A Universal LB-MSIA workflow using Freedom EVO platform for the Quantitative Analysis of Intact Therapeutic Antibodies of Different Allotype Subclasses

Application Note

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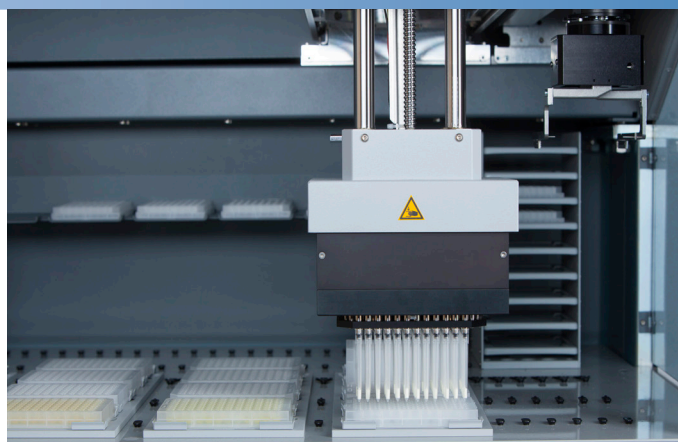
Key Words

Q Exactive Plus, monoclonal antibodies, deconvolution, HRAM, high resolution, accurate mass, MSIA, mass spectrometric immunoassay, high throughput, Tecan™, Freedom EVO®, MSIA Streptavidin-EVO



Goal:

To demonstrate the effectiveness of quantitatively analyzing therapeutic mAbs of different constructs and allotype subclasses from rodent plasma using the Thermo Scientific™ enhanced capacity MSIA™ Streptavidin EVO microcolumns; a pre-clinical bioanalytical solution, on the Tecan Freedom EVO® platform, based on mass spectrometric detection, specific for the bioanalysis of humanized, fully human and chimeric therapeutic monoclonal antibodies.

Introduction:

Classical protein analytical techniques, such as LBAs (Ligand Binding Assays) are not able to meet the data needs for pharmacokinetics, biotransformation assessment, and antibody functional determination studies. For example, an LBA would not provide the unique data requirements necessary for the establishment of DARs (Drug-Antibody Ratios) for Antibody-Drug Conjugates (ADCs). Many of these data requirements are met by the use of mass spectrometric (MS) based assays.

The Ligand Binding-Mass Spectrometric Immunoassay (LB-MSIA™) is a universal workflow for targeted pre-clinical analysis of biotherapeutics that combines the robust nature of traditional ligand binding assays with High Resolution/Accurate Mass (HRAM) spectrometric detection. This hybrid bioanalytical workflow is specifically enabled by MSIA Streptavidin EVO micro column technology; a proprietary product that is designed for the Tecan Freedom EVO platform and contains molecular trapping microcolumns covalently derivatized with streptavidin embedded within a pipette tip. Mounted onto Tecan's reliable and robust Freedom EVO equipped with a MCA96 liquid handling arm, the MSIA Streptavidin EVO microcolumns provide high throughput automated sample processing.

The functional design of the MSIA Streptavidin EVO microcolumns combined with the consistency of the Tecan Freedom EVO provides a high level of ease-of-use and reproducibility that is not present in bead-based methodologies. When the MSIA Streptavidin EVO microcolumns are coupled with a high affinity reagent, such as biotinylated anti-human IgG Fc affinity ligands, the workflow is able to selectively analyze for mAbs of different human IgG subclasses.

Below is a demonstration of the selectivity of this LB-MSIA for mAbs of different constructs and different allotype subclasses that provides quantitative data for each of the intact therapeutic mAbs spiked within rodent plasma. In order to quantitate the intact therapeutic mAbs the MS data was deconvolved resulting in peak intensities that represent the mAbs and their glycosylated forms. This next generation LB-MSIA workflow automated by the Freedom EVO platform, for the quantitative analysis of adalimumab (Humira®), infliximab (Remicade®), natalizumab (Tysabri®) and trastuzumab (Herceptin®), demonstrated to be reproducible (percent coefficients of variation < 20%), accurate (accuracy within 19%) and with a dynamic range over three orders of magnitude.

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Materials:

- Thermo Scientific™ MSIA™ Streptavidin-EVO, PN: 992STR96
- Tecan™ Freedom EVO® 150 Liquid Handling Robotic Platform equipped with a MCA96 head
- Thermo Scientific™ Finnpipette™ F1 Adjustable-Volume Pipettes, PN: 4700850
- CaptureSelect™ Biotin Anti-IgG-Fc (Human) Conjugate, PN: 7103262100
- adalimumab (Humira®, Abbvie™)
- infliximab (Remicade®, Janssen™)
- natalizumab (Tysabri®, Biogen®)
- trastuzumab (Herceptin®, Genentech™)
- Sigma-Aldrich® SILu™Lite SigmaMAb Universal Antibody Standard human, PN: MSQC4
- Mouse Plasma (K2 EDTA)
- Thermo Scientific™ BupH™ Modified Dulbecco's Phosphate Buffered Saline (PBS) Packs, PN: 28374
- MSIA™ Elution Buffer
- 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 96-Well Plates, Polypropylene, PN: 12-565-368
- Thermo Scientific™ ProSwift™ RP-4H Monolith Column, 1.0 x 250 mm, PN: 066640
- Thermo Scientific™ Vanquish™ UHPLC System
- Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer
- Thermo Scientific™ Xcalibur™ Software, Version 3.0
- Thermo Scientific™ Protein Deconvolution Software, Version 4.0 with the ReSpect™ algorithm

Method

The LB-MSIA workflow for the bioanalysis of therapeutic mAbs may be broken down into five major steps as illustrated in Figure 1. A Tecan Freedom EVO 150 Liquid Handling Robotic Platform equipped with the MCA96 head was used to provide automated repetitive bi-directional pipetting (aspirating and dispensing cycles) through the use of the Mix Command during script generation. The MSIA Streptavidin EVO microcolumns 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, humanized or chimeric therapeutic mAb from rodent plasma samples by incubating the samples with the anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns. The affinity bound mAbs are subsequently released from the microcolumn by treatment with elution buffer. The ensuing eluates containing the intact therapeutic mAbs are then analyzed using LC-MS (HRAM). Utilizing Thermo Scientific's™ Xcalibur™ (Version 3.0) and Protein Deconvolution (Version 4.0) Software, the resulting raw HRAM MS data are processed to provide high content quantitative data.

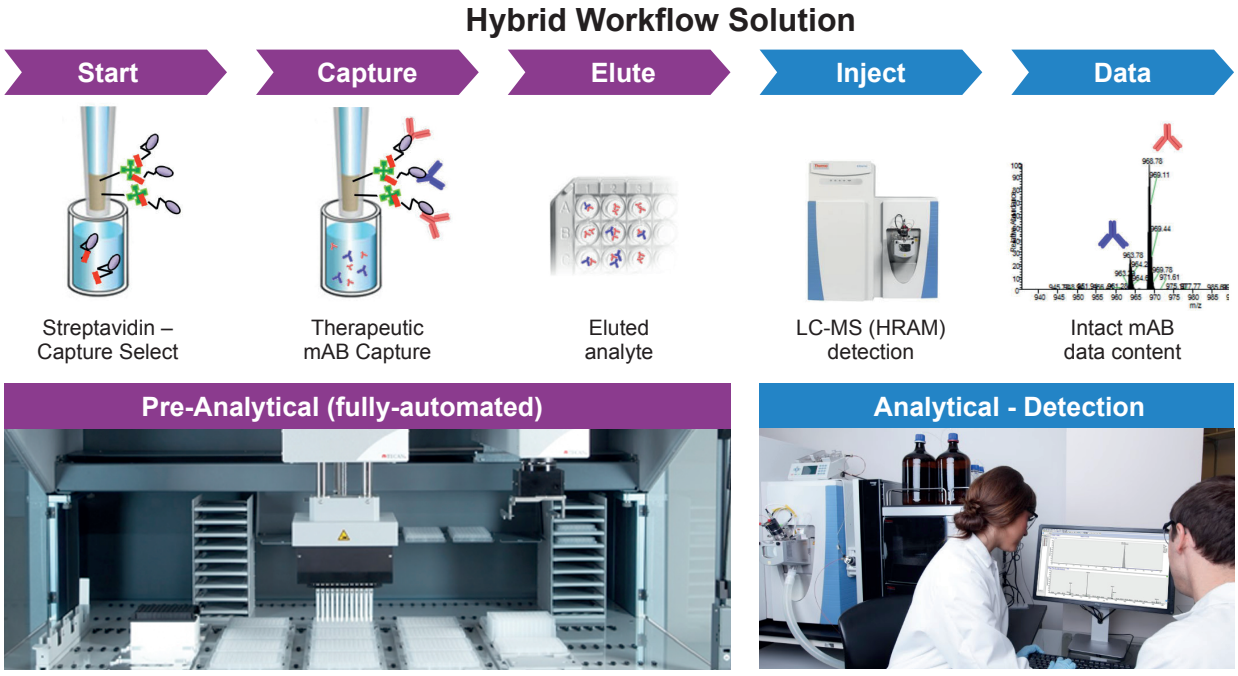


Figure 1– A schematic showing the five major steps of the LB-MSIA workflow

Pre-Analytical

Derivatization of MSIA Streptavidin EVO Microcolumns with Affinity Ligand

To enable the MSIA Streptavidin-EVO to have a specific affinity for the Fc region of human IgG subclasses, each of the streptavidin derivatized microcolumns were loaded with 125 µL of 12 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 Tecan Freedom EVO 150 Liquid Handling Robotic Platform with a MCA96 Head equipped with MSIA Streptavidin EVO microcolumns.

	Assay Step	Assay Solution	Total Well Volume (µL)	Mix Volume (µL)	Mix Cycles	Flow Rate (µL/sec)
1	Buffer Pre-Rinse	PBS	200	150	10x	115
2	Immobilization of anti-IgG-Fc	Biotin anti-IgG Fc conjugate antibody	125	70	500x	45
3	Buffer Rinse	PBS	200	150	10x	115
4	Buffer Rinse	PBS	200	150	10x	115

Table 1 – Derivatization of Streptavidin MSIA EVO microcolumns with Biotinylated Anti-Human IgG Fc; Freedom EVO® Protocol in Descending Order

Sample Preparation

All samples prepared consisted of 20 µL of mouse plasma supplemented with varying concentrations of one of the four therapeutic mAbs (adalimumab, infliximab, natalizumab or trastuzumab) as referenced in Table 2. The therapeutic mAb control sets were each prepared in replicates of five and the dynamic curve was prepared in duplicate. Then the curve and control sets were performed three times over three days.

Therapeutic mAb Samples	Concentration (µg/mL)
Adalimumab Dynamic Range	0.3125–320
Infliximab Dynamic Range	0.3125–160
Trastuzumab Dynamic Range	0.3125–320
Natalizumab Dynamic Range	0.3125–160
Control Set 1	3.75
Control Set 2	15
Control Set 3	120

Table 2 – Therapeutic mAb Sample Concentrations. Each control set was prepared individually for each mAb and consisted of 5 replicate samples and each dynamic curve was run in duplicate. The control sets and dynamic curve sets were repeated three times over three days.

Prior to incubation of the samples with the anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns each sample was further diluted with 80 µL of PBS supplemented with 0.7 µg of SILu™Lite SigmaMAb IS (Internal Standard). Using the Freedom EVO, the following steps outlined in Table 3 were performed to capture the therapeutic mAbs from the samples.

	Assay Step	Assay Solution	Total Well Volume (µL)	Mix Volume (µL)	Mix Cycles	Flow Rate (µL/sec)
1	Therapeutic mAb Capture*	Sample Solution	400	200	625x	46
2	Buffer Rinse	PBS	200	150	10x	115
3	Buffer Rinse	PBS	200	150	10x	115
4	Water Rinse	Water	200	150	10x	115
5	Water Rinse	Water	200	150	10x	115

*The Therapeutic mAb Capture is performed by Anti-IgG Fc MSIA Streptavidin EVO microcolumns.

Table 3 – Therapeutic mAb capture; Freedom EVO protocol in descending order

Sample Elution

Following the selective capture of the therapeutic mAb with the anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns, each device was treated with 100 µL of the MSIA Elution Buffer liberating the mAb. Reference Table 4 for the specifics of the repetitive pipetting used to elute the captured mAb from the MSIA Streptavidin EVO microcolumns. The intact mAb was then detected by LC-MS (HRAM).

	Assay Step	Assay Solution	Total Well Volume (µL)	Mix Volume (µL)	Mix Cycles	Flow Rate (µL/sec)
1	Elution	Elution Buffer	100	40	20x	48

Table 4 – Freedom EVO® Protocol for Eluting Affinity-Captured mAb from Anti-IgG-Fc-Derivatized MSIA Streptavidin EVO microcolumns

Analytical-Detection

Liquid Chromatography

The affinity-purified mAb eluates were separated on a Thermo Scientific™ Vanquish™ UHPLC system utilizing a Thermo Scientific™ ProSwift™ RP-4H (1 x 250 mm) column heated to 60 °C. Separation was performed utilizing a gradient of 10-48% 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 2000-3400 m/z in positive-ion mode on a Thermo Scientific™ Q Exactive™ Plus 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™ Xcalibur™ Software, Version 3.0. Thermo Scientific™ Protein Deconvolution ™ Software Version 4.0 utilizing the SlidingWindow feature in the ReSpect™ algorithm was used to process the MS raw data. The intensities of the deconvolved peaks were used to determine the peak intensity ratio (mAb peaks/SILuLite IS peaks) for each sample analyzed.

Results and Discussion

Presented here is a study of the quantitative analysis of four therapeutic mAbs of different constructs and allotype subclasses (Reference Table 5) from rodent plasma. The LB-MSIA workflow provided a streamlined solution that was universally applicable to all four of the therapeutic mAbs.

mAb Analyzed	Construct	Allotype Subclass
Adalimumab (Humira®)	Humanized	IgG1-kappa
Infliximab (Remicade®)	Chimeric	IgG1-kappa
Trastuzumab (Herceptin®)	Fully Human	IgG1-kappa
Natalizumab (Tysabri®)	Humanized	IgG4-kappa

Table 5 – Description of therapeutic mAbs with differing constructs and allotype subclasses used to test the LB-MSIA™ workflow

Deconvolution Data Analysis for the Quantitation of the Therapeutic mAbs

In this application, peak intensities obtained from deconvolved mass spectra were used for plotting the mAb calibration curves. The deconvolution software was utilized in this application to reduce the complexity in the raw MS data for the mAbs (Figure 2). The deconvolution data in Figure 2 shows multiple variants consisting of different amounts of glycosylated SiLuLite and adalimumab. The highest deconvolved peak intensity for the SILuLite and each mAb was pre-determined and utilized for all samples (Figure 2, SILuLite: 146,824 Da, adalimumab: 148,081 Da). Each therapeutic mAb sample was normalized to the SILuLite (IS) peak producing the peak intensity ratio for each sample (mAb peaks/SILuLite peaks). Referring back to the sample preparation of the pre-analytical section of the method, note SILuLite is added to each sample prior to the affinity capture (Figure 2). Normalization of all steps beginning with the affinity capture step helps to increase sample analysis accuracy and reproducibility.

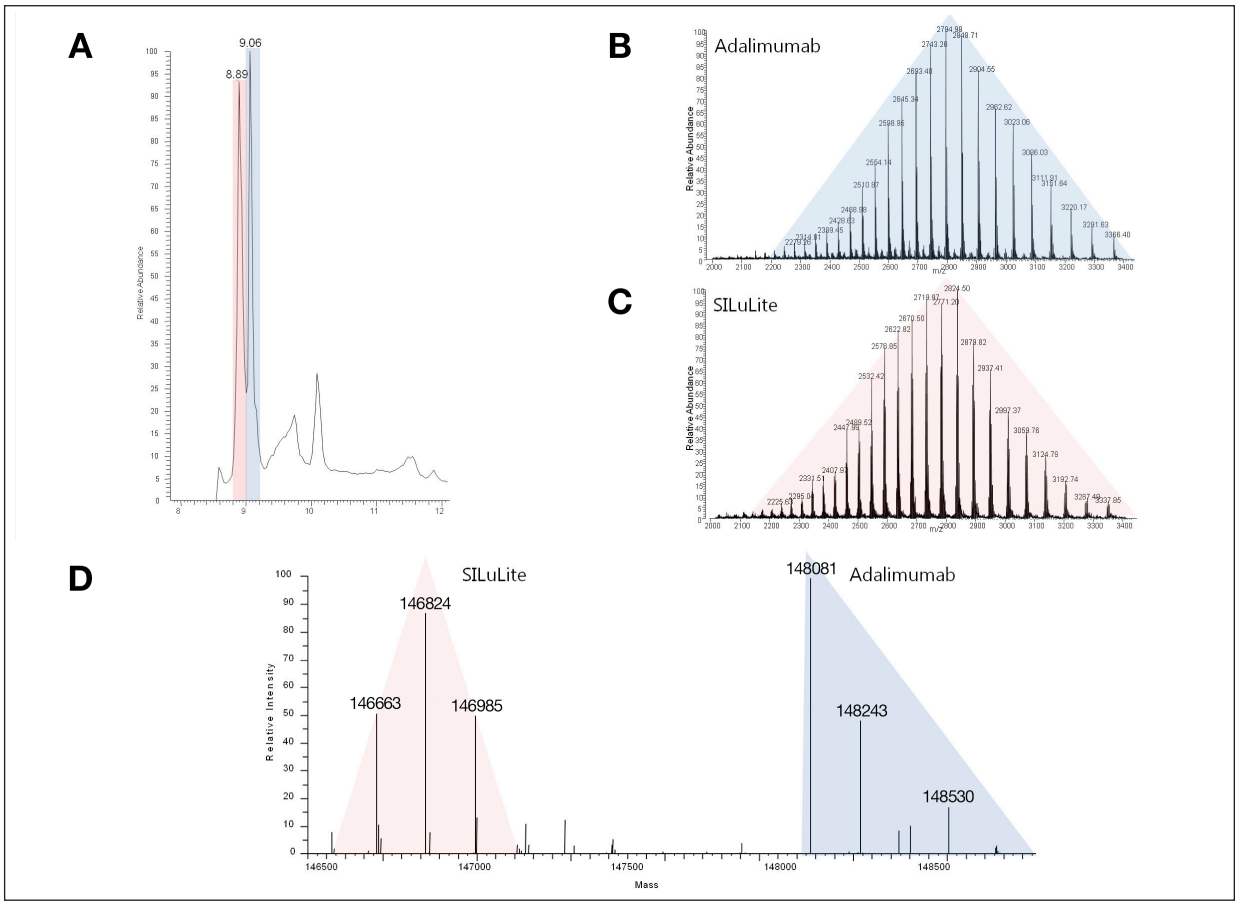
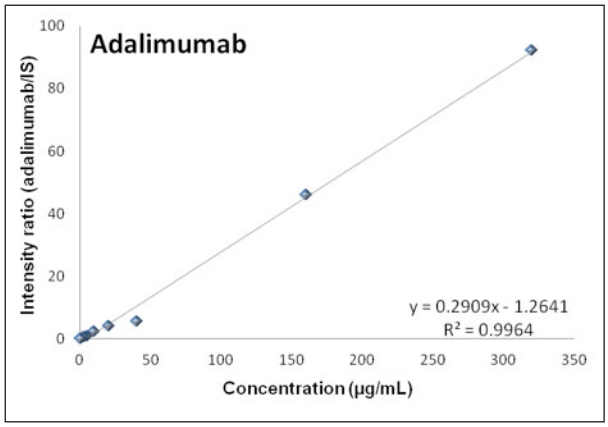
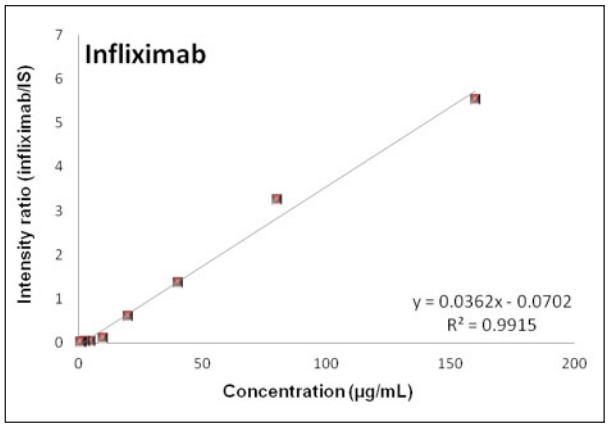


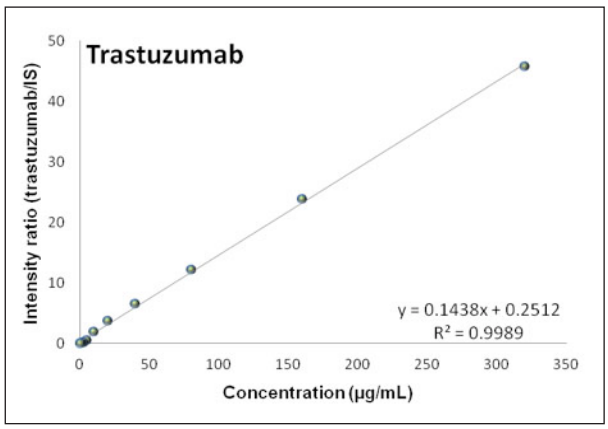
Figure 2 – The results of the LB-MSIA workflow performed on a 20 µL rodent plasma sample containing 10 µg/mL of adalimumab and 35 µg/mL SILuLite. A) Total ion chromatogram of the adalimumab sample showing the elution profile of intact adalimumab and SILuLite IS. B) Mass spectrum showing multiple charge states of adalimumab from the sample. C) Mass spectrum showing multiple charge states of the SILuLite IS from the sample. D) Deconvolved spectrum showing the masses and peak intensities for SILuLite (IS) (146824 Da) and adalimumab (148081).



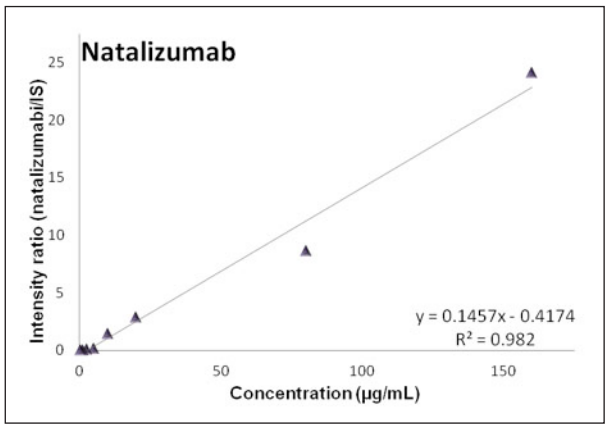
Adalimumab Control Sets			
Actual Conc. (µg/mL)	Experimental Conc. (µg/mL)	CV (%) n=5	Accuracy (%) n=5
120	97.7	13	-19
15	12.8	9	-15
3.75	4.1	8	8



Infliximab Control Sets			
Actual Conc. (µg/mL)	Experimental Conc. (µg/mL)	CV (%) n=5	Accuracy (%) n=5
120	104.7	11	-13
15	13.6	5	-9
3.75	3.4	5	-10



Trastuzumab Control Sets			
Actual Conc (µg/mL)	Experimental Conc. (µg/mL)	CV (%) n=5	Accuracy (%) n=5
120	110.5	7	-8
15	15.7	17	5
3.75	3.3	11	-13



Natalizumab Control Sets			
Actual Conc. (µg/mL)	Experimental Conc. (µg/mL)	CV (%) n=5	Accuracy (%) n=5
120	110.5	7	-8
15	15.7	17	5
3.75	3.3	11	-13

Figure 4 – Deconvolved Quantitation of four therapeutic mAbs analyzed with LB-MSIA from rodent plasma over a discontinuous period of 3 days: A) Adalimumab Working Curve Range of 0.3125-320 µg/mL. B) Infliximab Working Curve Range of 0.3125-160 µg/mL. C) Trastuzumab Working Curve Range of 0.3125-320 µg/mL.
D) Natalizumab Working Curve Range of 0.3125-320 µg/mL.

Conclusion

The demonstrated universal LB-MSIA workflow for the Freedom EVO platform utilizing MSIA Streptavidin EVO microcolumns with biotin-conjugated anti-IgG Fc provided an unmatched, highly sensitive, robust, and reproducible method for the quantitative analysis of several therapeutic mAbs of differing constructs and allotype subclasses. The combination of the CaptureSelect™ biotin anti-IgG-Fc (human) conjugate and the molecular trapping technology of the MSIA Streptavidin EVO microcolumns provides an ideal method of high selectivity that is able to assay low abundant (ng/mL) intact mAbs from rodent plasma with a dynamic range spanning three orders of magnitude. Furthermore, the workflow supported a high throughput application by performing the pre-analytical steps on the Tecan Freedom EVO 150 Liquid Handling Robotic Platform with a MCA96 Head. As a hybrid approach, the use of the Q Exactive Plus for HRAM detection helped provide additional analytical flexibility and data content by providing, in addition to the quantitative data, characterization data of intact therapeutic mAbs. Through the deconvolution of the MS data the biological complexity of the mAb samples was reduced allowing for consistent quantitative results. As shown, the combined benefits of the LB-MSIA enabled the quantitation of several therapeutic mAbs of different constructs and allotype subclasses with reproducibility that resulted in % coefficients of variation of less than 20% and accuracy within 19%.

Ordering Information

MSIA Streptavidin-EVO for Immunoaffinity Capture		
Compatible with the Tecan™ Freedom EVO® series of Platform equipped with a MCA96 liquid handling arm		
Cat. No.	**Description**	**Packaging**
992STR96	500µl MSIA Streptavidin-EVO	Pack of 96 units
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™ Pinpoint Software		
Thermo Scientific™ XCalibur™ Software		
Thermo Scientific™ Protein Deconvolution Software, Version 4.0 with the ReSpect™ algorithm		

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