Ligand Binding Mass Spectrometric Immunoassay (LB-MSIA™) Workflow with Deglycosylation for Therapeutic Antibodies

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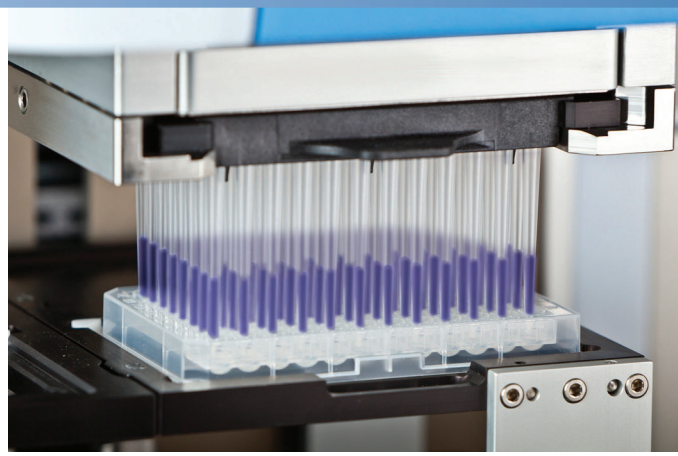
Goal:

To illustrate how pre-analytical deglycosylation of the antibody decreases data complexity and increases sensitivity that further improves upon the LB-MSIA work flow; a pre-clinical bio-analytical solution, based on mass spectrometric detection, specific for the bioanalysis of humanized and fully human therapeutic monoclonal antibodies using adalimumab in rodent plasma as a model biological system.

Introduction

A systematic study was performed to develop a universal workflow solution for the targeted analysis of humanized and fully human therapeutic mAbs that provides characterization information necessary to keep pace with new mAb therapeutic innovation and increased biological complexity. Focusing on the enablement of preclinical discovery and development research, the resultant automated and high throughput Ligand Binding-Mass Spectrometric Immunoassay (LB-MSIA™) combines the robust nature of traditional ligand binding assays with HRAM (High Resolution/Accurate Mass) mass spectrometric detection of intact mAbs. This hybrid bio-analytical workflow is specifically enabled by Streptavidin MSIA D.A.R.T.'S technology; a proprietary product that contains molecular trapping micro-columns covalently derivatized with streptavidin within a pipette housing. When the MSIA D.A.R.T.'S are coupled with a high affinity reagent, such as biotinylated anti-human IgG Fc affinity ligands, the workflow is able to selectively analyze for fully human and humanized therapeutic mAbs within rodent model plasma. Furthermore, the addition of a pre-analytical deglycosylation to this hybrid workflow also helps to meet the demanding data requirements for biotransformation assessments. For instance, a new class of mAb based therapeutics, Antibody-Drug Conjugates (ADC), has unique data requirements for the establishment of Drug-Antibody Ratios (DAR) that are lacking standardized methodologies.

This LB-MSIA that includes the deglycosylation demonstrated percent coefficients of variation (% CV) and sensitivity akin to a traditional ligand binding assay.



Specifically, the reproducible detection of the therapeutic mAb, adalimumab, was achieved at concentrations aslow as 20 ng/mL directly from mouse plasma, an improvement over the 125 ng/mL limit-of-detection obtained with the non-deglycosylated intact workflow demonstrated in the previous application note (APAAMSIALB0615).

Materials

- Thermo Scientific™ Streptavidin MSIA™ D.A.R.T.'S, PN: 991STR12
- Thermo Scientific™ Finnpipette™ Novus i Multichannel Electronic Pipette, PN: 991SP12
- Alternative High-Through-Put Option: Thermo Scientific™ Versette™ Automated Liquid Handler
- Thermo Scientific™ Finnpipette™ F1 Adjustable-Volume Pipettes, PN: 4700850
- CaptureSelect™ Biotin Anti-IgG-Fc (Human) Conjugate, PN: 7103262100
- Abbvie™ Humira® (adalimumab)
- Mouse Plasma (K2 EDTA)
- Thermo Scientific™ BupH™ Modified Dulbecco's Phosphate Buffered Saline (PBS) Packs, PN: 28374
- MSIA™ Elution Buffer

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Materials cont.

- Fisher Chemical™ Optima™ Ammonium Hydroxide, PN: A470
- Fisher BioReagents™ Ammonium Bicarbonate, P N: BP2413500
- New England BioLabs™ glycerol-free PNGase F, PN: P0705
- Thermo Scientific™ HERAtherm™ Advanced Protocol Microbiological Incubator (37 °C)
- Fisher Chemical™ Optima™ LC/MS Grade Water, PN: W6
- Fisher Chemical™ Optima™ LC/MS Grade Formic Acid, PN: A117
- Fisher Chemical™ Optima™ LC/MS Grade Acetonitrile, PN: A955
- Thermo Scientific™ Nunc™ 500µL 95-Well Plates, Polypropylene, PN: 12-565-368
- Thermo Scientific™ Nunc™ 1.3 and 2.0mL DeepWell Plates with Shared-Wall Technology, 96 DeepWell, Polypropylene, PN: 12-565-650
- Thermo Scientific™ ProSwift™ RP-4H Monolith Column, 1.0 x 250 mm, PN: 066640
- Thermo Scientific™ Dionex™ UltiMate® 3000 UHPLC System
- Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer
- Thermo Scientific™ XCalibur™ Software, Version 2.2
- Thermo Scientific™ Protein Deconvolution Software, Version 3.0 with the ReSpect™ algorithm

Method

The LB-MSIA workflow for the bio-analysis of therapeutic antibodies may be broken down into five major steps as illustrated in Figure 1. A Thermo Scientific™ Novus i electronic pipette was used to provide the repetitive bi-directional pipetting (aspirating and dispensing cycles) necessary to pass solutions through the micro-column housed within each of the MSIA D.A.R.T.'S. The Streptavidin MSIA D.A.R.T.'S are first derivatized with a biotin-conjugated anti-IgG Fc, an affinity ligand that specifically binds to the Fc portion of all four human IgG subclasses. The next step is to assay for the fully human therapeutic monoclonal antibody (adalimumab) from rodent plasma samples by incubating the samples with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S. The affinity bound adalimumab is subsequently released from the micro-column by treatment with the elution buffer and then deglycosylated using PNGase F. The ensuing eluate containing deglycosylated intact adalimumab is then analyzed using LC-MS (HRAM). Utilizing Thermo Scientific's™ XCalibur™ (Version 2.2) and Protein Deconvolution (Version 3.0) Software the resulting raw HRAM MS data is processed to provide high content qualitative data.

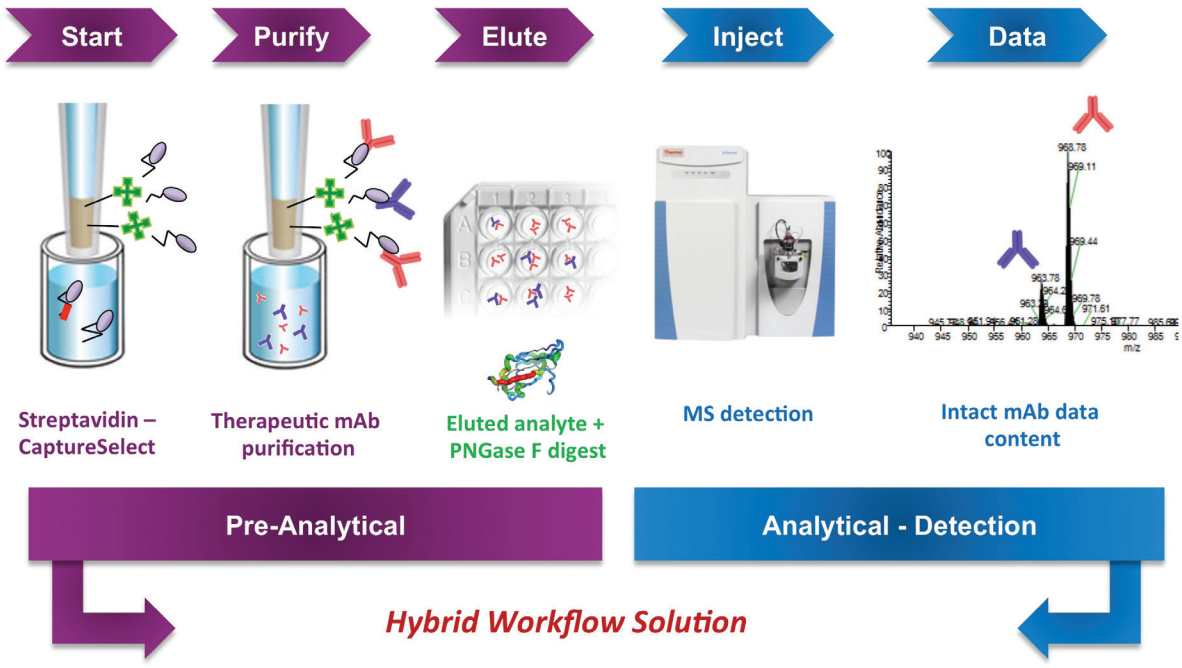

Figure 1: A schematic showing the five major steps of the LB-MSIA Workflow

Method cont.

Pre-Analytical

Derivatization of Streptavidin MSIA D.A.R.T.'S with Affinity Ligand

To enable the Streptavidin MSIA D.A.R.T.'S to have a specific affinity for humanized and fully human mAbs, each of the streptavidin derivatized micro-columns were loaded with 125 µL of 4 µg/mL CaptureSelect™ biotin anti-IgG-Fc (Human) conjugate, a single domain antibody (Life Technologies), prepared in PBS (BupH™ Modified Dulbecco's PBS). This was accomplished by following the steps provided in Table 1 utilizing a Thermo Scientific™ Novus i electronic pipette equipped with Streptavidin MSIA D.A.R.T.'S.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	▲ / ▼ Speed
1	Buffer Pre-Rinse	PBS	200	150	10x	4
2	Immobilization of anti-IgG-Fc	Biotin anti-IgG Fc conjugate antibody	125	70	500x	1
3	Buffer Rinse	PBS	200	150	10x	4
4	Buffer Rinse	PBS	200	150	10x	4

Table 1 – Derivatization of Streptavidin MSIA D.A.R.T.'S with Biotinylated Anti-Human IgG Fc; Novus i Protocol in Descending Order

Sample Preparation

All samples prepared consisted of 200 µL of mouse plasma supplemented with varying concentrations of adalimumab within the range of 20 - 8000 ng/mL. Prior to incubation of the samples with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S each sample was further diluted with 200 µL PBS. Using the Novus i, the following steps outlined in Table 2 were performed to capture adalimumab from the samples.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	▲ / ▼ Speed
1	Adalimumab Capture by Anti-IgG-Fc MSIA D.A.R.T.'S	Sample Solution	400	300	500x	1
2	Buffer Rinse	PBS	200	150	10x	4
3	Buffer Rinse	PBS	200	150	10x	4
4	Water Rinse	Water	200	150	10x	4
5	Water Rinse	Water	200	150	10x	4

Table 2 – Adalimumab Capture; Novus i Protocol in Descending Order

Sample Elution

Following the selective capture of adalimumab with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S, each device was treated with 50 µL of the MSIA™ Elution Buffer liberating the adalimumab. Reference Table 3 for the specifics of the repetitive pipetting used to elute captured adalimumab from the D.A.R.T.'S.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	▲ / ▼ Speed
1	Elution	Elution Buffer	50	30	20x	4

Table 3 – Novus i Protocol for Eluting Affinity-Captured Adalimumab from Anti-IgG-Fc-Derivatized D.A.R.T.'S.

Deglycosylation

To deglycosylate the adalimumab present in each eluate, 7 µl of 50% ammonium hydroxide was added followed by 5 µL of 50 units/µL PNGase F prepared in 100mM ammonium bicarbonate, pH 8.5. The microplate containing the eluates was then sealed and incubated at 37 °C overnight for 15-18 hours. To quench the deglycosylation process and have the sample ready for LC-MS analysis, an additional 38 µL of elution buffer was added to each eluate in order to reduce the pH below 7. The deglycosylated intact adalimumab was then detected by LC-MS (HRAM).

Analytical Detection

Liquid Chromatography

The affinity-purified adalimumab eluates were separated on a Thermo Scientific™ Dionex™ UltiMate® 3000 RSLC (Rapid Separation Liquid Chromatography) system utilizing a Thermo Scientific™ ProSwift™ RP-4H (1 x 250 mm) column heated to 60 °C. Separation was performed utilizing a gradient of 10% - 32% of 0.2% formic acid in acetonitrile over 12 minutes at a flow rate of 200 µL/min.

Mass Spectrometry

For all samples, full-scan MS data were acquired over the range of m/z 2,000 - 4,500 in positive-ion mode on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer with a resolving power of 17,500 (FWHM) at m/z 200 and the AGC (Automatic Gain Control) set to a target value of 3.00E6.

Data Analysis

All LC-MS raw data was collected using Thermo Scientific's XCalibur™ Software, Version 2.2. From the raw MS data an extracted ion chromatogram was generated for the five most abundant charge states of the deglycosylated intact adalimumab (Figure 2B and 2C), which were then integrated to obtain the AUC (Area Under the Curve) value for each sample analyzed.

Further characterization of adalimumab, specifically in reference to the presence of glycosylation, was obtained from processing the MS raw data using Thermo Scientific's™ Protein Deconvolution™ Software Version 3.0 utilizing the ReSpect™ algorithm.

Results and Discussion

The MSIA workflow provides a unique push button and automated solution for therapeutic mAb bio-analytics. By combining the performance characteristics of traditional ligand binding assays with the benefits of HRAM MS detection, high value data content is produced that is highly sensitive, robust, and reproducible. The molecular trapping technology of the MSIA D.A.R.T.'S creates an ideal scenario to assay therapeutics from plasma utilizing high affinity anti-IgG-Fc binders specific for fully human mAbs. The additional pre-analytical deglycosylation further improves upon the ability to identify post-translational modifications that may be present in complex in vivo biotransformation studies and Drug Antibody Ratio (DAR) determination. The integration of the Q Exactive for HRAM detection helps provide additional analytical flexibility over other developing triple quadrupole methods reliant on peptide analysis.

Detection of Deglycosylated Intact Adalimumab

The data presented in Figure 2 shows the analysis of deglycosylated intact adalimumab purified from a sample containing 100ng adalimumab at a concentration of 500 ng/mL in mouse plasma. The LC elution profile shown in Figure 2A indicates a retention time of eleven minutes for the deglycosylated intact adalimumab. No mouse IgG or other mouse proteins were detected when the entire LC elution gradient (2-14 minutes) was scanned in the MS and data deconvolved using the Protein Deconvolution software (data not shown). This indicates the high selectivity that can be achieved with the LB-MSIA workflow. Figures 2B and 2C respectively show the resulting mass spectrum of deglycosylated intact adalimumab and the corresponding zoom-in of m/z 2600-2880 to highlight the five most abundant charge states.

The mass spectrum was then deconvolved resulting in Figure 3A. The deconvolved data showed two masses with the 145191 mass representing deglycosylated intact adalimumab, while the other mass, 145318, is shifted by ~127 Da, suggestive of the presence of additional two potassium and three oxidations. As a comparison to the deglycosylated intact adalimumab data, Figure 3B shows deconvolved mass spectrum data for the non-deglycosylated intact adalimumab. The non-deglycosylated adalimumab data shows three masses, with 148080, 148244, and 148407 representing the addition of hexose groups (162 Da) to adalimumab.

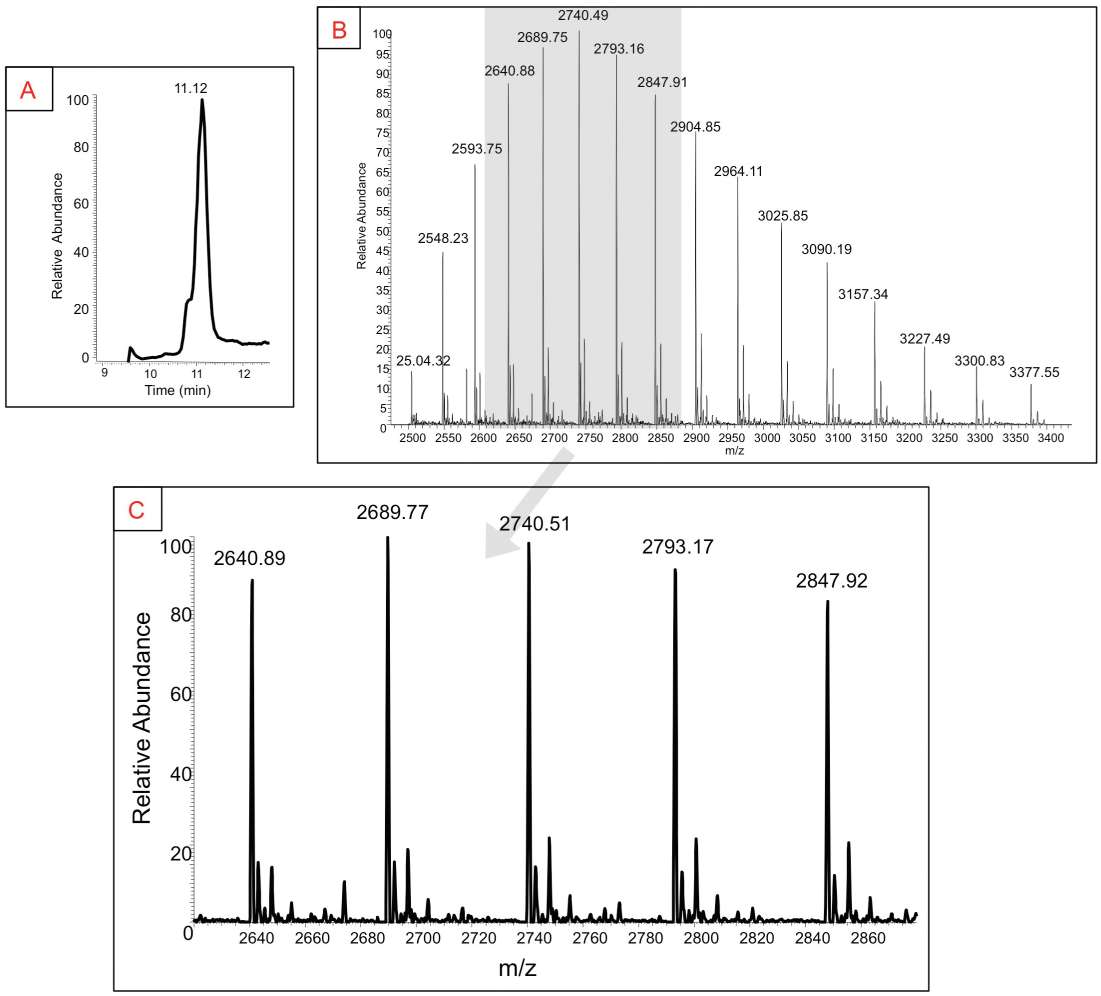

Figure 2.Deglycosylated analysis of adalimumab: 100ng adalimumab sample at a concentration of 500 ng/mL purified from mouse plasma. A) Base peak chromatogram of adalimumab showing the elution profile of deglycosylated adalimumab. B) and C) Raw MS spectra and corresponding zoom-in of the region m/z 2600-2880 around the five most abundant charge states, respectively.

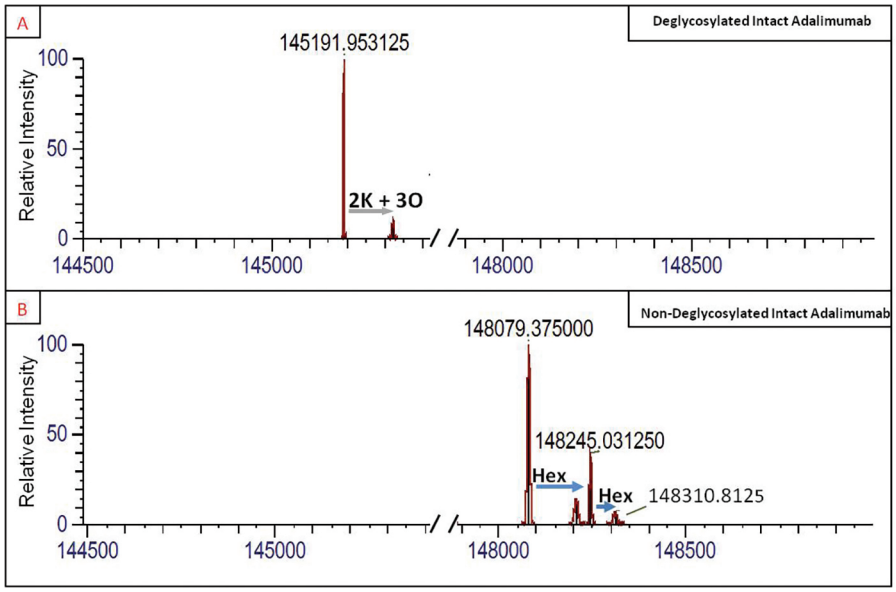

Figure 3 A). The deconvolved average mass (M+H) of deglycosylated intact adalimumab. **B)** The deconvolved average mass (M+H) of non-deglycosylated intact adalimumab.

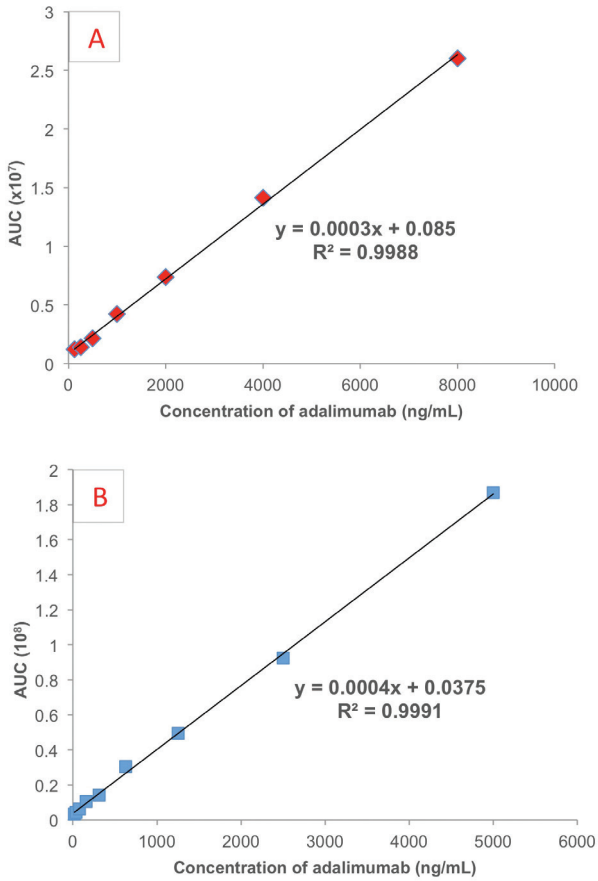

Figure 4 - Dynamic range of therapeutic mAb MSIA: Dynamic range of non-deglycosylated intact adalimumab; 125-8000 ng/mL. **B)** Dynamic range of deglycosylated intact adalimumab; 20-5000 ng/mL.

Dynamic Range Comparison Between Non-Deglycosylated and Deglycosylated Intact Adalimumab

To test the sensitivity of the developed universal LB-MSIA workflow for the pre-clinical bio-analysis of therapeutic mAbs (specifically adalimumab) from mouse plasma, samples were run producing a plot akin to a dosing curve represented in Figure 4. Triplicate samples were prepared and analyzed for each concentration. For each sample the AUC from the extracted ion chromatogram for the top five charge states (Refer to Figure 2C) were summed and the average for each concentration was then used to generate the plots in Figure 4. The assay achieved a linear dynamic range of 125 - 8000 ng/mL for the detection of non-deglycosylated intact adalimumab with CVs within the acceptable range for traditional ligand binding assays. However, when the intact adalimumab was deglycosylated an improved lower limit of detection with a linear dynamic range of 20-5000 ng/mL was achieved with coefficients of variation of 6% and accuracies within 7% (Table 4). These improvements are the results of reducing the heterogeneity and complexity of the therapeutic mAb. An improved linearity (R2 =0.9991) was also observed with the deglycosylated intact adalimumab data when compared with the data from the non-deglycosylated intact adalimumab (R2 = 0.9988).

mAb Analyzed	Control Conc. (ng/mL)	Average Experimental Conc. (ng/mL)	STDEVP	CVs
Non-deglycosylated Intact Adalimumab	1000	650	4.84E-02	7.4%
Deglycosylated Intact Adalimumab	500	465	1.35E-02	6.0%

Table 4. Summary of the adalimumab control samples studied. Control samples also utilized 200 µL of mouse plasma.

Conclusion

A hybrid approach for the universal bio-analysis of fully human and humanized therapeutic mAbs in pre-clinical research was demonstrated and improved upon by the addition of a pre-analytical deglycosylation. The developed LB-MSIA provided a highly sensitive, robust, and reproducible method for the generation of high value data content for the bio-analysis of therapeutic antibodies. Both the raw and the deconvolved HRAM mass spectra generated in this study clearly showed the presence of the post-translational modifications of adalimumab, thus making MSIA amiable for additional bio-analyses, including complex in vivo biotransformation studies and Drug Antibody Ratio (DAR) determination. The high selectivity of the CaptureSelect™ biotin anti-IgG-Fc (human) conjugate combined with the molecular trapping technology of the MSIA D.A.R.T.'S creates an ideal scenario to assay low abundant (ng/mL) intact human therapeutic mAbs from rodent plasma. As a hybrid approach, the use of the Q Exactive for HRAM detection helps provide additional analytical flexibility and data content over other developing triple quadrupole methods that are reliant on peptide analysis. As shown, the combined benefits of the LB-MSIA enable the characterization of a deglycosylated mAb over a wide dynamic range (20-5000 ng/mL) while maintaining coefficients of variation of <15% and accuracies within 20%.

Ordering Information

MSIA D.A.R.T.'S for Immunoaffinity Capture		
Compatible with the Thermo Scientific Versette Automated Liquid Handler and Thermo Scientific Finnpipette® Novus i Multichannel Electronic Pipette		
Cat. No.	**Description**	**Packaging**
991CUS02	300µl MSIA D.A.R.T.'S, Custom	Pack of 96 units
991PRT11	300µl MSIA D.A.R.T.'S, Protein A	Pack of 96 units
991PRT12	300µl MSIA D.A.R.T.'S, Protein A	Pack of 24 units
991PRT13	300µl MSIA D.A.R.T.'S, Protein G	Pack of 96 units
991PRT14	300µl MSIA D.A.R.T.'S, Protein G	Pack of 24 units
991PRT15	300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 96 units
991PRT16	300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 24 units
991STR11	300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 96 units
991STR12	300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 24 units
991001096	300µl MSIA D.A.R.T.'S, Insulin	Pack of 96 units
991001024	300µl MSIA D.A.R.T.'S, Insulin	Pack of 24 units
991R	300 µL MSIA D.A.R.T.'S, Reloadable Rack	1 reloadable rack, D.A.R.T.'S are not included
Automated Liquid Handling Platform		
Cat. No.	**Description**	
650-MSIA	MSIA Versette Automated Liquid Handler	
Multichannel Pipettes and Pipette Stand		
Cat. No.	**Description**	**Packaging**
991S	Finnpipette Novus i Adjustable Pipette Stand	1 pipette stand
991SP12	Finnpipette Novus i Electronic 12-Channel Pipette, 30-300µl and Pipette Stand	1 pipette and 1 pipette stand
Liquid Chromatography		
Cat. No.	**Description**	
	Thermo Scientific™ Dionex™ UltiMate® 3000 UHPLC System	
066640	ProSwift™ RP-4H Monolith Column, 1.0 x 250 mm	
Mass Spectrometry and Software		
Description		
Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer		
Thermo Scientific™ TSQ Vantage Triple Stage Quadrupole Mass Spectrometer		
Thermo Scientific™ Pinpoint Software		
Thermo Scientific™ XCalibur™ Software		
Thermo Scientific™ Protein Deconvolution Software, Version 3.0 with the ReSpect™ algorithm		

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