# Application of LC-MS for Characterization and Bioanalysis of Therapeutic Antibodies

Kevin Bateman
Distinguished Scientist
Merck Research Laboratories

Benno Ingelse
Director Bioanalysis
Quintiles, Bioanalytical and ADME labs

EBF 2014

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# Acknowledgements


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- Yaoli Song

# Method Development for LC-MRM-MS Based Monoclonal Antibody Quantitation

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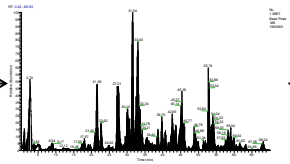
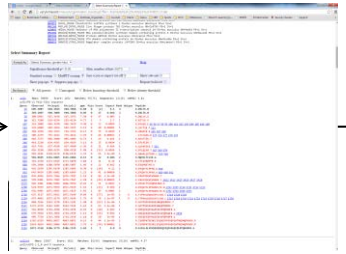
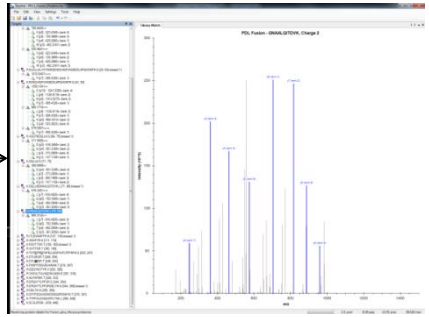
# mAb/Protein Assay Development

Nanoacquity Orbi Velos







~ 2 days

TQS w/ iKey

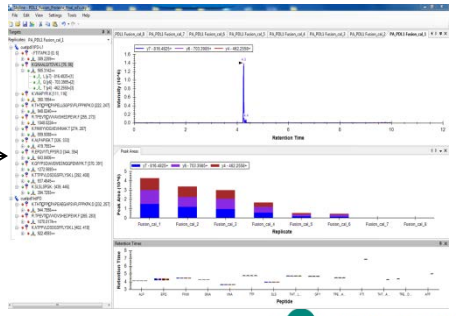
→ Peptide selection → CE Opt → Matrix Check →

≤10 min Methods

~ 1 day

AssayMAP

→ Std Curve → Immuno Capture → Digestion →



~ 2 days

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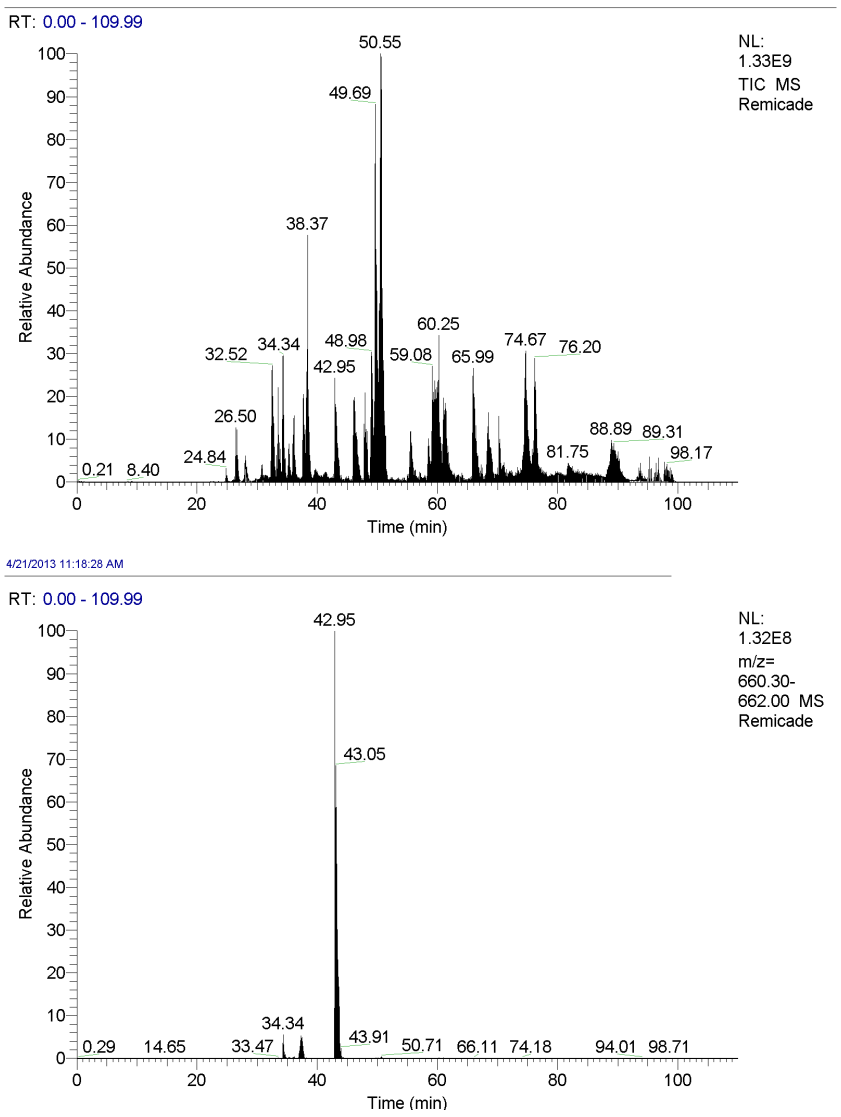
# HRMS peptide mapping for mAb




TIC, 100 min gradient, hundreds of peptides

One of the target peptides: STSGGTAALGCLVK

Waters nanoAcquity Velos-Orbitrap, 1 ul injection of 0.5 ug/ul digested MAb

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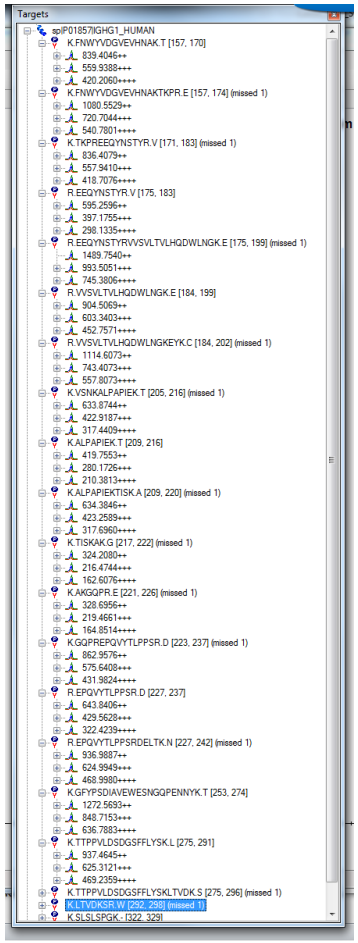
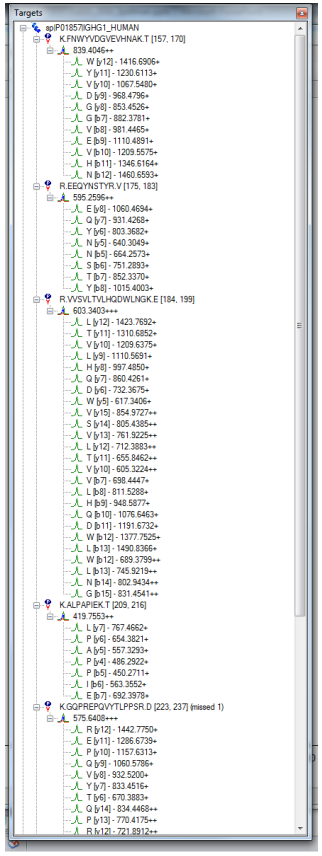
# Method Refinement and Optimization with Skyline

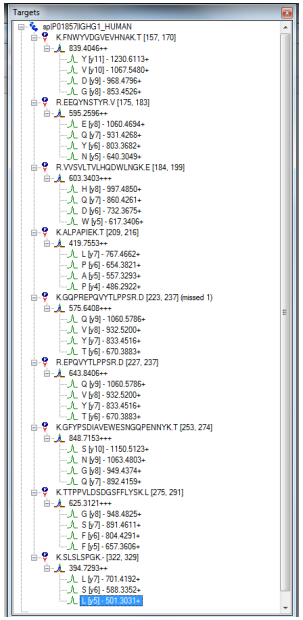
Start with long list of peptides:
Multiple Charge States
Many transitions




Refine to best peptide signals




Further Refine to best transitions




CE optimization



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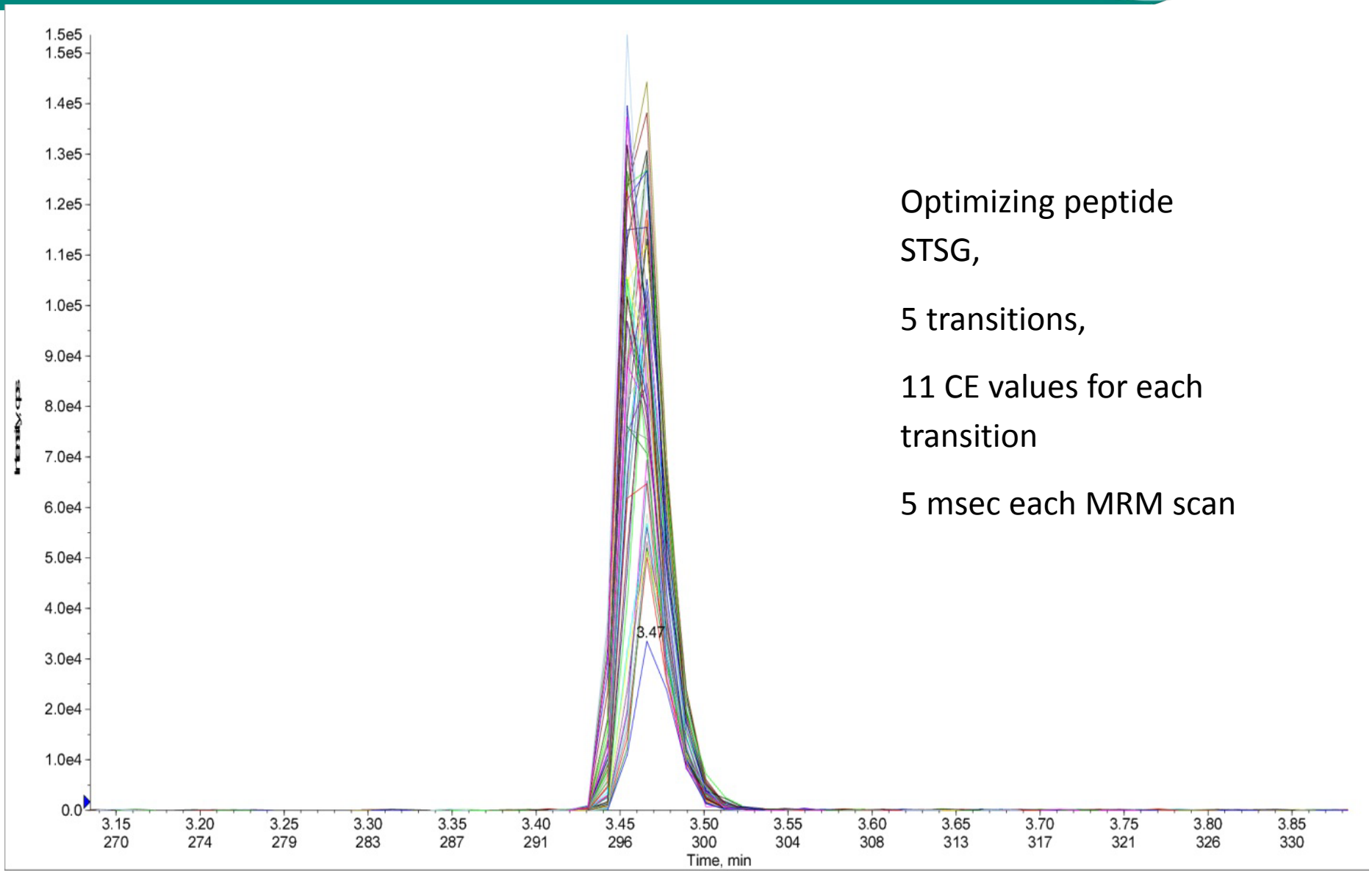
# Example CE Optimization



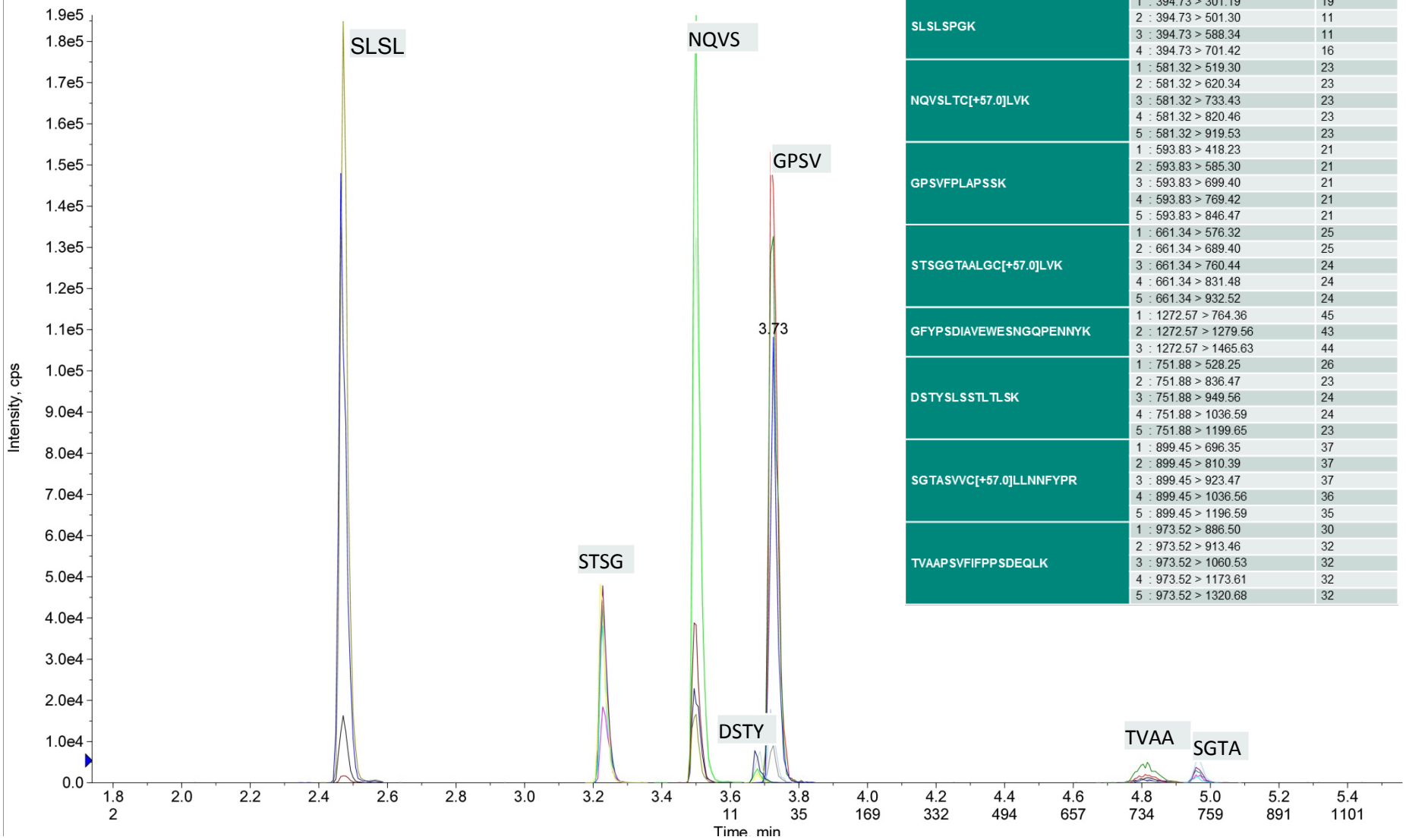

Optimizing peptide STSG,

5 transitions,

11 CE values for each transition

5 msec each MRM scan

# The optimized, scheduled MRM for selected peptides




| Peptide | Transition | Col.Energy |
|---|---|---|
| SLSLSPGK | 1 : 394.73 > 301.19 | 19 |
| | 2 : 394.73 > 501.30 | 11 |
| | 3 : 394.73 > 588.34 | 11 |
| | 4 : 394.73 > 701.42 | 16 |
| NQVSLTC[+57.0]LVK | 1 : 581.32 > 519.30 | 23 |
| | 2 : 581.32 > 620.34 | 23 |
| | 3 : 581.32 > 733.43 | 23 |
| | 4 : 581.32 > 820.46 | 23 |
| | 5 : 581.32 > 919.53 | 23 |
| GPSVFPLAPSSK | 1 : 593.83 > 418.23 | 21 |
| | 2 : 593.83 > 585.30 | 21 |
| | 3 : 593.83 > 699.40 | 21 |
| | 4 : 593.83 > 769.42 | 21 |
| | 5 : 593.83 > 846.47 | 21 |
| STSGGTAALGC[+57.0]LVK | 1 : 661.34 > 576.32 | 25 |
| | 2 : 661.34 > 689.40 | 25 |
| | 3 : 661.34 > 760.44 | 24 |
| | 4 : 661.34 > 831.48 | 24 |
| | 5 : 661.34 > 932.52 | 24 |
| GFYPSDIAVEWESNGQPENNYK | 1 : 1272.57 > 764.36 | 45 |
| | 2 : 1272.57 > 1279.56 | 43 |
| | 3 : 1272.57 > 1465.63 | 44 |
| DSTYSLSSTLTLSK | 1 : 751.88 > 528.25 | 26 |
| | 2 : 751.88 > 836.47 | 23 |
| | 3 : 751.88 > 949.56 | 24 |
| | 4 : 751.88 > 1036.59 | 24 |
| | 5 : 751.88 > 1199.65 | 23 |
| SGTASVVC[+57.0]LLNNFYPR | 1 : 899.45 > 696.35 | 37 |
| | 2 : 899.45 > 810.39 | 37 |
| | 3 : 899.45 > 923.47 | 37 |
| | 4 : 899.45 > 1036.56 | 36 |
| | 5 : 899.45 > 1196.59 | 35 |
| TVAAPSVFIFPPSDEQLK | 1 : 973.52 > 886.50 | 30 |
| | 2 : 973.52 > 913.46 | 32 |
| | 3 : 973.52 > 1060.53 | 32 |
| | 4 : 973.52 > 1173.61 | 32 |
| | 5 : 973.52 > 1320.68 | 32 |

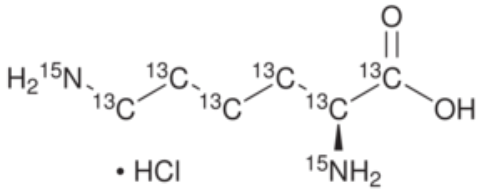
# Internal standards for MS quantitation

- Typically incorporated heavy amino acid labeled by C13 or N15

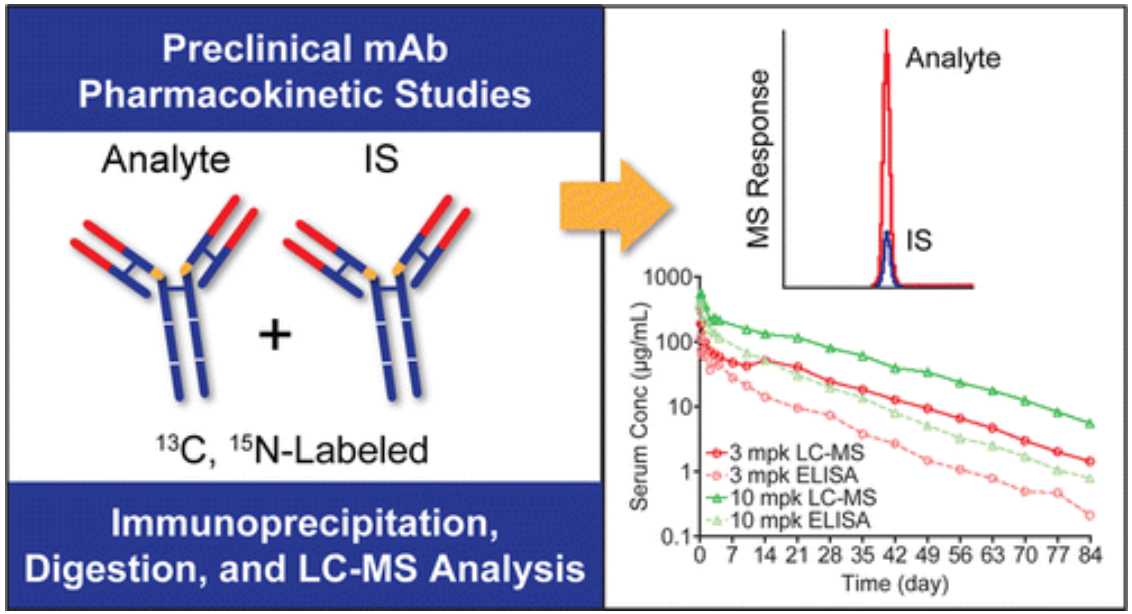
- Correcting for variations in :
  1. Sample preparation
  2. LC performance
  3. Ionization efficiency
  4. MS response

# Our internal standard is the intact mAb molecule

Sigma SILUmAb: full length MAb with heavy K and R




A heavy Lys, with +8 Da mass




Zhang, et al., Anal Chem. 2014 Aug 14. [Epub ahead of print]

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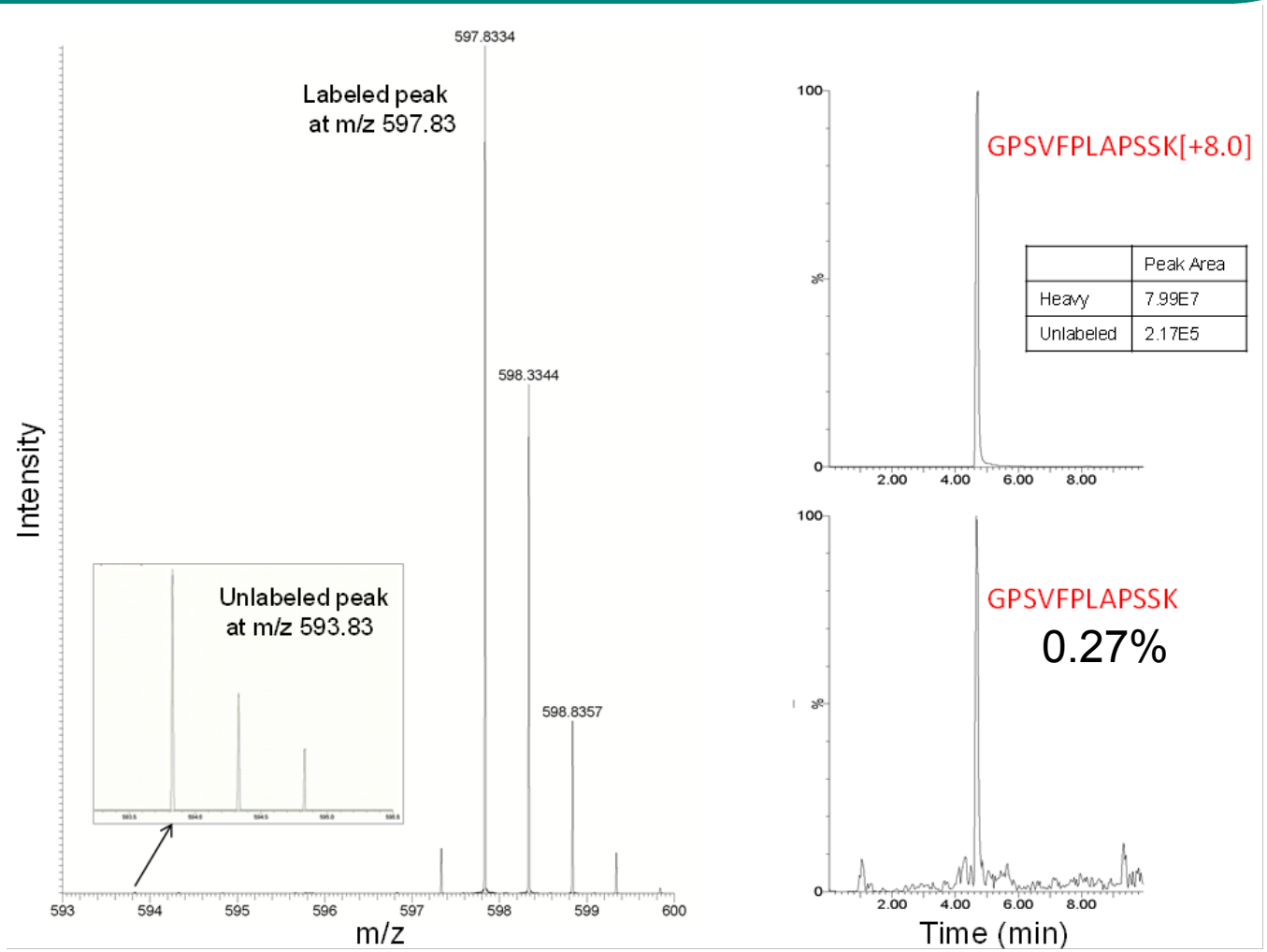
# SILUmAb generates identical surrogate peptides to target mAbs




The mass spectrum (left) and MRM quantitation (right) for the selected peptide GPSVFPLAPSSK in SILUMAb. The signal of the labeled form (+8 Da) is more than 100 fold higher than the unlabeled form. The low abundance of the unlabeled peak indicates negligible interference from the internal standard.

Zhang, et al., Anal Chem. 2014 Aug 14. [Epub ahead of print]



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# Evaluation of Generic Sample Preparation Approaches*

- 1. Pellet digestion of total plasma proteins

  Detected 5-10 ug/ml

- 2. Protein A/G (columns) pull-down of IgGs

  Detected 40 ug/ml

- 3. Anti-human Fc antibody pull-down of target IgG1

  Detected <0.25 ug/ml

*50 ul plasma

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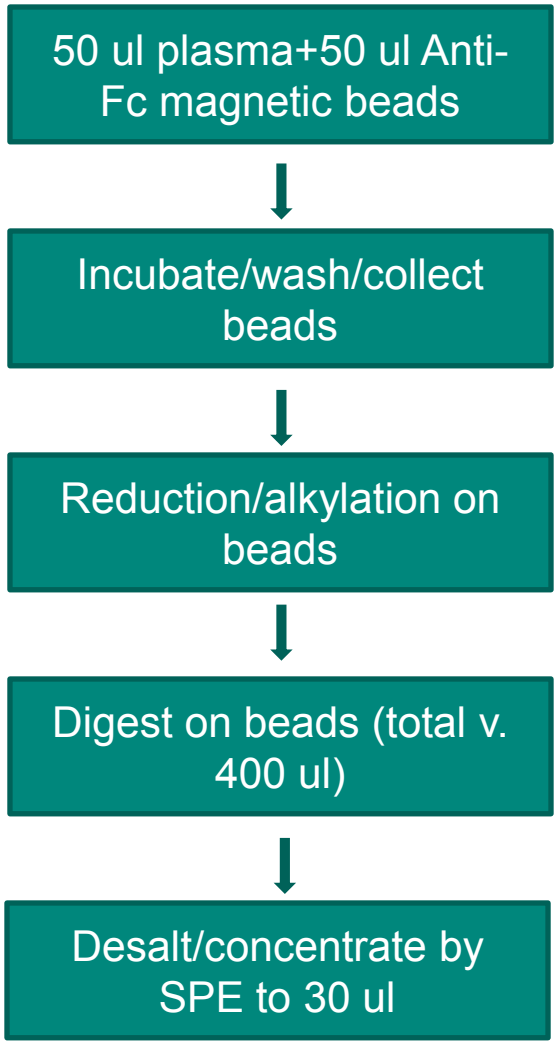
# Anti-human Fc antibody pull-down of target IgG1




MagneZoom™ Goat Anti-Human IgG (FC) Kit

Oasis HLB 96-well µElution Plate, 2 mg Sorbent per Well, 30 µm Particle Size

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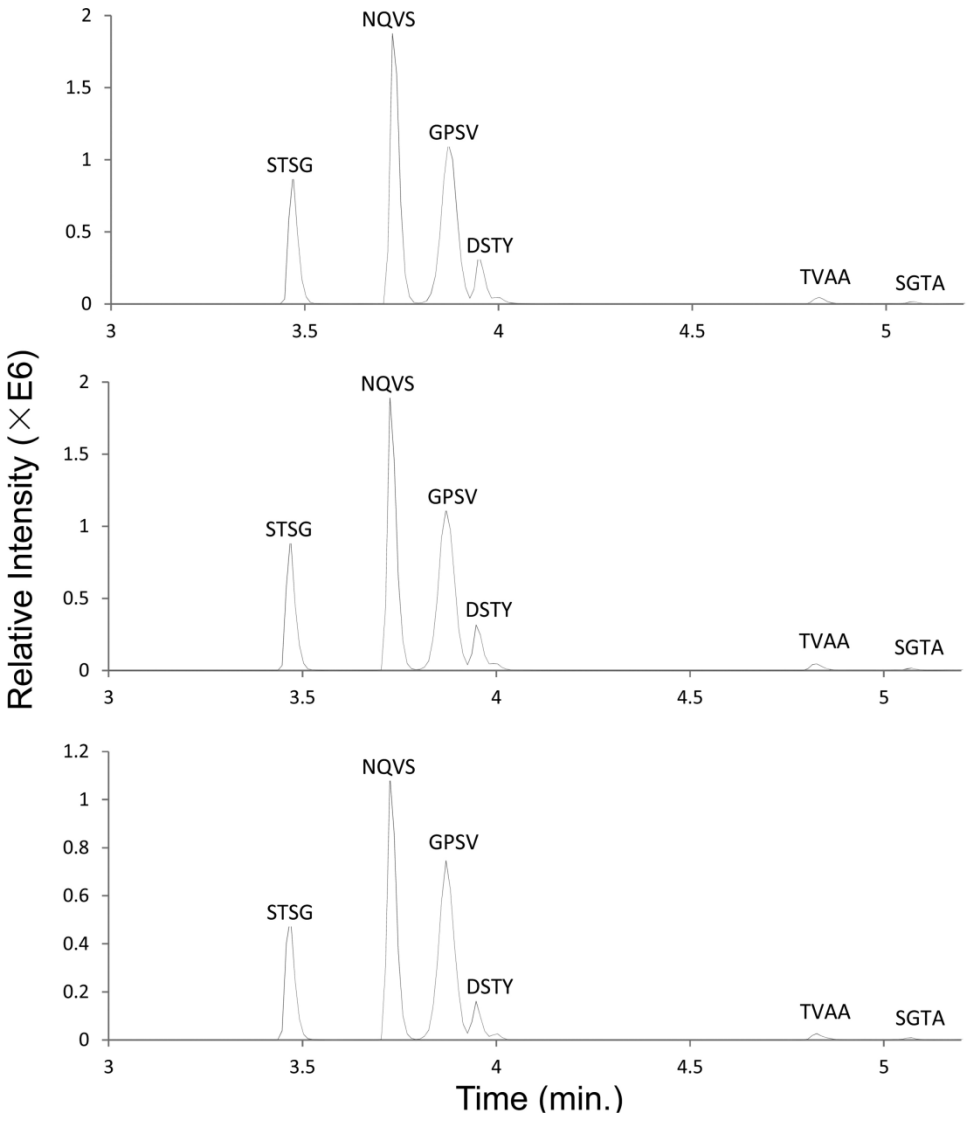
# Selected Peptides Represent Reliable Surrogate Measures Across Different Antibodies




aIL-10

aIL-17

Remicade

MRM experiment for three different mAbs. The total ion chromatogram of the monitored peptides indicates that the peptides can reproducibly represent different antibodies.



Zhang, et al., Anal Chem. 2014 Aug 14. [Epub ahead of print]

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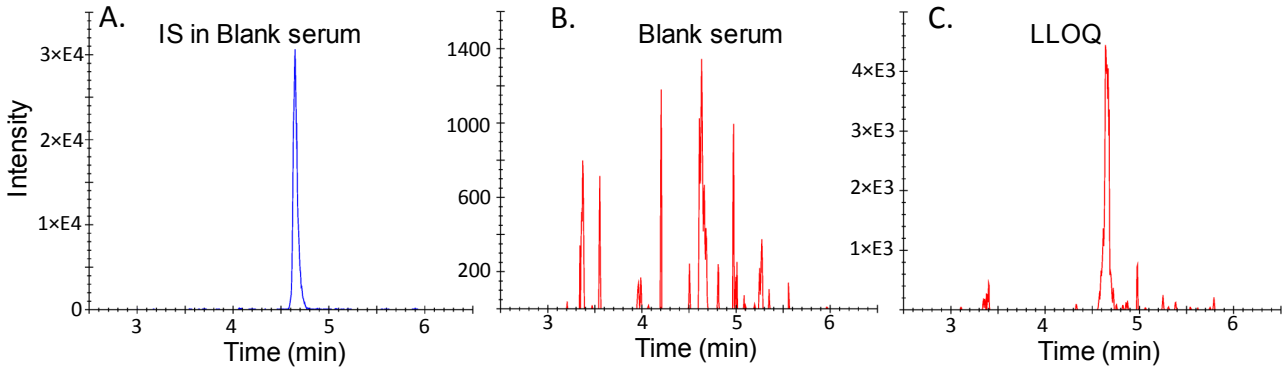
# Interference peaks identified from different animal plasma

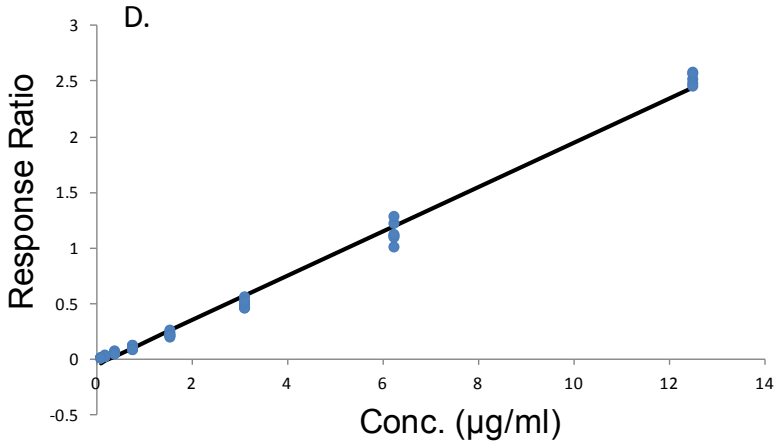
| MRM transition ID | Q1 m/z | Q3 m/z | Mouse | Rat | Guinea pig | Rabbit | Beagle | Monkey |
|---|---|---|---|---|---|---|---|---|
| HC.GPSVFPLAPSSK.+2b6 | 593.83 | 585.30 | | ● | ● | | ● | ● |
| HC.GPSVFPLAPSSK.+2b8 | 593.83 | 769.42 | | | | | ● | ● |
| HC.GPSVFPLAPSSK.+2y4 | 593.83 | 418.23 | | | | | ● | ● |
| HC.GPSVFPLAPSSK.+2y7 | 593.83 | 699.40 | | | | | ● | ● |
| HC.GPSVFPLAPSSK.+2y8 | 593.83 | 846.47 | | | | | ● | ● |
| HC.NQVSLTC[+57_0]LVK.+2y4 | 552.81 | 462.27 | | | | ● | ● | ● |
| HC.NQVSLTC[+57_0]LVK.+2y5 | 552.81 | 563.32 | | | ● | ● | ● | ● |
| HC.NQVSLTC[+57_0]LVK.+2y6 | 552.81 | 676.41 | | | | ● | ● | ● |
| HC.NQVSLTC[+57_0]LVK.+2y7 | 552.81 | 763.44 | | | ● | ● | ● | ● |
| HC.NQVSLTC[+57_0]LVK.+2y8 | 552.81 | 862.51 | | | | ● | ● | ● |
| HC.SLSLSPGK.+2y3 | 394.73 | 301.22 | | ● | | ● | | ● |
| HC.SLSLSPGK.+2y5 | 394.73 | 501.29 | | | | | ● | ● |
| HC.SLSLSPGK.+2y6 | 394.73 | 588.32 | ● | | | | | ● |
| HC.SLSLSPGK.+2y7 | 394.73 | 701.39 | | ● | ● | ● | ● | ● |
| HC.STSGGTAALGC[+57_0]LVK.+2y5 | 632.83 | 519.30 | | | ● | | | ● |
| HC.STSGGTAALGC[+57_0]LVK.+2y6 | 632.83 | 632.38 | ● | ● | | ● | ● | ● |
| HC.STSGGTAALGC[+57_0]LVK.+2y7 | 632.83 | 703.42 | | | ● | | | |
| HC.STSGGTAALGC[+57_0]LVK.+2y8 | 632.83 | 774.45 | | | | | ● | |
| HC.STSGGTAALGC[+57_0]LVK.+2y9 | 632.83 | 875.50 | | ● | ● | | ● | |
| HC.GFYPSDIAVEWESNGQPENNYK.+2y12 | 1272.57 | 1465.63 | | | | | | |
| HC.GFYPSDIAVEWESNGQPENNYK.+2y11 | 1272.57 | 129.55 | | | | | | |
| HC.GFYPSDIAVEWESNGQPENNYK.+2y16 | 1272.57 | 764.36 | | | | | | |
| LC.DSTYSLSSTLTLSK.+2y10 | 751.88 | 1036.59 | | | | ● | | |
| LC.DSTYSLSSTLTLSK.+2y11 | 751.88 | 1199.65 | | | | ● | | |
| LC.DSTYSLSSTLTLSK.+2y8 | 751.88 | 836.47 | | | | ● | | |
| LC.DSTYSLSSTLTLSK.+2y9 | 751.88 | 949.56 | | | | ● | | |
| LC.SGTASVVC[+57_0]LLNNFYPR.+2y5 | 870.94 | 696.35 | | | | | | |
| LC.SGTASVVC[+57_0]LLNNFYPR.+2y6 | 870.94 | 810.39 | | | | | ● | |
| LC.SGTASVVC[+57_0]LLNNFYPR.+2y7 | 870.94 | 923.47 | | | | | | |
| LC.SGTASVVC[+57_0]LLNNFYPR.+2y8 | 870.94 | 1036.56 | | | | | | |
| LC.SGTASVVC[+57_0]LLNNFYPR.+2y9 | 870.94 | 1139.57 | | | | | | |
| LC.TVAAPSVFIFPPSDEQLK.+2b9 | 973.52 | 886.50 | | | | | | |
| LC.TVAAPSVFIFPPSDEQLK.+2y10 | 973.52 | 1173.62 | | | | | | |
| LC.TVAAPSVFIFPPSDEQLK.+2y11 | 973.52 | 1320.68 | | | | | | |
| LC.TVAAPSVFIFPPSDEQLK.+2y8 | 973.52 | 913.46 | | | | | | |
| LC.TVAAPSVFIFPPSDEQLK.+2y9 | 973.52 | 1060.53 | | | | | | |

'HC' means the peptide is from the IgG heavy chain and 'LC' means the peptide is from the IgG light chain. '•' indicates an identified interference peak for the specific transition.

Zhang, et al., Anal Chem. 2014 Aug 14. [Epub ahead of print]

# Performance characteristics of the LC-MRM-MS method




| % CV | | |
|---|---|---|
| HQC | MQC | LQC |
| 1.3 | 8.7 | 18.0 |

| % bias | | |
|---|---|---|
| HQC | MQC | LQC |
| 8.6 | 8.5 | -5.53 |

A) IS signal from single blank, B) Analyte signal from single blank, C) LLOQ of analyte, D) Standard curve from 6 independent replicates. Percent CV and Bias are shown for low (0.5 ug/mLµg/mL), medium (5 ug/mLµg/mL) and high (20 ug/mLµg/mL) QC (n=3) samples.

Zhang, et al., Anal Chem. 2014 Aug 14. [Epub ahead of print]



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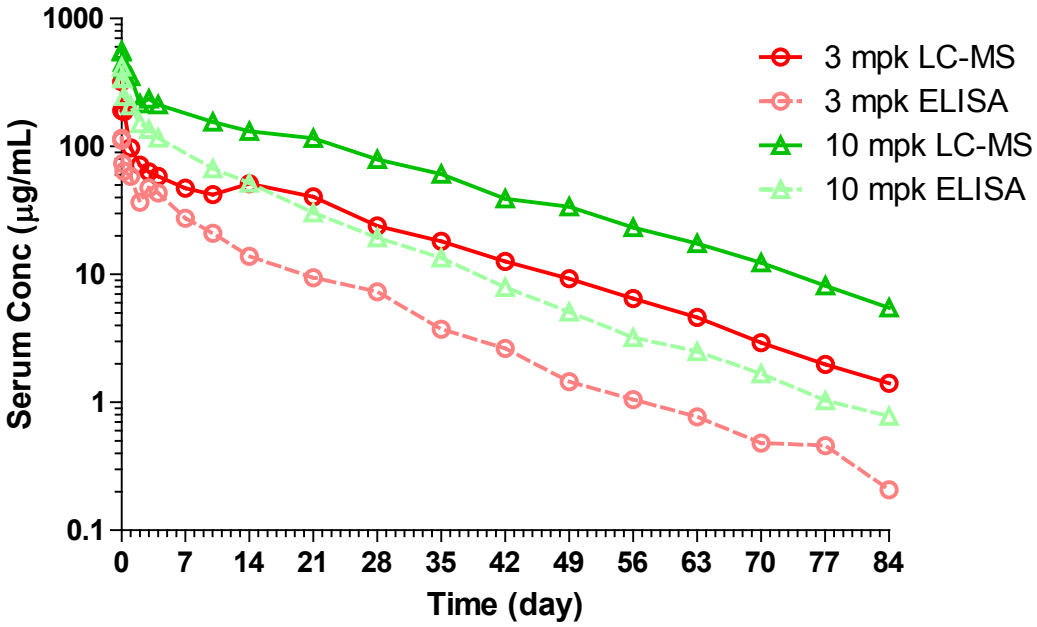
# LC-MS vs. LBA based mAb quantitation




**The comparison of LBA data and LC-MS data for two doses of aPCSK9 mAb in Cyno Monkey**

- **Why are they different?**
- **How do we interpret the data?**
- **Which one is right?**

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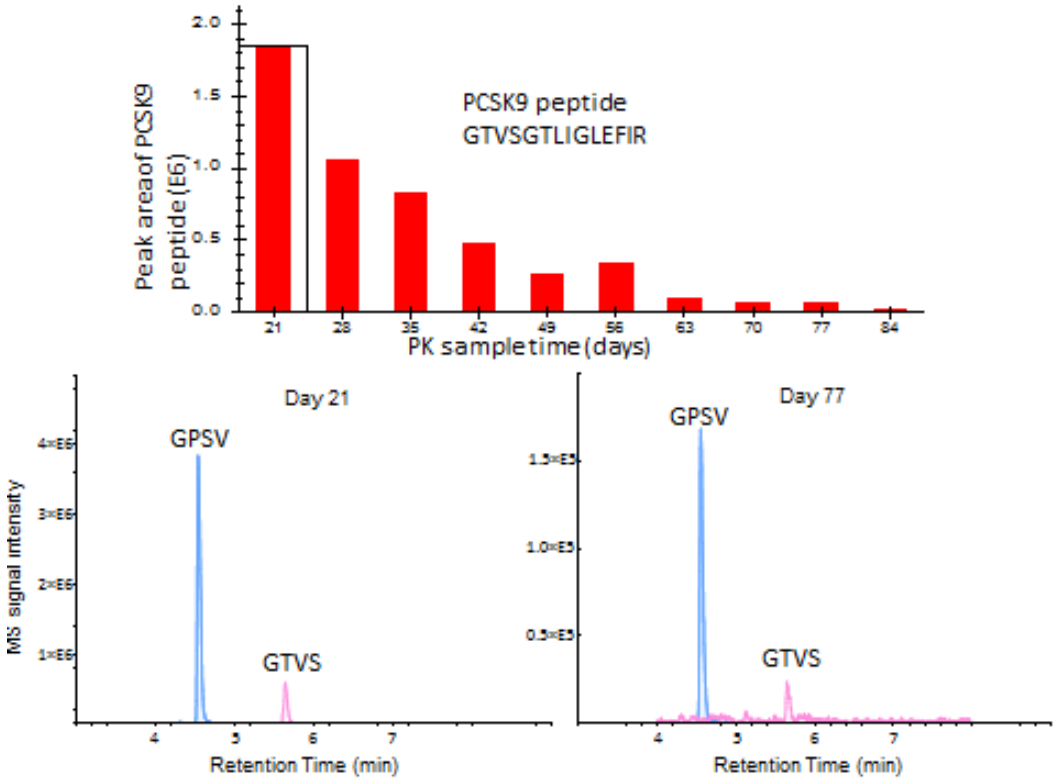
# LC-MS vs. LBA based mAb quantitation




**The PCSK9 ligand is present in the anti-Fc immuno-captured aPCSK9 samples**

- **We can detect and quantify both the drug and the ligand target at the same time in the same sample.**

**Generic Automated Method for Liquid Chromatography–Multiple Reaction Monitoring Mass Spectrometry Based Monoclonal Antibody Quantitation for Preclinical Pharmacokinetic Studies**

Qian Zhang,[†] Daniel S. Spellman,[†] Yaoli Song,[‡] Bernard Choi,[§] Nathan G. Hatcher,[†] Daniela Tomazela,[‡] Maribel Beaumont,[‡] Mohammad Tabrizifard,[‡] Deepa Prabhavalkar,[‡] Wolfgang Seghezzi,[‡] Jane Harrelson,[†] and Kevin P. Bateman*[,†]

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# Increased Throughput for mAb Quantitation:

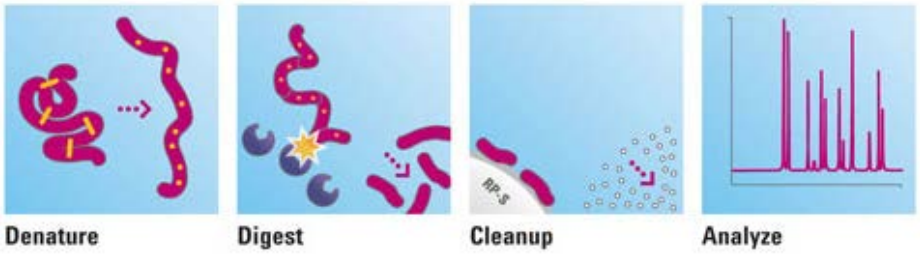
## Agilent AssayMap Bravo Platform and Transfer of LC-MS/MS assay to Acquity UPLC/TQS MS Platform

# Agilent AssayMAP

The Agilent AssayMAP technology is an open access, walkaway automation solution specifically designed for biomolecule sample preparation

Protein A-based Affinity Enrichment


In-solution Enzymatic Digestion

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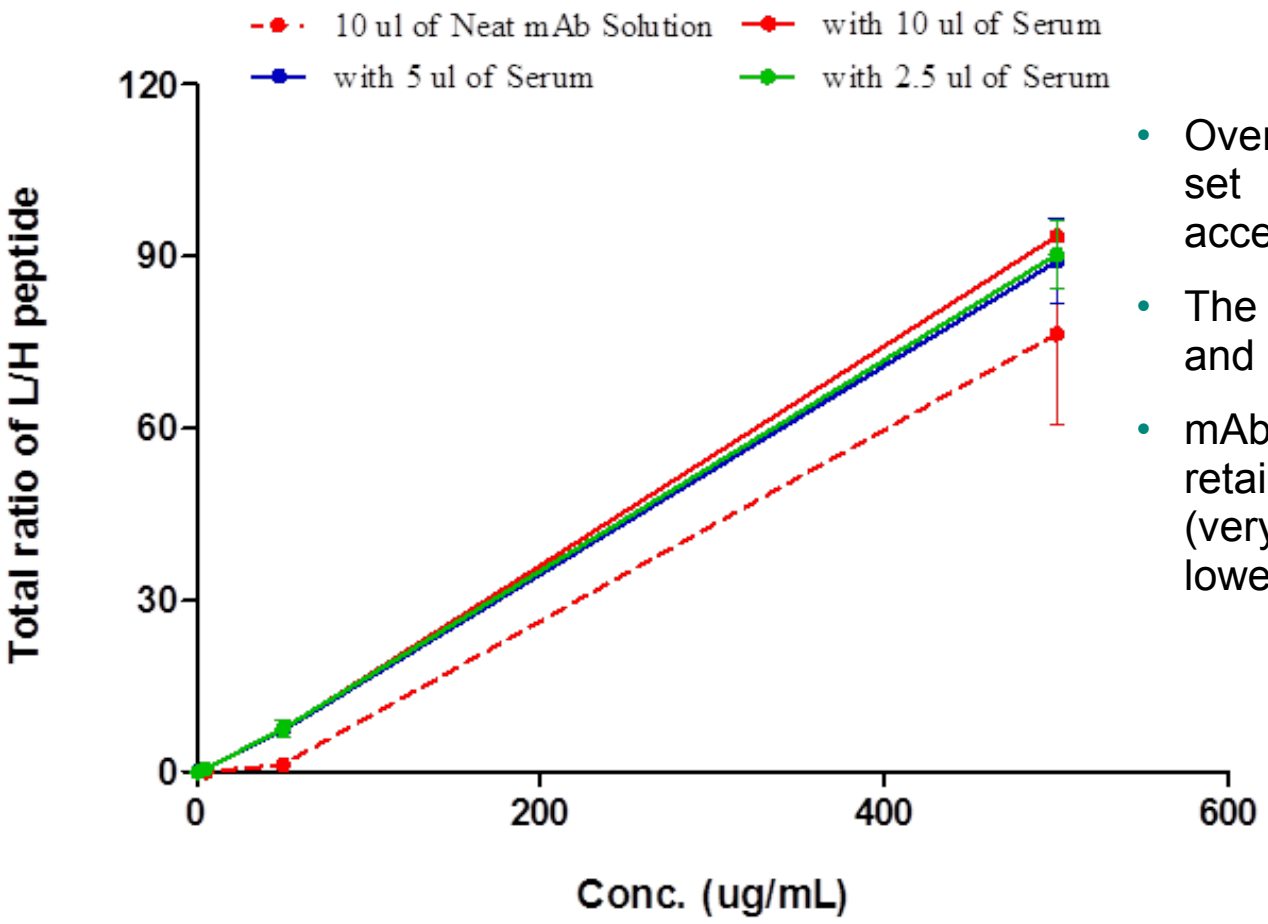
# BRAVO Evaluation

- Objective: To test the automation capability for preclinical mAb PK.
  - Test the maximum and minimum loading for mAb, biomatrices, mAb in matrix
  - Reproduce preclinical mAb PK protocol on automation platform and test performance.

# Experimental Detail of Evaluation

- Mouse serum filtered with 0.2 um filter before used
- Internal Standard peptides – 2 ul of  isotopic labeled of targeted peptide
- Concentration of mAb and Matrix :
  - 4 levels of mAb Concentration: 500, 50, 5, and 0.5 ug/mL (10 ul total volume/per sample)
  - 3 levels of Matrix: 10, 5, 2.5 ul/ per sample (final total sample volume brought to 20uL)
  - 4 levels of neat mAb solution were used as control
  - All samples run in triplicate
- Prepared samples with AssayMap Ab-purification and in solution digestion protocols
- Analyzed via Waters TQ-S instrument with LC/MS-MS (MRM) method
- Data Process with Skyline software by sum of peak area of every transition from targeted peptides

# Standard Curve of Targeted peptide in Mouse Serum




- Overall, the variability from this set of samples is within acceptable range ($R^2$ >95).
- The linearity of loading of sample and matrix were good
- mAb alone appears to be retained by protein A column (very low signal or absent at lowest concentration)



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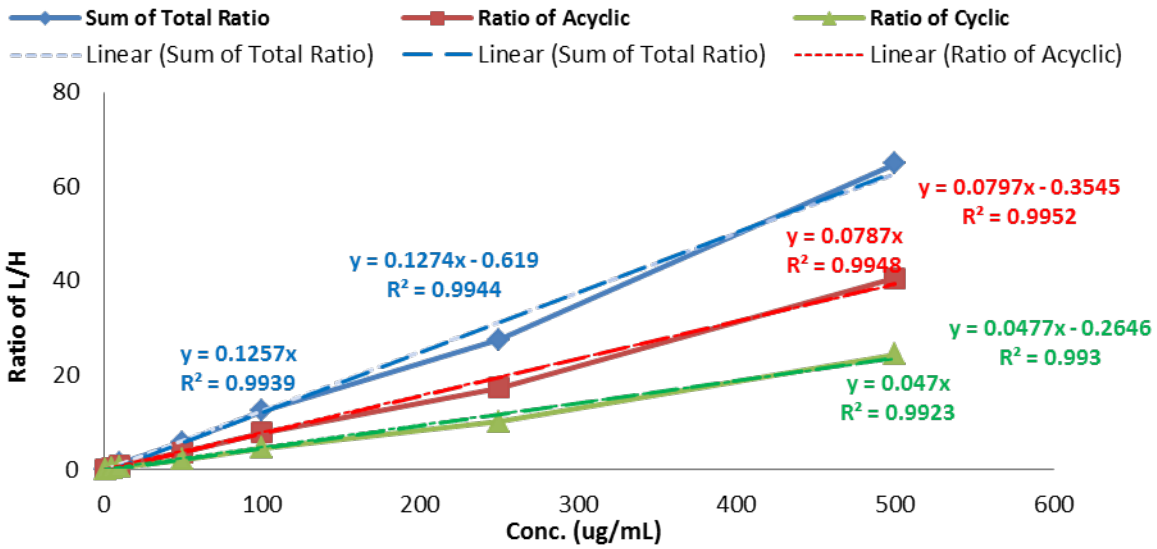
# Standard Curve of Targeted peptide in Mouse Serum: High Throughput vs. Standard Protocol

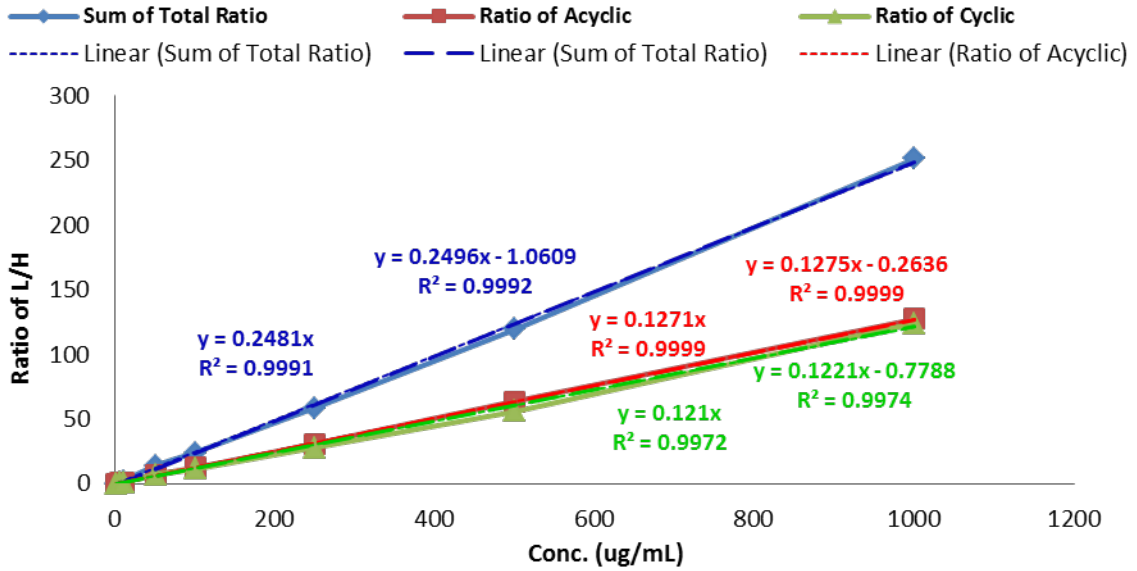
## Standard Protocol

- LLOQ @ 17pM of starting protein
- 10 uL serum
- 8 uL injection

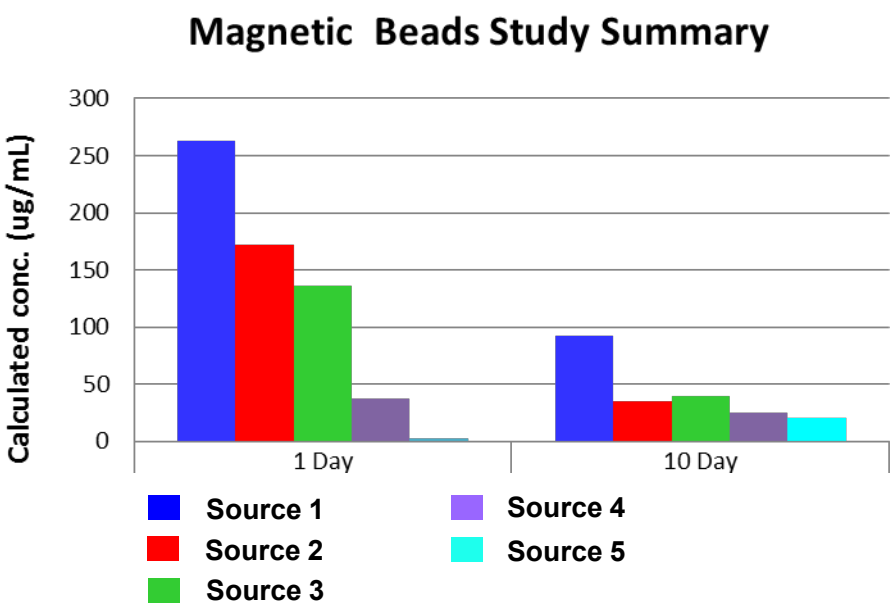



## High Throughput Protocol

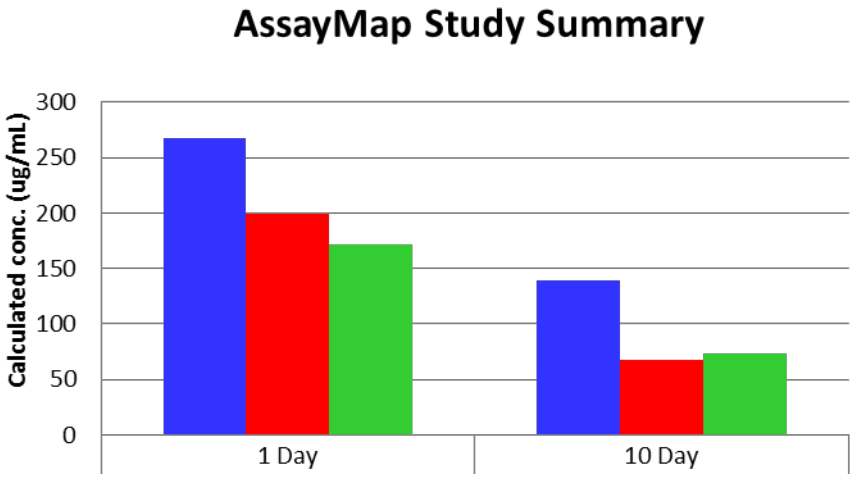
- LLOQ @ <3.4 pM of starting protein
- 5 µl serum
- 5 uL injection
- Improved linear range, and response



# The Calculated Concentration of Targeted Peptide: High Throughput vs. Standard Protocol




Calculated concentration is within the linear range (1-500 ug/mL);
PBS control might have some interfere




Calculated concentration is within the linear range (1-1000 ug/mL)

Equivalent data generated by either method



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# Summary Comparison for Standard vs. HT

| | Original Method | | HT Method | |
|---|---|---|---|---|
| | Method | Cost | Method | Cost |
| tested serum volume | 20 ul | | 10 - 2.5 ul | |
| Linear range | 2.44-625 ug/mL | | 1-1000 ug/mL | |
| IP- pull down | Magnetic beads | ~$33/per sample | Protein A Tips | ~$4/per sample |
| IP-Instrument | Manual | priceless | Agilent Bravo | $~100,000 |
| maximum sample # /per process | 32 individual samples | | 96 well plate | |
| Time (hrs) /per process | ~2-4 hrs | *sample tranfer | ~2-3 hrs | No transfer for next step |
| Protease Digestion | trypsin | ~$ 7 | trypsin | ~$ 7 |
| Protease-Instrument | Manual | | Agilent Bravo | |
| maximum sample # /per process | 32 individual samples | | 4 x 96 well plates | |
| Time (hrs) /per process | ~7-9 hrs | ** continuous protease digestion | ~1-2 hrs before and after digest | **overnight protease digestion |
| Internal standard volume (ul) | 15 | | 2 | |
| Final volume (ul) | ~ 90-100 | | ~90-100 | |
| Instrument time for LC/MS/MS | 20 min/per sample | | 7 min/per sample | |
| Total Assay time for 96 wells | ~ 2 weeks | | 2.5 days | |

# Increased Throughput for mAb Characterization:
## Methionine Oxidation by LC-MRM vs. LC-UV-MS

# Chromatogram of Peptide maping (UV)

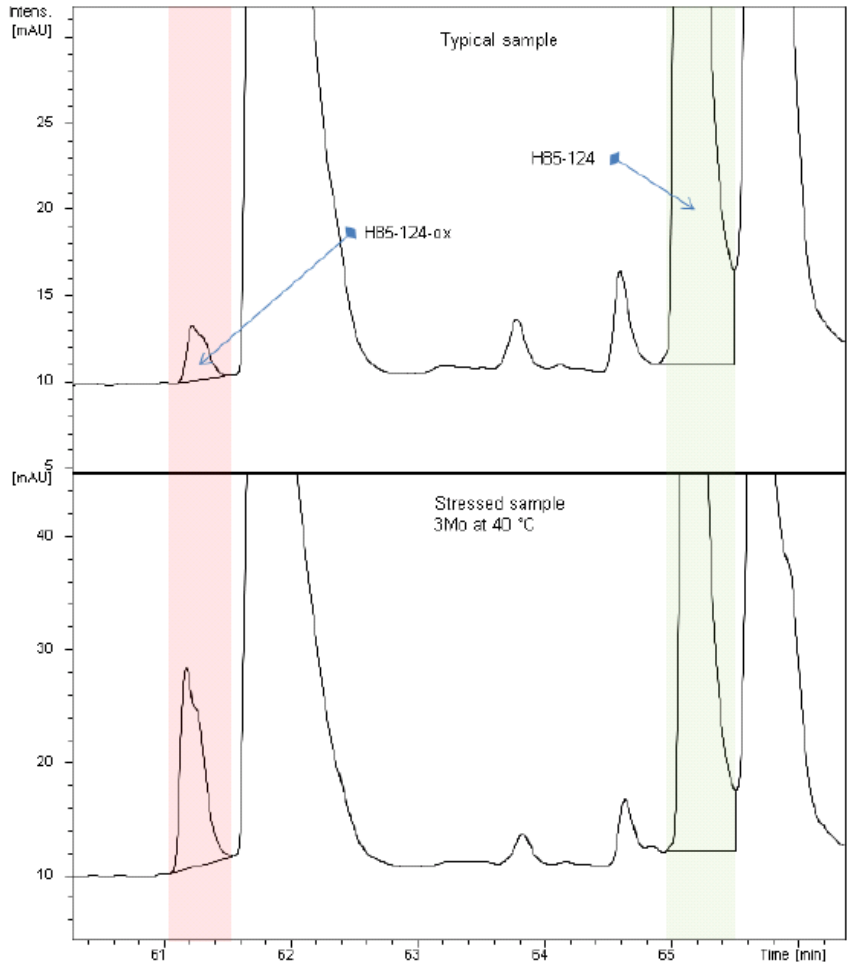


**Figure 7:** **Typical integration of oxidized peptide H85-124 and non-oxidized peptide on the 214 nm UV trace for a typical and a stressed sample.**

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# Oxidized Methionine$^{105}$ results at ADV deparment

- The ADV analysis results of 2 batches MK-XXXX :

| Samples | Gln-1 conversion (EIC) | Met-105 oxidation (EIC) | Met-105 oxidation (UV) | Met-252 oxidation (EIC) | Met-358 oxidation (EIC) | Met-428 oxidation (EIC) | Asn-384, Asn-389 deamidation | Asn-384, Asn-389 Succinimide | Asn55 deamidation | Asn55 Succinimide |
|---|---|---|---|---|---|---|---|---|---|---|
| L00036374 T=0Mo | 93.7% | **6.6%** | **4.0%** | 4.4% | 0.4% | 0.5% | 6.3% | 1.7% | 0.5% | 1.7% |

| Samples | Gln-1 conversion (EIC) | Met-105 oxidation (EIC) | Met-105 oxidation (UV) | Met-252 oxidation (EIC) | Met-358 oxidation (EIC) | Met-428 oxidation (EIC) | Asn-384, Asn-389 deamidation | Asn-384, Asn-389 Succinimide | Asn55 deamidation | Asn55 Succinimide |
|---|---|---|---|---|---|---|---|---|---|---|
| 0000361374 Comparability | 93.9% | **5.9%** | **3.9%** | 3.2% | 0.3% | 0.8% | 6.9% | 1.9% | 0.6% | 1.9% |

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# Oxidized Methionine$^{105}$

***Digestion:***

-25µL (0.5 mg/mL) of MK-XXXX in 0.5M Tris pH 7.5 + 25µL 8M Urea + 10 mM DTT : mix, incubate for 45 min at 55°C

-Add 12.5 µL iodoacetamide to the mixture : mix, incubate for 45 min at 55°C

-Add 150µL Trypsin (2 µg/mL) in 0.25M Tris pH 7.5 : mix and incubate o/n at 37°C

***UPLC Methode :***

Run time : 4min

Gradient :

-0-2 min : 0-30% ACN

-2-3 min : 30-90% ACN

Solvent : A _0.05% FA in Milli Q; B_0.05% in ACN

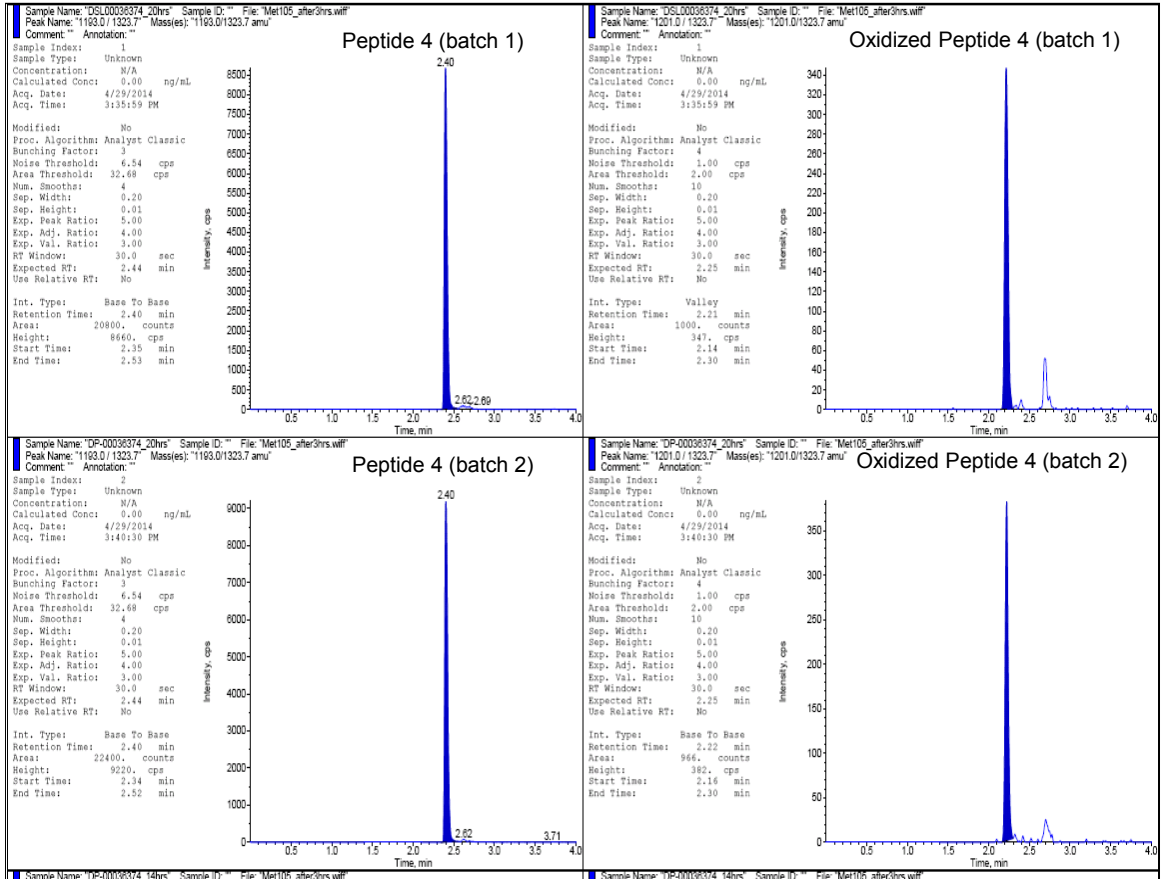
Injection : 1µL

Column : Acquity UPLC BEH Shield RP C18 2.1 mm × 50 mm x1.7 µm

***MS conditions :***

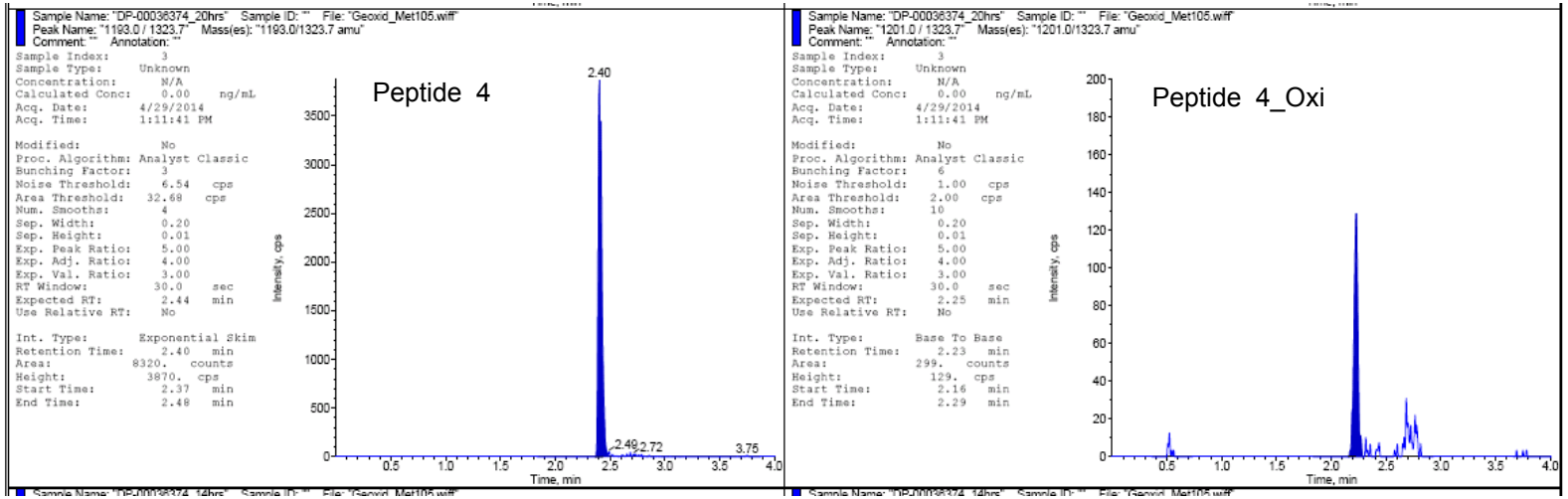
CE and DP were calculated by Skyline

TIS mode

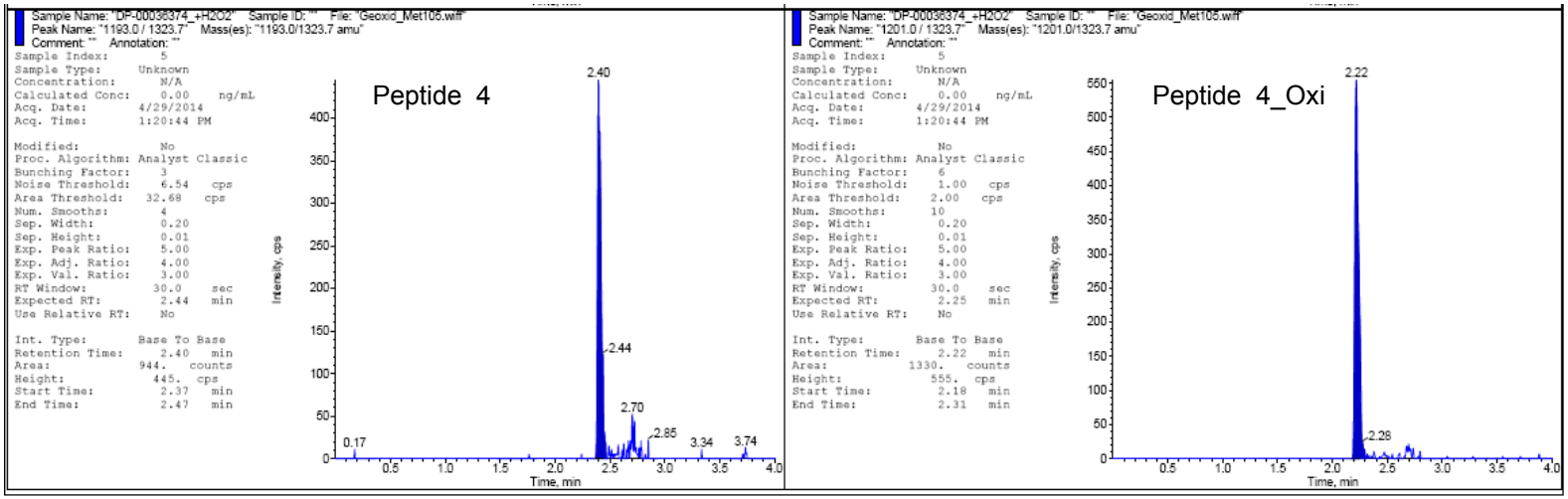


# Trypsin Digestion after Treatment with $H_2O_2$

- Without treatment




- Treatment with $H_2O_2$

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# Oxidized Methionine$^{105}$ (peptide 4)

| Date | Batch | Conc. 1 (mg/mL) | Peak area | Peak area Ox | Mean percentage of the Oxidized Met | % CV | Digestion n= | Injections | ADV (EIC) | ADV (UV) | Q1-Q3 | MS | Inject (µL) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 27-May-14 | O000361374 | 0.054 | 172627 | 8109 | 4.5 | 4.0 | 3 | 15 | | | 1193->580 | 5500 | 5 |
| 03-Jun-14 | O000361374 | 0.054 | 152280 | 8350 | 5.2 | 9.7 | 3 | 15 | | | 1193->580 | 5500 | 5 |
| 24-Apr-14 | O000361374 | 0.054 | 22400 | 966 | 4.1 | | 1 | 1 | 5.9 | 3.9 | 1193->1323 | 4000 | 1 |

| Date | Batch | Conc. 1 (mg/mL) | Peak area | Peak area Ox | Mean percentage of the Oxidized Met | % CV | Digestion n= | Injections | ADV (EIC) | ADV (UV) | Q1-Q3 | MS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 27-May-14 | L00036374 | 0.054 | 119040 | 5968 | 4.8 | 3.8 | 3 | 15 | | | 1193->580 | 5500 | 5 |
| 03-Jun-14 | L00036374 | 0.054 | 124436 | 6370 | 4.9 | 7.2 | 3 | 15 | | | 1193->580 | 5500 | 5 |
| 24-Apr-14 | L00036374 | 0.054 | 20800 | 1000 | 4.6 | | 1 | 1 | 6.6 | 4.0 | 1193->1323 | 4000 | 1 |

(*) Each digestion was 5x injected

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# Characterization Summary

- Targeted protein quantitation for specific modifications can improve throughput for protein characterization
- Improved throughput also enables more complex experiments to be completed with reduced turnaround time while improving overall precision

# Future Directions

- Going beyond ligand binding assays
  - Modified protein quantification
- Microsampling approaches for protein quantification
  - Reducing animal usage
  - Improving data quality

# Going Beyond LBA Approaches

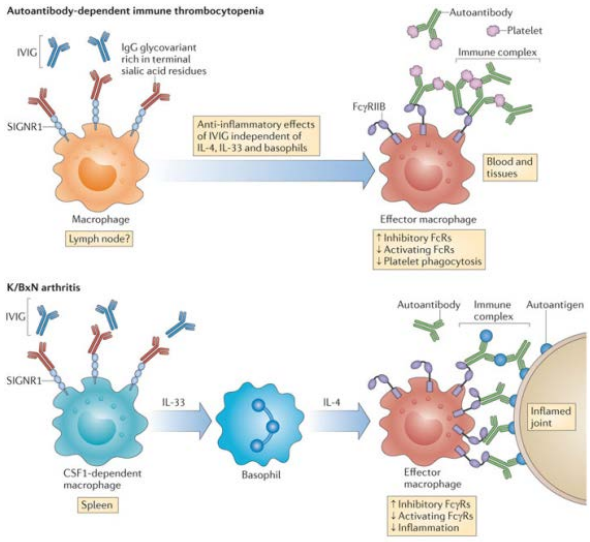
BaoJen Shyong

**Goal:** Provide sensitive (0.1-1ug/mL LLOQ), high throughput LC-MS-based pharmacokinetic measure of therapeutic protein with additional measures of the glycosylated peptide as a secondary requirement

## Background

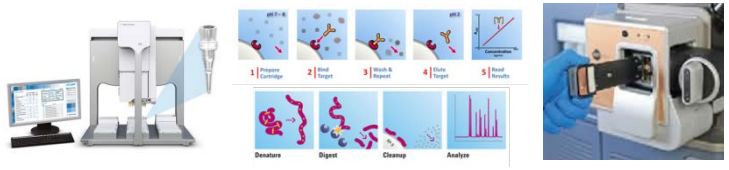
- Indicated for anti-inflammatory activity (arthritis, ITP, etc..)
- Recombinant alternative to plasma-derived Immunoglobulins (Ig)
- Anti-inflammatory activity of commercial intravenous Ig (IVIg) is derived in part from the presence of a small fraction of specific α2,6 sialylated glycoforms in the Fc region of IgGs.
- An α2,6 sialylated Fc has been engineered to provide an Ig with greater sialic acid content

**How glycosylation of IgG affects IVIG activity? SIGNR1 as a novel IgG glycoform-specific FcγR1**

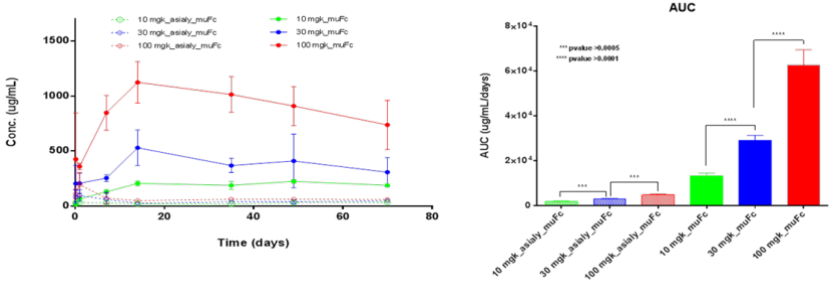



Nature Reviews Immunology 13, 176-189 (March 2013)

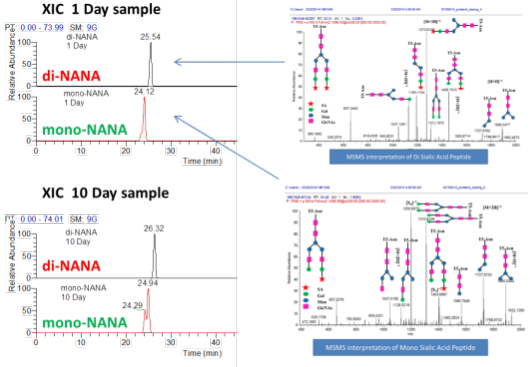
**Full Automated Assay Implemented with Nanotile coupled LC-MS**



**PK measures for Protein**




**Monitoring and Relative Quantitation of Glycoforms**

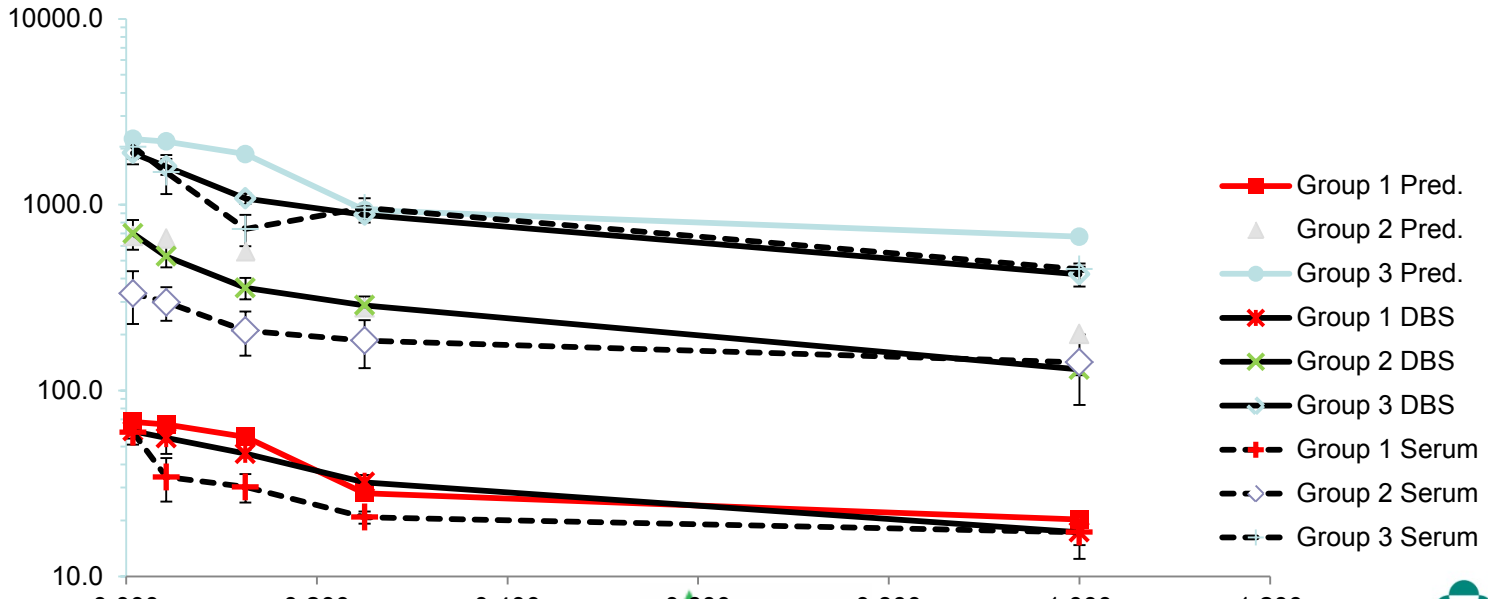



Di-sialyated glycopeptide was less than 70% of total glycopeptides

# DBS for mAb

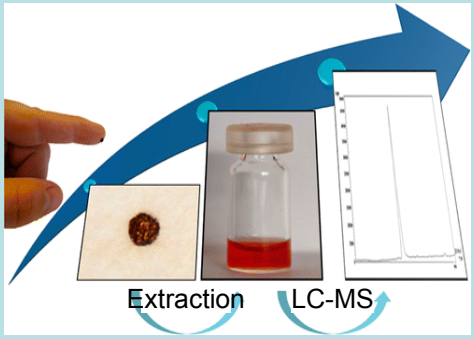
- Serum Study
- 3 dose groups (3, 30, 100 mpk)
- 3 mice per time point with 5 time points (45 total animals)
- 50 mg of mAb required

- DBS Study
- 3 dose group (3, 30, 100 mpk)
- 3 mice per group with 5 time points (9 total animals)
- 10 mg mAb required



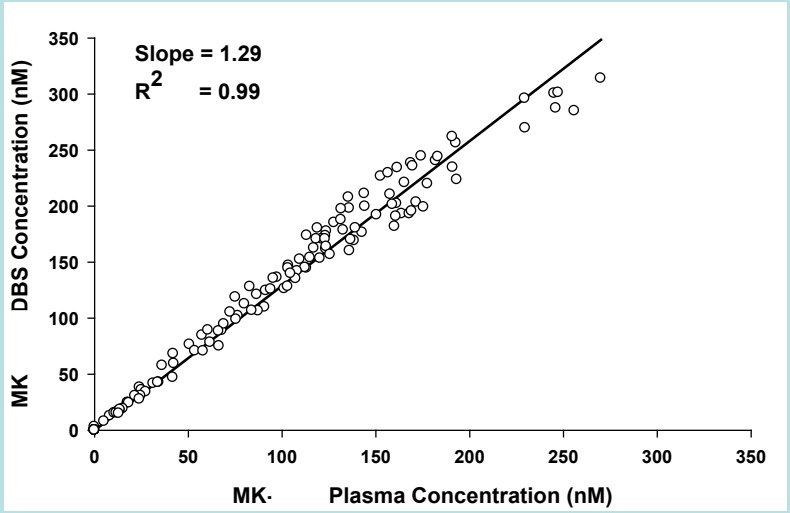
# Dried Blood Spots: From Lab to Clinic

## Bioanalytical Innovation



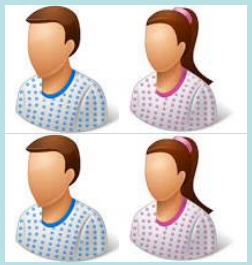

Dried Blood Spots:
Collect small blood volumes (uL)
Assay meets FDA guidelines
Simplified shipping/handling

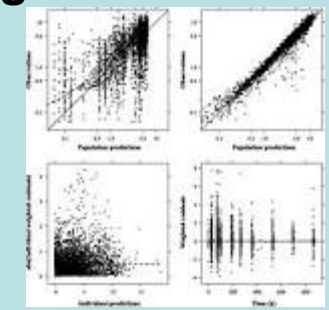
## From Lab to Clinic




Implemented in Phase 2/3 trial:
Strong body of data presented to regulators to show it correlates well with plasma assay.

## Enabling Phase III





Richer pop PKPD Datasets
Less operational complexity

## Enabling the Future


Home sampling integrated with eMed technologies, improving patient compliance and clinical datasets

Public

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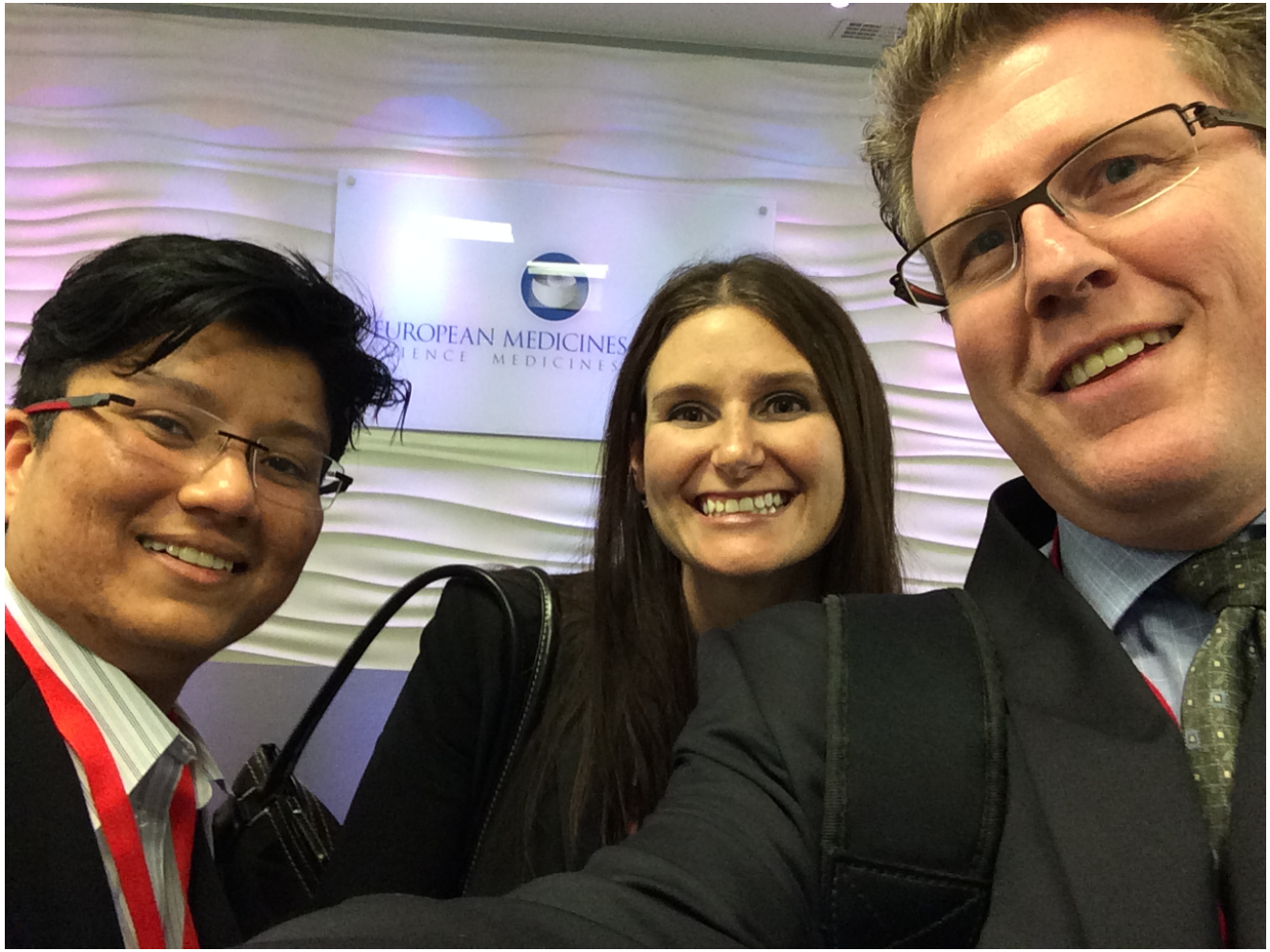
# EMA Feedback on DBS in the Clinic

- For MK-XXXX, specifically: "it is considered that the approach to support the use of DBS as the sole source of PK data for the remainder of the MK-XXXX Phase 3 program is **robust and acceptable under the conditions described**"
- For general bridging approach, including use of pop PK modeling: "The implementation of DBS requires unique considerations which are not readily translatable to other development programs. However, **the overall strategy is endorsed**."
- For general content of BA validation package: "The presented approach could serve as a basis for a validation of DBS (under the conditions discussed) and depending on results, **could support the use of the DBS in other clinical programs**. In absence of a defined regulatory framework to guide this process, the Applicant could request a follow up once data will be available for analysis."
- More general feedback: "Any candidate substance for DBS will need to be evaluated for being suitable for this approach (bioanalytically, PK wise, etc) and the qualification approach may need to be adjusted accordingly."

**Merck is implementing DBS for both small and large molecule sample collection in clinical trials**

# EMA Selfie



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