

# A universal LB-MSIA workflow using Freedom EVO platform for the pre-clinical analysis of therapeutic antibodies of differing allotypes in rodent plasma

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## Key Words

Ligand Binding Mass Spectrometric Immunoassay, Therapeutic Monoclonal Antibodies, MSIA Streptavidin EVO microcolumns, Anti-IgG-Fc, Ligand Binding Assays, High Resolution/Accurate Mass, Freedom EVO, liquid handling, automation

## Goal:

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

## Introduction:

Presented is a demonstration of the developed Ligand Binding-Mass Spectrometric Immunoassay (LB-MSIA), a universal workflow solution for the targeted pre-clinical analysis of therapeutic mAb, to enable the analyses of therapeutic mAb of differing allotypes, providing characterization data necessary to keep pace with new mAb therapeutic innovation. Focusing on the enablement of preclinical discovery and development research, the resultant automated and high throughput LB-MSIA workflow for Freedom EVO platform 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 MSIA Streptavidin EVO microcolumns technology; a proprietary high capacity product that contains molecular trapping microcolumns covalently derivatized with streptavidin within a pipette housing. Mounted onto Tecan's reliable and robust Freedom EVO platform equipped with a MCA96 head, the MSIA Streptavidin EVO microcolumns provide an unparalleled, high throughput automated sample processing. 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 fully-human, humanized, and chimeric mAbs spiked within rodent plasma. Below is a demonstration of the selectivity of this LB-MSIA for mAbs of three different allotype subclasses; IgG1-kappa, IgG2-kappa and IgG4-kappa.

This next generation LB-MSIA workflow for Freedom EVO platform demonstrated percent coefficients of variation (% CV) and sensitivity akin to a traditional ligand binding assay for adalimumab (Humira®), infliximab (Remicade®), denosumab (Prolia®), natalizumab (Tysabri®) and trastuzumab (Herceptin®). In addition, the assay established the sensitive reproducible detection of intact therapeutic mAb as low as 244 ng/mL in mouse plasma.

## Materials:

- Thermo Scientific™ MSIA™ Streptavidin EVO microcolumns
- Tecan™ Freedom EVO® 150 Liquid Handling Robotic Platform equipped with a MCA96 head
- Thermo Scientific™ Finnpiptette™ F1 Adjustable-Volume Pipettes, PN: 4700850
- Thermo Scientific™ CaptureSelect™ Biotin Anti-IgG-Fc (Human) Conjugate, PN: 7103262100
- Abbvie™ adalimumab (Humira®)
- Janssen™ infliximab (Remicade®)
- Amgen® denosumab (Prolia®)
- Biogen® natalizumab (Tysabri®)
- Genentech™ trastuzumab (Herceptin®)
- 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™ 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™ 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
- Greiner Microplate 96, PN 655101

## 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 Tecan Freedom EVO 150 Liquid Handling Robotic Platform with the MCA96 head was used to provide the 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 monoclonal antibody from rodent plasma samples by incubating the samples with the anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns. The affinity bound mAb is subsequently released from the microcolumn by treatment with the elution buffer. The ensuing eluate containing the therapeutic mAb is 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 is processed to provide high content qualitative data.

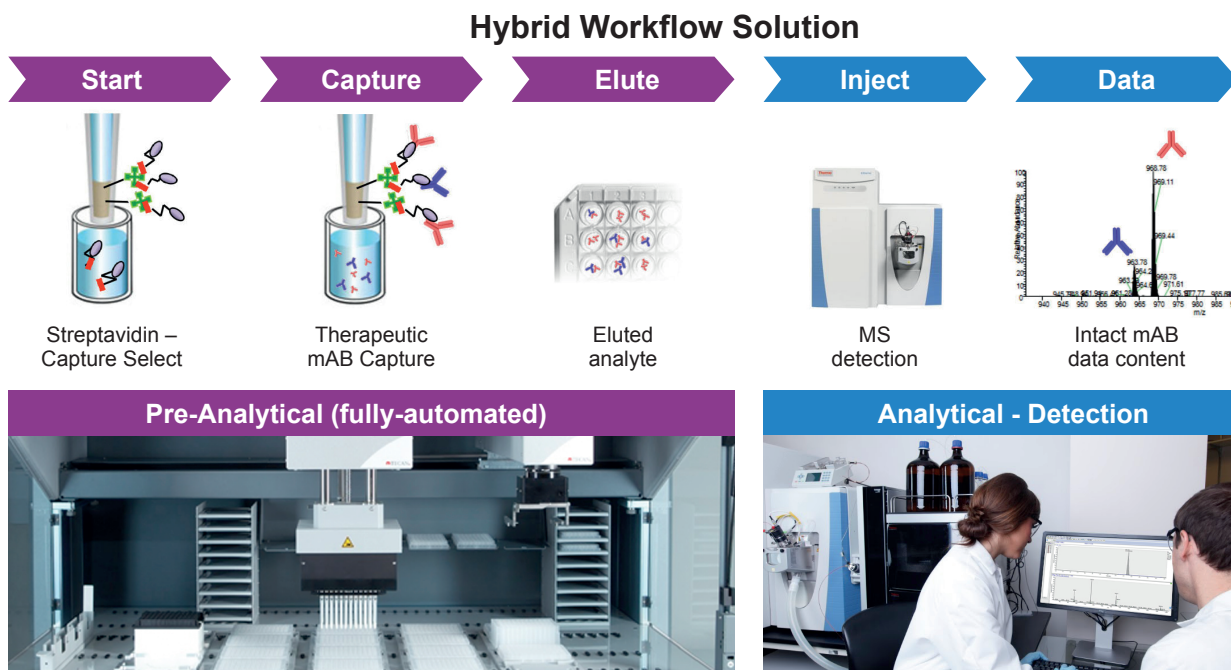


Figure 1— A schematic showing the five major steps of the LB-MSIA workflow

### Pre-Analytical

#### Derivatization of MSIA Streptavidin EVO Microcolumns with Affinity Ligand

To enable the MSIA Streptavidin EVO microcolumns to have a specific affinity for humanized, fully human and chimeric mAbs, each of the streptavidin derivatized microcolumns were loaded with 125  $\mu\text{L}$  of 12  $\mu\text{g/mL}$  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 ( $\mu\text{L}$ )	Mix Volume ( $\mu\text{L}$ )	Mix Cycles	Flow Rate ( $\mu\text{L}/\text{sec}$ )
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 MSIA Streptavidin EVO microcolumns with biotinylated anti-human IgG Fc; Freedom EVO protocol in descending order

#### Sample Preparation

All samples prepared consisted of 200  $\mu\text{L}$  of mouse plasma supplemented with varying concentrations of one of five therapeutic mAbs (adalimumab, infliximab, denosumab, natalizumab or trastuzumab). The dosing curve ranges utilized for each mAb are listed in the Table 2.

Therapeutic mAb	Dynamic Range ( $\mu\text{g/mL}$ )
Adalimumab (Hurmira)	0.244–125
Infliximab (Remicade)	0.244–31.25
Trastuzumab (Herceptin)	0.244–125
Denosumab (Prolia)	0.293–150
Natalizumab (Tysabri)	0.244–125

Table 2 – mAb dosing curve ranges for LB-MSIA sample preparation

Prior to incubation of the samples with the anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns each sample was further diluted with 200  $\mu\text{L}$  PBS. 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 ( $\mu\text{L}$ )	Mix Volume ( $\mu\text{L}$ )	Mix Cycles	Flow Rate ( $\mu\text{L}/\text{sec}$ )
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

\*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  $\mu\text{L}$  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 ( $\mu\text{L}$ )	Mix Volume ( $\mu\text{L}$ )	Mix Cycles	Flow Rate ( $\mu\text{L}/\text{sec}$ )
1 Elution	Elution Buffer	100	40	20x	48

Table 4 – Freedom EVO protocol for eluting affinity-captured therapeutic mAb from anti-IgG-Fc-derivatized MSIA Streptavidin EVO microcolumns

## Analytical-Detection

### Liquid Chromatography

The affinity-purified therapeutic mAb eluates were separated on a Vanquish UHPLC system utilizing a ProSwift RP-4H (1 x 250 mm) column heated to 60 °C. Separation was performed utilizing a gradient of 10% – 38% 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 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. From the raw MS data an extracted ion chromatogram was generated for the five most abundant charge states of the intact therapeutic mAbs, which were then integrated to obtain the AUC (Area Under the Curve) value for each sample analyzed.

Further characterization of the mAbs, specifically in reference to the presence of glycosylation, was obtained from processing the MS raw data using Thermo Scientific Protein Deconvolution Software Version 4.0 utilizing the ReSpect algorithm.

## Results and Discussion

The LB-MSIA workflow for Freedom EVO platform provides a unique push button and automated solution for therapeutic mAb bio-analytics for a broad variety of therapeutic mAbs with differing allotype subclasses. The allotype subclasses of the five therapeutic mAbs used in this study are presented in Table 5.

mAb Analyzed	Construct	Allotype Subclass
Adalimumab (Humira)	Humanized	IgG1-kappa
Infliximab (Remicade)	Chimeric	IgG1-kappa
Trastuzumab (Herceptin)	Fully Human	IgG1-kappa
Denosumab (Prolia)	Fully Human	IgG2-kappa
Natalizumab (Tysabri)	Humanized	IgG4-kappa

Table 5 – Description of therapeutic mAbs with differing allotypes used to test the LB-MSIA workflow

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 Streptavidin EVO microcolumns creates an ideal scenario to assay the different allotypes from plasma utilizing high affinity anti-IgG-Fc binders specific for fully human, humanized and chimeric mAbs. Utilization of the MSIA Streptavidin EVO microcolumns on a Tecan Freedom EVO enables robust high-throughput automation for the generation of reproducible results. The integration of the Q Exactive Plus for HRAM detection helps provide additional analytical flexibility over other developing triple quadrupole methods reliant on peptide analysis.

### Dynamic Range Comparison of Five Therapeutic Monoclonal Antibodies of Differing Allotypes

To test the sensitivity of the LB-MSIA workflow for each of the five therapeutic mAbs of differing allotypes from mouse plasma, samples were run producing a plot akin to a dosing curve represented in Figure 2. Prior to beginning work on the dosing curves the corresponding deconvolved masses of each of the intact therapeutic mAbs were established by analyzing the mAb standards. The therapeutic mAbs deconvolved masses are represented in Table 6. For each sample the AUC from the extracted ion chromatogram for the top five charge states were summed and the average for each concentration was then used to generate the plots in Figure 2. The assay achieved a linear dynamic range for each mAb analyzed with CVs of the triplicate control samples within the acceptable range for traditional ligand binding assays represented in Table 7.

mAb Analyzed	Deconvolved Mass (Da)
Adalimumab (Humira)	148,081
Infliximab (Remicade)	148,514
Trastuzumab (Herceptin)	148,057
Denosumab (Prolia)	147,355
Natalizumab (Tysabri)	148,813

Table 6 – Therapeutic mAb  $m/z$  values

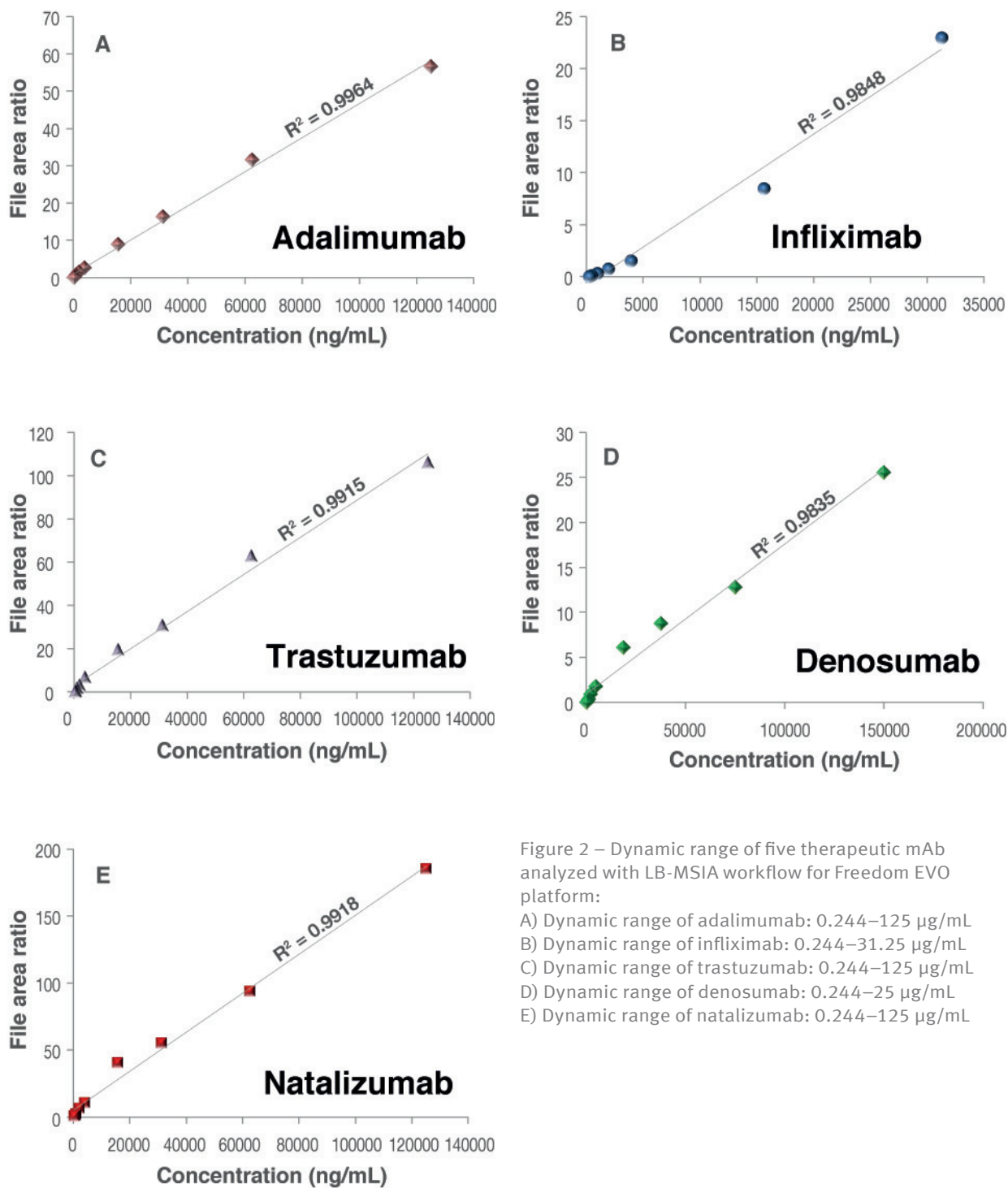


Figure 2 – Dynamic range of five therapeutic mAb analyzed with LB-MSIA workflow for Freedom EVO platform:

A) Dynamic range of adalimumab: 0.244–125  $\mu\text{g/mL}$

B) Dynamic range of infliximab: 0.244–31.25  $\mu\text{g/mL}$

C) Dynamic range of trastuzumab: 0.244–125  $\mu\text{g/mL}$

D) Dynamic range of denosumab: 0.244–25  $\mu\text{g/mL}$

E) Dynamic range of natalizumab: 0.244–125  $\mu\text{g/mL}$

mAb Analyzed	Dynamic Range (µg/mL)	Control Concentration (ng/mL)	Control CVs (%)
Adalimumab (Humira)	0.244–125	7812.5	2.55
Infliximab (Remicade)	0.244–31.25	7812.5	4.71
Trastuzumab (Herceptin)	0.244–125	7812.5	3.50
Denosumab (Prolia)	0.293–150	9375	2.01
Natalizumab (Tysabri)	0.244–125	7812.5	4.10

Table 7 – Summary of the control samples studied for each of the five therapeutic mAbs. Control samples also utilized 200 µL of mouse plasma.

## Conclusion

The demonstrated universal LB-MSIA workflow for 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 generation of high value data content for the bio-analysis of each of the fully human, humanized and chimeric therapeutic antibodies with differing allotypes. The high selectivity of the CaptureSelect biotin anti-IgG-Fc (human) conjugate combined with the molecular trapping technology of the MSIA Streptavidin EVO microcolumns 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 Plus 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 workflow for Freedom EVO platform with the enhanced capacity of the MSIA Streptavidin EVO microcolumns enable the characterization of multiple intact mAbs over a wide dynamic range capable of three orders of magnitude while maintaining coefficients of variation of <5% for control samples.

## Ordering Information

MSIA Microcolumns for Immunoaffinity Capture		
Compatible with the Tecan Freedom EVO series with MCA96 head		
Cat. No.	Description	Packaging
992STR96	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™ Finnpiptette™ 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	Finnpiptette Novus i Adjustable Pipette Stand	1 pipette stand
991SP12	Finnpiptette 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		
Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer		
XCalibur Software		
Protein Deconvolution Software, Version 4.0 with the ReSpect algorithm		

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