay onto the SMC platform, we achieved sufficient sensitivity to measure GFAP also in serum and plasma with low endogenous levels of this biomarker...

these kits use the same antibody pair



Assay Platform	Dynamic Range
ELISA	1563 – 100,000 pg/mL
MILLIPLEX	12 - 50,000 pg/mL
SMC	0.5 – 500 pg/mL



Thank you for your
attention

Stanislav An

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