A Robust and Generic Method for Quantification of Monoclonal Antibody Therapeutics in Biological Matrices

Biologics analysis simplified with the BioBA kit, a hybrid strategy for therapeutic antibody assays

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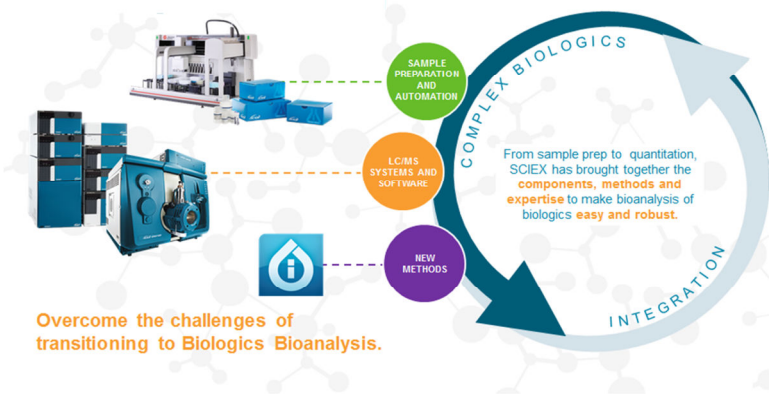
Key Challenges of Biologics Bioanalysis

- **Structurally complex analytes** – Growing compound class complexity requires rigorous method development for every new biologic in the development pipeline.
- **Tedious sample extraction methodology –** Lengthy and complicated procedures for measuring active or free circulating drug are more challenging in complex matrices.
- **Poor data reproducibility -** Rigorous sample extraction methods are needed to achieve reproducible assay statistics.
- **Numerous assay possibilities -** Choosing the right assay platform (*e.g.,* LBA, LC/MS/MS) to achieve accurate answers more rapidly can be overwhelming when faced with too many options.
- **Poor selectivity** – Multiple interferences from complex matrices (e.g., antidrug antibodies, neutralizing antibodies, free, target bound, and partially bound drugs) can lead to poor assay performance and inaccurate results.
- **Demanding assay performance requirements** - High selectivity, sensitivity and robust quantitation is needed for the accurate analysis of biotherapeutics in complex matrices (e.g., plasma, serum, tissue).

Key Benefits and Features of BioBA Solution

- **Superior assay performance -** Optimized immunoaffinity sample extractions and accurate MRM signature peptide quantification surpass the free-drug measurement and assay statistics of LBA-based assays.
- **Ready-to-use kit –** Assay reagents are compiled into one kit that provides a generalized approach for the immuno-capture and signature-peptide release of any free, unbound monoclonal antibody (and its active isoforms).
- **Comprehensive and universal solution**: Six key instrument and software components are needed for easy transition from small molecule bioanalysis to biologics bioanalysis: 1) BioBA High-Capacity Enrichment Sample Preparation Kit, 2) Biomek Fx automated liquid handling workstation, 3) Exion LC system, 4) QTRAP® 6500+ System, 5) MultiQuant™ Software, and 6) Biopharma vMethods.
- **Improved selectivity –** Signature-peptide-based LC/MS/MS analysis enhances the selectivity, specificity and overall assay statistics for mAb quantification.
- **Expanded linear range -** Inclusion of high-capacity magnetic beads produced a wider dynamic range (up to 4-5 orders) for monoclonal antibody measurements in complex matrices.
- **Meets or exceeds bioanalytical assay requirements -** Highly sensitive and selective MRM-based signature peptide LC/MS/MS analysis of monoclonal antibodies delivers GLP level assay statistics
- **Synergism of combined methodologies -** The hybrid LBA-LC/MS assay complements ELISA-only-based assays by providing additional selectivity and specificity.



BioBA kit - comprehensive monoclonal antibody quantification solution.

INTRODUCTION

Over the past 20 years, monoclonal antibody (mAb) drug development has advanced significantly, becoming a rapidly growing segment of the biotherapeutics market that has generated treatments for a wide variety of diseases ranging from inflammatory disorders to cancer. This expansion of biologics-based medicines has pushed drug manufacturers to adopt new analytical approaches to quantify and characterize multiple mAb variants efficiently, with particular focus on the high-throughput needs of early-stage pharmacokinetic (PK) studies. Traditionally, PK methods have employed ligand-binding assays (LBAs) to assess mAb performance; however, LBAs require a specific antibody reagent to be developed for each mAb variant, a process that is often not compatible with the compressed timeframes encountered during the initial stages of drug development.

More recently, LC/MS/MS-based methods have come to the forefront as a feasible approach for the quantification of mAbs in biological matrices, with many of these methods relying on proteolytic digestion of the target mAb and quantification of multiple unique signature peptides, which are equivalent to levels of the whole protein. The LC/MS/MS method complements the traditional LBA approach and provides the specificity and sensitivity necessary for overcoming LBA limitations—such as matrix interferences and time-consuming reagent development. Further incorporation of immunoaffinity-enrichment steps, which concentrate the target mAb prior to proteolysis and lower the background complexity, has increased the sensitivity of LC/MS/MS-based quantitation.

Fine-tuning and developing MRM transitions for each unique signature-peptide can also encumber early-stage mAb characterization. Seeking a general quantification protocol that could be applied to any mAb species across all IgG subclasses, researchers developed and optimized a universal peptide sequence approach—where signature peptides derived from the conserved Fc (fragment-crystallizable) region of the mAb—are quantitated after immuno-enrichment and proteolysis steps. Specifically, the majority of mAb subclasses used in biotherapeutics are based on humanized IgG subtype 1 (IgG_1) antibodies and share broadly homogenous Fc sequences that are unvaried even among mAbs derived from other subtypes 2-4. Targeting the Fc region during immuno-capture allows for the selective pull-down of humanized mAbs from non-human biological matrices using a singular immunoaffinity approach (an anti-human Fc antibody conjugated to a streptavidin-coated bead) without resorting to the time-consuming production of new reagents. In addition, MRM method development time is significantly condensed because mAb detection is based on pre-developed MRM transitions that consistently detect the same signature peptides from the conserved Fc region regardless of the mAb analyte (**Figure 1**).

To reduce the error and variability of peptide quantitation data resulting from variations in sample preparation and matrix effects, a stable isotope labeled (SIL) mAb IgG_1 is added to biological samples as an internal standard (IS) and contains the same conserved Fc region and similar physiochemical properties as unlabeled mAbs. Equivalent SIL signature peptides produced alongside target mAb-derived peptides are detected using pre-developed MRM transitions (**Figure 1**), providing a standardized way to compensate for incomplete protein digestion and sample losses during immuno-capture and proteolysis reactions.

Good quantitation statistics rely on reproducible measurements of target mAb levels, and are strongly dependent on: 1) complete immuno-capture of the total target mAb population in the biological sample evaluated, and 2) consistent and uniform peptide release and absolute protease cleavage site fidelity during proteolysis of the target mAb. Herein, we compare and optimize magnetic bead immunoaffinity strategies for the targeted selection of therapeutic mAbs from non-human

Universal Peptide Sequence	Location	Isotype Overlap	Species Homology
DTLMISR	HC	IgG_1, IgG_2, IgG_3, IgG_4	Rhesus monkey Cynomolgus monkey
FNWYVDGVEVHNAK	HC	IgG_1	
VVSVLTVLHQDWLNGK	HC	IgG_1, IgG_3, IgG_4	
GFYPSDIAVEWESNGQP ENNYK	HC	IgG_1, IgG_4	
AGVETTTPSK	LC	lambda	Rhesus monkey Cynomolgus monkey
YAASSYLSLTPEQWK	LC	lambda	

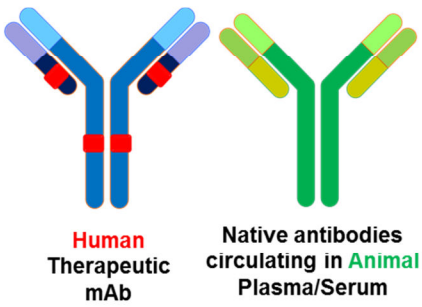


Figure 1. Universal peptide strategy. Tryptic peptide sequences resulting from SILu™Mab were blasted against the SwissProt and NCBI databases to ascertain uniqueness of the sequences against common preclinical mAbs. Selected peptides are shown in the table above, along with their location (highlighted in red) within the antibody.

biological fluids, as well as adjust proteolysis conditions that foster complete signature peptide release. This generalized and streamlined workflow is then applied to the quantification of two well-characterized, commercially available therapeutic mAbs, cetuximab (Erbitux) and infliximab (Remicade) producing concentration measurements that meet or exceed bioanalytical standards for precision, accuracy, and specificity.

These studies were intended to lay the groundwork for the development of standardized PK workflows for mAbs quantification in both pre-clinical and clinical studies—workflows that are now available commercially in a pre-packaged, optimized and generic approach for assaying mAbs in non-human matrices—the BioBA sample preparation kit.

MATERIALS AND METHODS

Sample Preparation

Immuno-capture assay

Immunoaffinity purification of the target mAbs from non-human plasma was carried out using a biotinylated antibody specific for the Fc region of human mAbs (Southern Biotech) that was immobilized on high-capacity magnetic beads coated with streptavidin. For comparative binding capacity experiments, the anti-human-Fc, biotinylated antibody was also coupled to the Dynabeads® brand of magnetic, streptavidin-coated beads (Life Technologies) according to the manufacturer's protocol. For immuno-enrichment assays, TBS-washed magnetic beads (25 µL) coupled to anti-human Fc were combined with animal serum (33% in TBS) spiked with a range of concentrations of target mAb or an unlabeled, universal, human mAb standard, SILu™Lite SigmaMAb (Sigma). A stable-isotope-labeled (SIL), universal mAb internal standard, SILu™Mab (5 µg/mL) was added as internal standard (IS) to each sample, and the incubations were shaken (2 hr, 20 °C). After discarding the supernatant, captured IgGs were eluted with 0.1% TFA (50 µL) from the TBS-washed mAb/bead conjugates and neutralized with 500 mM ammonium bicarbonate (5 µL).

Proteolytic digestion of immuno-enriched fraction

For proteolytic digestion of IgGs requiring strong denaturing conditions, eluted protein was denatured in urea (3.6 M) and reduced with TCEP (7.65 mM) at 37 °C for 60 min in 50 mM ammonium bicarbonate. Samples were alkylated with iodoacetamide (8 mM) for 45 min at 20 °C and then

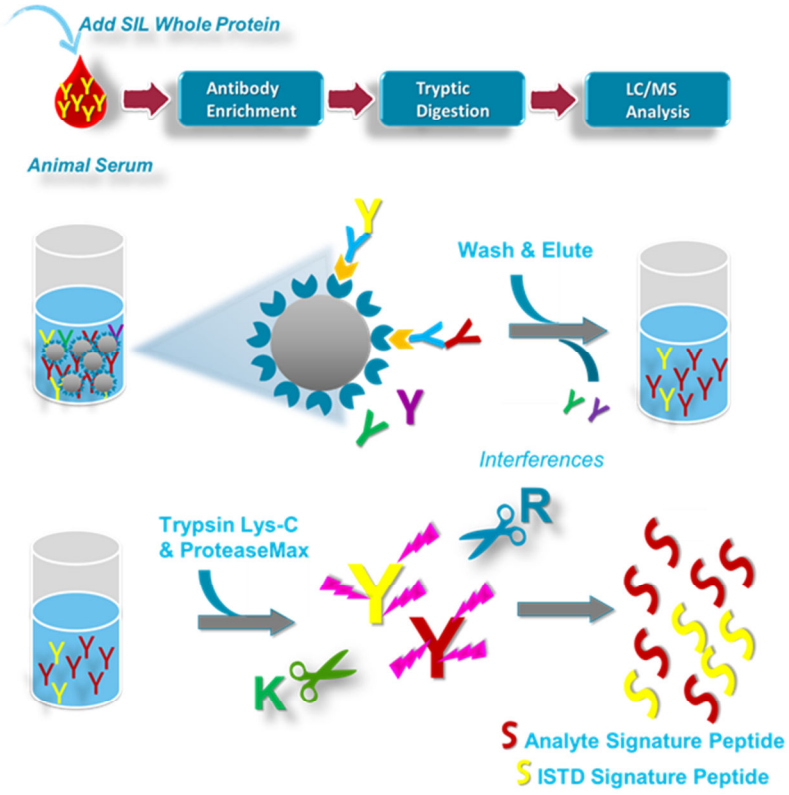


Figure 2. Immunocapture sample extraction workflow. The BioBA sample monoclonal antibody (mAb) extraction workflow can be performed in three easy steps when using complex biological samples such as plasma or serum. In Step 1, a whole-protein, stable-labeled (SIL) internal standard (IS) is added to assure quantitative rigor throughout the sample extraction process. In step 2, both internal standard and the target mAb are selectively bound to high-capacity streptavidin-coated magnetic beads conjugated to a biotinylated anti-human-Fc antibody. In step 3, the immune-enriched fraction undergoes proteolysis using trypsin/LysC, with enhanced digestion efficiencies achieved by adding an anionic surfactant, for a more robust generation of signature peptides for LC/MS quantitation.

proteolytically digested with Trypsin/LysC (3.75 µg/mL) at 37 °C for 3 hr. The protease mixture was delivered in an anionic surfactant that maximizes digestion efficiency by slightly denaturing tightly folded proteins. The released peptides were acidified with 98% formic acid (2 µL) prior to analysis by LC/MS/MS on a hybrid quadrupole ion trap instrument.

Urea was omitted from the reducing/denaturing step if target mAbs only required mild denaturing conditions for proteolysis. (Partial protein unfolding was achieved using 0.025% BioBA anionic surfactant added with the protease mixture.)

LC/MS conditions

Quantitative signature peptide analysis was performed on a SCIEX QTRAP® LC/MS/MS system coupled to an Exion LC system. Three unique fragment ions were monitored for each signature peptide for both the target mAb and the IS, requiring high-flow chromatographic conditions that improved throughput and minimized matrix interferences while permitting the simultaneous monitoring of multiple MS/MS transitions.

Chromatography

LC System:	Exion System
Column:	Phenomenex Kinetex C18 (50 × 2.1 mm), 2.6 µM
Column Temp.:	40 °C
Injection:	5.0 µL
Flow Rate:	600 µL/min
Mobile Phase:	A) Water, 0.1% formic acid
	B) Acetonitrile, 0.1% formic acid

Gradient:

Time/min	A%	B%
0	98	2
8.0	35	65
8.1	10	90
9.0	10	90
9.1	98	2
10.0	98	2

Mass Spectrometry

System:	QTRAP® 6500 LC/MS/MS System
Interface:	IonDrive™ Turbo V Source used in high-mass, positive-ion mode
Ion Source Gas 1 (GS1):	85
Ion Source Gas 2 (GS2):	80
Curtain Gas (CUR):	25
Temperature (TEM):	500 °C
IonSpray Voltage:	5500
Scan Type:	MRM in high mass mode

Peptide MRM Optimization for SILu™Lite and SILu™Mab using DiscoveryQuant™ 3.0 Software

Choosing the best MRM for a peptide within a protein digest is a challenge because multiple charge states are possible for each tryptic peptide, and the abundance of product ion possibilities

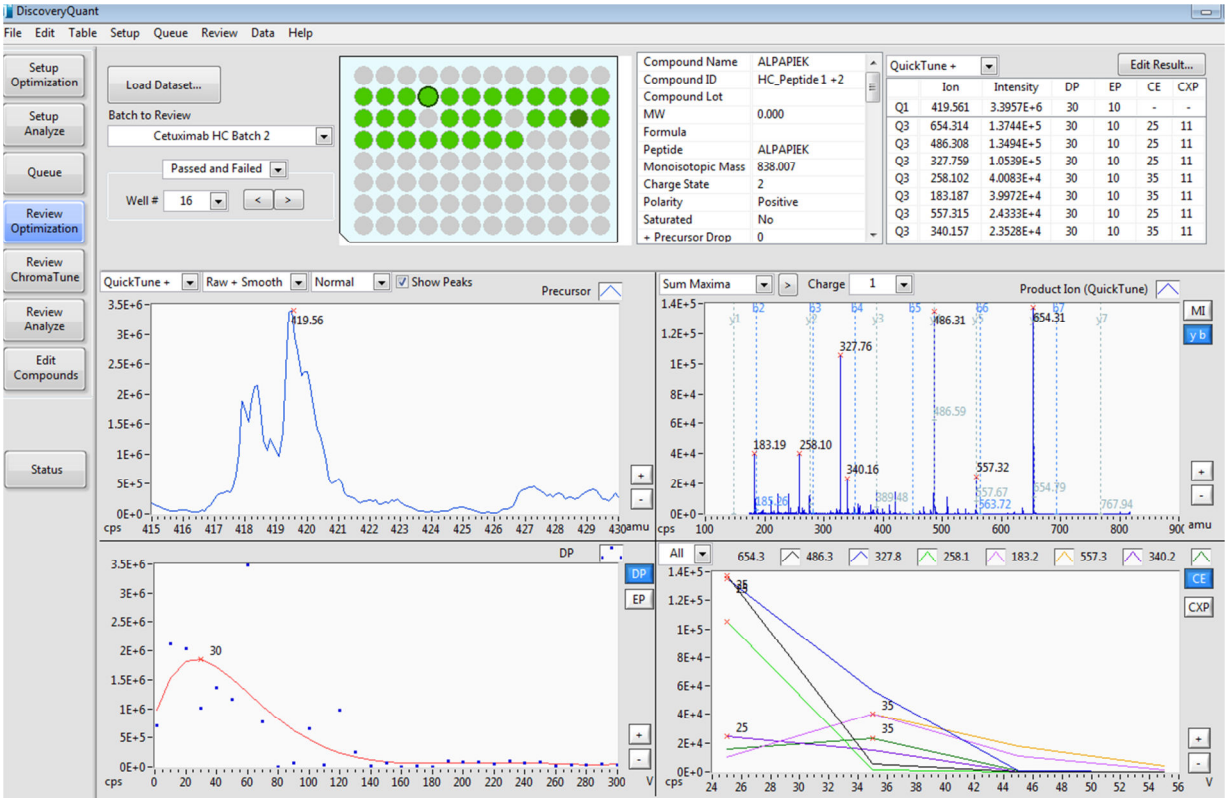


Figure 3. Optimize review panel of the mAb digest tune. Each tryptic peptide is tuned for precursor mass, product ion spectra, CE and DP. An overlay of theoretical *y* and *b* product ion masses are used to choose selective MRMs, and green lights indicate a passing peptide tune.

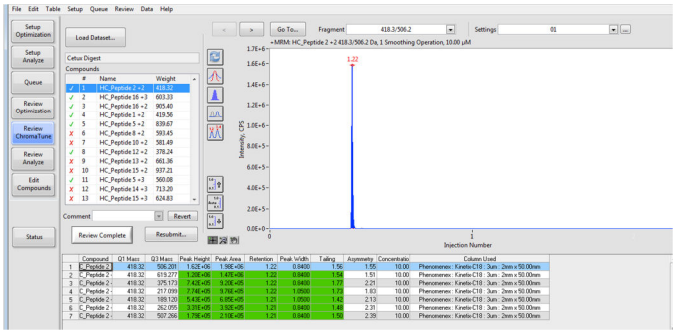


Figure 4. ChromaTune module in DiscoveryQuant™ Software. ChromaTune review panel of the mAb digest. After tuning with Optimize the mAb digest is injected on-column and each tryptic peptide charge state and MRM is reviewed for: sensitivity and chromatographic properties such as: retention time, peak width and peak tailing. Green lights and check marks indicated tryptic peptides that meet acceptance criteria

leave a significant number of MRMs to be screened. DiscoveryQuant™ 3.0 Software, an automated MRM-tuning and optimization application, is the ideal tool for evaluating and selecting peptide MRM transitions for increased throughput and maximum sensitivity. The Optimize module in DiscoveryQuant Software (**Figure 3**) collects product ion spectra and allows for the enhancement of compound-dependent mass spectrometer parameters (DP, CE, CXP and EP) in under 1.5 minutes via infusion or flow-injection analysis. The MRM transitions along with the adjusted MS parameters are stored in the DiscoveryQuant Software database. These transitions are available for incorporation into LC-MRM methods to 1) perform automated LC screening and validation using the ChromaTune module and 2) build quantitative methods and batches within Analyst® Software.

Heavy and light chain sequences of mAbs were digested *in silico* using a web-based tool (PeptideMass, ExPASy). Thirty tryptic peptides between 7–25 amino acids in length were chosen for optimization. The sequences were entered into the DiscoveryQuant Software Optimize setup table, and a QuickTune optimization was performed for the +2 and +3 charges states of each peptide (**Figure 3**). Digested mAb (5.0 µg/ml) in 30% acetonitrile/0.1% formic acid was infused at 3.0 µL/min during DiscoveryQuant Software optimization. After reviewing the precursor and product ion QuickTune data, peptide MRMs that passed the intensity acceptance criteria were optimized using FineTune, where DP, CE, CXP were adjusted for maximum sensitivity. After optimization by infusion, the peptide digest was diluted 5-fold and aliquoted into a 96-well plate. The ChromaTune module of DiscoveryQuant Software (**Figure 4**). was then used to inject mAb digests on-column and assess the sensitivity and chromatographic properties of each peptide charge-state and associated MRM under the LC conditions on page 4. The peptides with the best sensitivity were then assessed for selectivity by creating MRM methods from the DiscoveryQuant Software database and analyzing blank rat plasma digests.

Data processing

Scheduled MRM™ Algorithm

Tracking MRM transitions for a large number of compounds—including both labeled and unlabeled in the same sample—can slow down processing speeds, drag down the duty cycle and impact reproducibility. To improve throughput during multi-analyte detection, the MS quantitation workflows for measuring multiple signature peptide MRM transitions included a time-saving feature—the *Scheduled* MRM™ Pro Algorithm. This allowed for MRM transitions for particular analytes to be slated only when column elution was expected (for a short window of time), reducing the need for ongoing, multi-period analysis of every analyte (**Figure 5**) for maximal acquisition efficiency and quantitative accuracy.

Optimal MRM transitions for at least three fragment ions were selected for each signature peptide and were monitored using the *Scheduled* MRM Algorithm. Standard concentration curves were generated using MultiQuant™ Software. All samples were analyzed in triplicate. Lower limits of quantification (LLOQ) and quantitative data statistics were determined using MultiQuant™ Software.

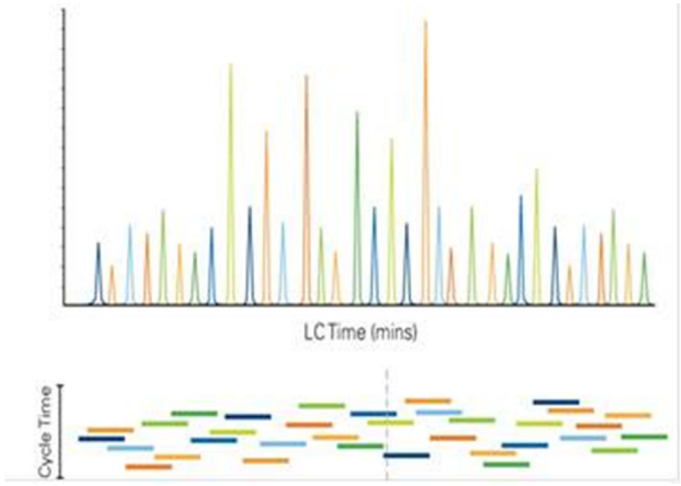


Figure 5. Scheduled MRM™ Algorithm. Multiple peptides can be quantitated simultaneously in the same run by monitoring the MRM transition for each peptide only during the a short window of time centered around its elution from the LC system into the MS. Selective analyte monitoring periods reduce the need for ongoing multi-period analysis of every analyte.

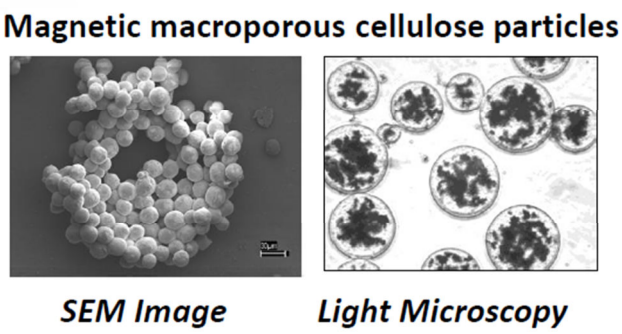


Figure 6. Images of magnetic beads. Paramagnetic affinity beads (30–50 µm) composed of iron encapsulated by macroporous cellulose were analyzed by scanning electron microscopy (SEM) and light microscopy, revealing uniform spheres that offer substantial surface area for efficient binding of target proteins.(*Figure courtesy of Promega*.)

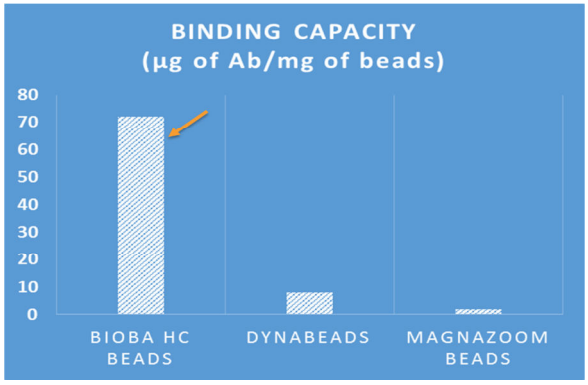


Figure 7. Binding capacity of magnetic beads. The binding capacities of commercially available magnetic beads were compared by measuring the total protein (using a BCA protein assay) before and after incubating excess mAb with different beads conjugated to anti-human-Fc antibodies (at equivalent bead masses). The binding capacity is expressed as the difference between the mAb protein concentrations of the load and flow-through fractions.

Results and Discussion

Binding capacity comparisons of high-capacity, streptavidin-coated magnetic beads

The macro-porous structure of the BioBA high-capacity beads offers a significant increase in surface area over other commercially available magnetic beads used for immunoaffinity experiments and provides several advantages, including low, non-specific binding, uniform size (30–50 µm, **Figure 6**), and fast magnetic response for easy handling during sample preparation during the wash and elution steps.

To ensure the complete capture of all target mAb in the plasma sample, an excess of on-bead, mAb binding sites compared to free analyte is required. Bead oversaturation due to poor binding site availability could lead to underestimation of target mAb

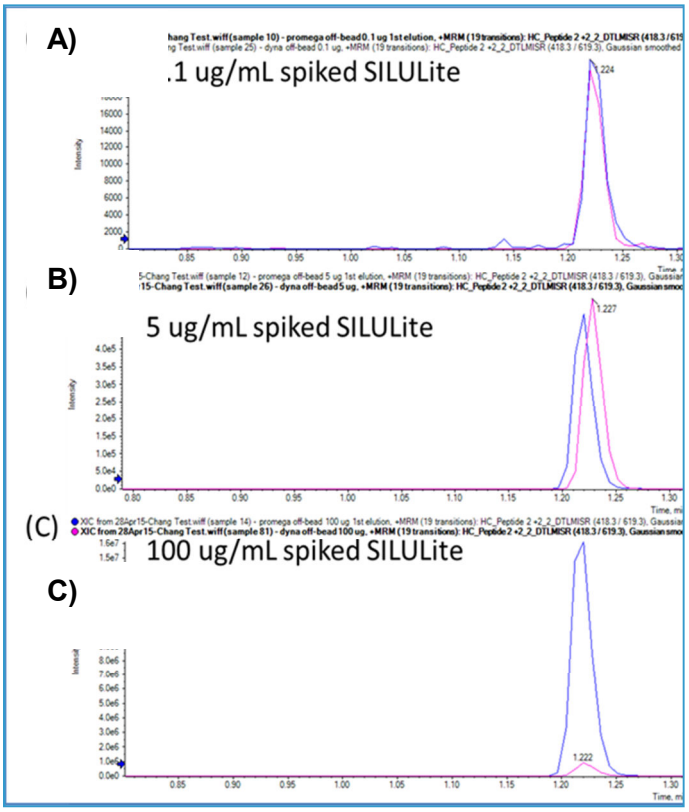


Figure 8. Comparative binding capacity of high-capacity magnetic beads. The XIC of peptide DTLMISR is shown for rat plasma samples containing (A) 0.1 µg/mL, (B) 5 µg/mL, (C) 100 µg/mL of SILu™Lite after incubation with either the new BioBA beads (*blue*) or with Dynabeads® (*pink*) and subsequent LC/MS/MS analysis of digested SILuLite eluents. Peak heights were compared to determine binding capacity.

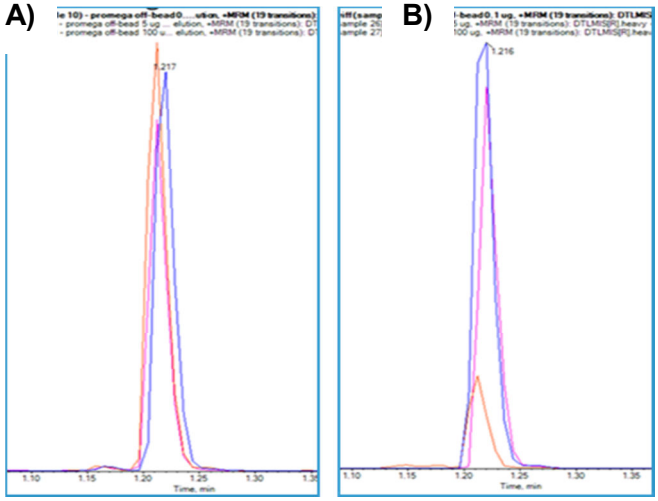


Figure 9. Peak height comparisons of XICs of isotope-labeled DTLMISR from SILu™Mab. Equivalent masses of BioBA beads (*left panel*) or Dynabeads (*right panel*) were incubated with a fixed amount of stable-isotope-labeled (SIL) internal standard, SILu™Mab, in the presence of varying concentrations of an unlabeled, universal, human mAb preparation, SILu™Lite: 0.1 µg/mL (*blue*), 5 µg/mL (*pink*), and 100 µg/mL (*orange*). Captured mAbs were eluted and proteolytically digested. The SIL signature peptide DTLMISR from SILu™Mab was quantitated using an MRM method, and peak heights were compared to determine binding capacity.

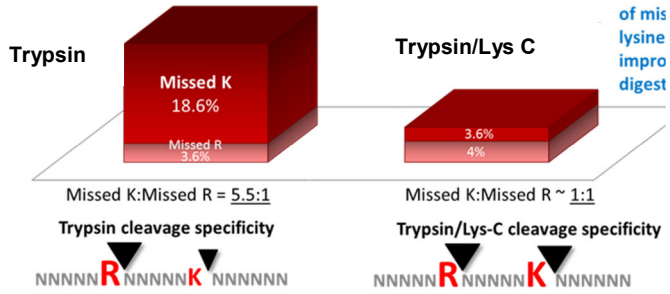


Figure 10. Reduced number of missed cleavages with trypsin/LysC. A) Proteins were digested overnight using trypsin or trypsin/LysC, at 37 °C under conventional, non-denaturing conditions. Use of the protease mixture trypsin/LysC eliminated the majority of missed lysine sites and improved the overall digestion efficiency. (*Figure courtesy of Promega*.)

levels, resulting in undesirable data variability. Protein binding as assessed by BCA protein assay of total unbound protein before and after incubation with magnetic beads was significantly increased for the BioBA high-capacity beads by 3.5-fold and 7-fold compared to protein binding on the Magnasphere beads or the Dynabeads, respectively (**Figure 7**).

Further comparisons of the binding capacity of the BioBA beads to other magnetic beads were completed using rat plasma incubated with varying concentrations of an unlabeled, standardized, mAb preparation, SILu™Lite, prior to immuno-capture and subsequent proteolysis of bound target mAb. Measurements of the total amount of bound antibody recovered from the washed beads indicates that protein retention is the same for lower levels of incubated mAb (0.1–5 µg/mL) on either the high-capacity BioBA bead or the Dynabeads (**Figures 8A and B**). However, at elevated mAb levels (100 µg/mL), MRM peak areas for the signature peptide expand 10-fold when mAbs are incubated with the BioBA beads compared to the Dynabeads (**Figure 8C**), revealing a significant gain in protein retention at the upper mAb concentrations for the high-capacity beads.

Because fixed amounts of SIL internal standard, SILuMab, were added to each binding experiment, labeled signature peptide levels should remain unchanged in the presence of increasing concentrations of SILuLite if the binding capacity of each bead type were equivalent. Further evaluation of signature peptide XICs derived from SILu™Mab, showed that the total amount of recovered IS was unchanged when incubated with increasing levels of SILu™Lite in the presence of the BioBA magnetic beads (**Figure 9A**). However, incubations of Dynabeads with elevated SILuLite concentrations (100 µg/mL) revealed that IS binding was reduced 10-fold (**Figure 9B**). Combined together, measurement of a SIL and standard mAb-derived signature peptide (DTLMISR) indicate that the BioBA high-capacity beads have a 10-fold increased binding capacity compared to other

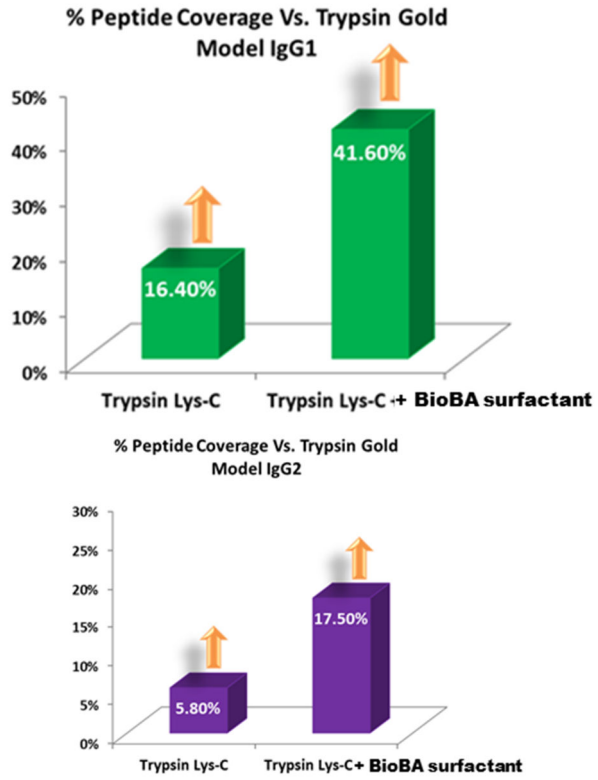


Figure 11. Improved percent peptide coverage with trypsin/LysC combined with BioBA surfactant. Improvements in percent peptide coverage as assessed in peptide mapping experiments of IgG_1 (*top panel*) or IgG_2 (*bottom panel*) under different digestion conditions are shown. A high-resolution, accurate-mass instrument analyzed protein digests after peptide separation under long-gradient, high-flow conditions on an Exion HPLC. Peptide mapping was completed using BioPharmaView™ Software. The percent peptide coverage is shown as a percentage fold increase in % peptide coverage for trypsin/LysC proteolysis relative to digestion with Trypsin Gold alone for conventional, non-denaturing conditions versus mild-denaturing conditions using the BioBA anionic surfactant.

commercially available magnetic beads, especially at higher mAb concentrations (in the range of 50–100 µg/mL).

Digestion efficiency of Trypsin/LysC

Trypsin, a protease that specifically cleaves at Lys and Arg sites, is subject to autolysis, which can result in broadened specificity and non-specific cleavage of peptides, complicating quantitative analyses that rely on uniform peptide release. Accurate mAb quantitation requires complete protease fidelity to cleavage sites for release of identical signature peptides from the parent protein, and this is typically assessed by tracking the number of missed cleavages at Lys sites on the target mAb. The combination of trypsin with an additional protease, LysC, reduces the number of missed Lys sites and improves cleavage site coverage during standard proteolysis conditions (**Figure 10**). To further increase digestion efficiency and improve the

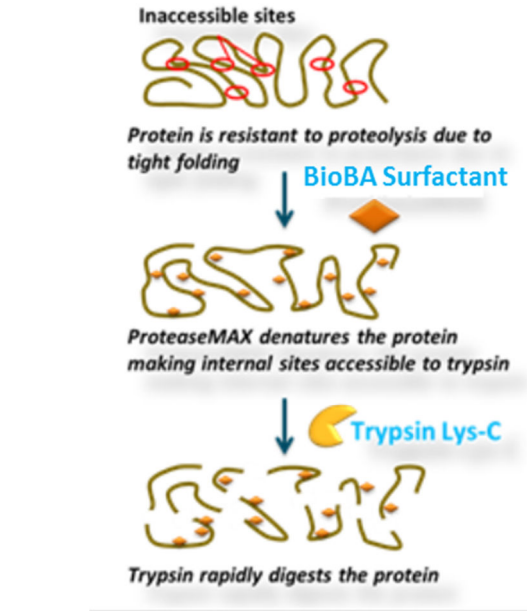
uniformity of peptide release, the BioBA anionic surfactant and protein solubilizing agent, was added to the reaction mixture, resulting in partial protein unfolding and improved protease access to cleavage sites.

To evaluate the cleavage site coverage of different proteases, peptide mapping experiments of mAbs from two representative IgG classes (IgG1 and IgG2) were performed under various denaturing conditions (**Figure 11**). Digested peptides were analyzed by high-resolution accurate mass analysis on a TripleTOF® 5600 System using mAb digests that were separated under long-gradient, high-flow conditions on an Exion HPLC. Peptide mapping was completed using BioPharmaView™, and the percentage of IgG sequence coverage after digestion with either Trypsin Gold (a recombinant version of trypsin engineered to resist auto-lysis) or with trypsin/LysC were compared for standard non-denaturing digestion conditions or for slightly denaturing conditions with BioBA surfactant.

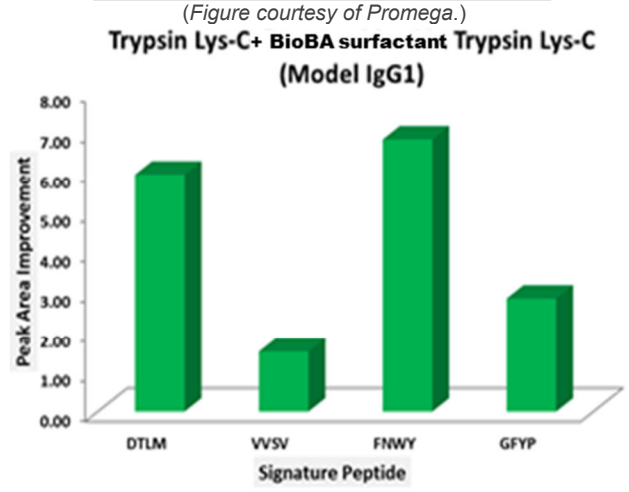
Digests with the least number of missed Lys sites showed increased % peptide coverage, which was assessed by calculating the percentage of peptide coverage with trypsin/LysC relative to proteolysis with Trypsin Gold alone. Digestions with trypsin/LysC supplemented with BioBA surfactant boosted peptide coverage from 16.4% to 41.6% for the model IgG_1 and from 5.8% to 17.5% for the model IgG_2 (**Figure 11**). This corresponds to an average 2.75-fold increase in sequence coverage by digesting with trypsin/LysC plus BioBA surfactant over trypsin/LysC in standard non-denaturing conditions.

In addition to increasing the peptide sequence coverage, trypsin/LysC plus BioBA surfactant also increased the yields of signature peptides compared to digestion with trypsin/LysC alone. To assess the extent of peptide release under mildly-denaturing conditions with BioBA surfactant, peak areas for multiple signature peptides released from IgG_1 and IgG_2 are shown for digests prepared with trypsin/LysC plus BioBA surfactant and compared to peptide levels produced with trypsin/LysC alone (**Figure 12**). Peak areas for each signature peptide increased for digests of IgG_1 (2-7 fold) and IgG_2 (8-10-fold) in the presence of BioBA surfactant, indicating that peptide release is more fully enhanced when target mAbs are slightly unfolded.

The BioBA surfactant, improves access to internal cleavage sites in resistant proteins by promoting partial protein unfolding. However, reaction conditions are mild enough so that full protease activity is maintained during incubations with BioBA surfactant. These mild denaturing conditions boost peptide release over conventional, non-denaturing conditions where internal cleavage sites that are resistant to proteases remain



(*Figure courtesy of Promega.*)



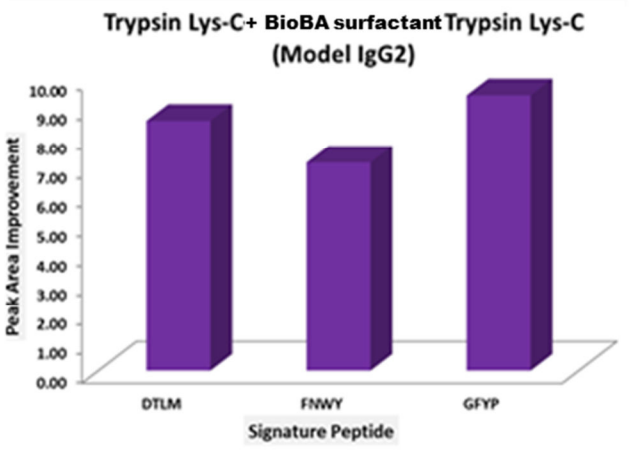


Figure 12. Enhanced peptide release with trypsin/LysC and BioBA surfactant. A) Addition of an anionic surfactant, BioBA surfactant improves access to resistant protease cleavage sites by providing mild denaturing conditions that promote partial protein unfolding and maintenance of full protease activity. Increased peak areas for multiple signature peptides released from IgG_1 (B) and IgG_2 (B) are shown for mAbs digests with trypsin/LysC that were conducted under mild (BioBA surfactant) or non-denaturing conditions.

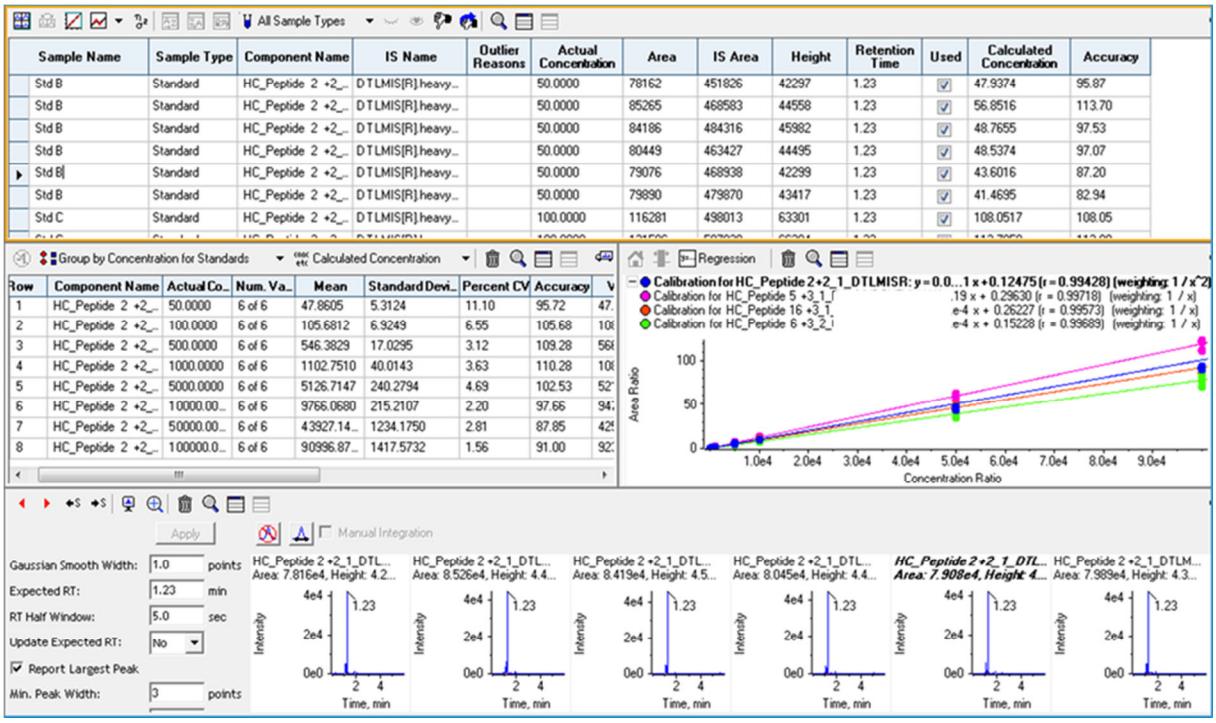


Figure 13. MultiQuant™ Software display of cetuximab signature peptide quantitation results. A screen shot is shown of cetuximab signature peptide quantitation data displayed in MultiQuant Software. Panels showing calibration curve concentrations, quantitation statistics, calibration curve overlays, and chromatographic traces are displayed on one screen for a comprehensive view of signature peptide results.

inaccessible (**Figure 12**). The use of trypsin/LysC also decreased the overall mAb digestion time to 2-3 hr, a significant increase in time savings compared to the overnight (10-12 hr) incubations required with trypsin alone.

Quantitation of generic and universal signature peptides by LC/MS/MS

Cetuximab (Erbitux), a therapeutic mAb used for the treatment of metastatic colorectal cancer, was quantitated using the BioBA sample preparation protocols for mAbs described here. Varying concentrations of cetuximab (0.05 – 100 µg/mL) were spiked into rat plasma, prior to incubation with the BioBA magnetic beads and subsequent proteolytic digestion of the immunoaffinity fraction containing the target mAb. Calibration curves of three representative peptides of cetuximab obtained were linear for 3 orders of magnitude and gave a LLOQ of 50 ng/mL. Cetuximab concentrations derived using the linear regression curves generated in these experiments displayed an accuracy of 85–115% and a %CV < 7% **(Figure 13**).

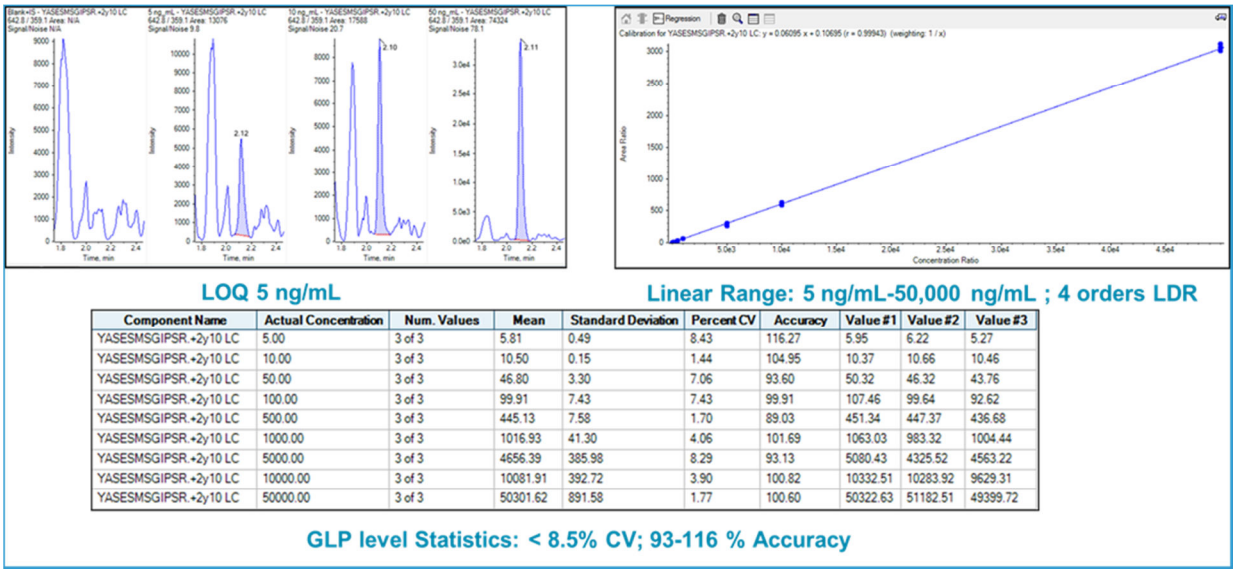
Because multiple MRM transitions are monitored for multiple signature peptides (both labeled and SIL), analysis and tracking of mAb quantitative data can be quite complex. To simplify the evaluation and review of multiple quantitative data sets, MultiQuant™ Software permits the user to view raw data files, statistical analysis of data, linear regression curves, and chromatographic traces for each target ion quantified by MRM all on the same software screen (**Figure 13**). A menu tool permits straightforward navigation to each signature peptide (and the multiple fragment ions evaluated), resulting in a software-user interface that not only consolidates data compilation, but eases the transition between data sets.

These experimental conditions were also used to quantitate infliximab (Remicade), a tumor necrosis factor alpha mAb that is used to treat autoimmune disorders. Signature peptides of infliximab were obtained through proteolysis of immunoaffinity-enriched rat plasma samples that had been spiked with varying concentrations of infliximab (0.005–50 µg/mL). The calibration curve generated had a linear dynamic range of 4 orders of magnitude, and an LLOQ of 5 ng/mL was obtained. Concentrations of infliximab derived from this curve displayed an accuracy of 89 – 116% and a %CV < 8.5% (**Figure 14**).

Conclusions

A general assay was developed that accurately and reproducibly quantified mAbs (both IgG_1 and IgG_2) from complex biological fluids.

- Signature peptide release was optimized through enhanced binding of target mAbs to immunoaffinity beads and improved proteolytic digestion conditions.



Component Name	Actual Concentration	Num. Values	Mean	Standard Deviation	Percent CV	Accuracy	Value #1	Value #2	Value #3
YASESMSGIPSR.+2y10 LC	5.00	3 of 3	5.81	0.49	8.43	116.27	5.95	6.22	5.27
YASESMSGIPSR.+2y10 LC	10.00	3 of 3	10.50	0.15	1.44	104.95	10.37	10.66	10.46
YASESMSGIPSR.+2y10 LC	50.00	3 of 3	46.80	3.30	7.06	93.60	50.32	46.32	43.76
YASESMSGIPSR.+2y10 LC	100.00	3 of 3	99.91	7.43	7.43	99.91	107.46	99.64	92.62
YASESMSGIPSR.+2y10 LC	500.00	3 of 3	445.13	7.58	1.70	89.03	451.34	447.37	436.68
YASESMSGIPSR.+2y10 LC	1000.00	3 of 3	1016.93	41.30	4.06	101.69	1063.03	983.32	1004.44
YASESMSGIPSR.+2y10 LC	5000.00	3 of 3	4656.39	385.98	8.29	93.13	5080.43	4325.52	4563.22
YASESMSGIPSR.+2y10 LC	10000.00	3 of 3	10081.91	392.72	3.90	100.82	10332.51	10283.92	9629.31
YASESMSGIPSR.+2y10 LC	50000.00	3 of 3	50301.62	891.58	1.77	100.60	50322.63	51182.51	49399.72

GLP level Statistics: < 8.5% CV; 93-116 % Accuracy

Figure 14. Quantification statistics for infliximab. Quantification results for the infliximab signature peptide YASESMSGIPSR are shown. Chromatographic traces for the infliximab peptide are shown for varying concentrations of infliximab (*upper left panel*). The calibration curve generated from varying concentrations of infliximab is linear for 4 orders of magnitude (*upper right panel*). The quantitation statistics are shown in the table provided, (*lower panel*) and meet the required bioanalytical acceptance criteria for accuracy and precision.

- An MRM-based peptide quantitation method using an LC/MS/MS approach provided accurate and precise measurements of mAb-based therapeutics in biological samples.
- New high-capacity streptavidin-coated magnetic beads showed good specificity and sample recovery, producing LLOQs and ULOQs for target mAbs of 5 ng/mL and 100 µg/mL, respectively.
- Calibration curves showed excellent linearity for 4 orders of magnitude.
- The combination of trypsin/Lys C with the BioBA surfactant improved overall peptide coverage up to 45% compared to mAb digestions with Trypsin Gold.
- Signature peptide peak area was also increased up to 10-fold when mAbs were treated with trypsin/LysC and BioBA surfactant.
- The hybrid LBA/LC/MS-based assay complements ELISA-only-based assays by providing additional selectivity and specificity.
- The use of sample extraction protocols with ready-to-use consumables will rapidly accelerate biologics bioanalysis and the quantitation of large-molecule protein-based therapeutics.

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