

Bioanalysis of Intact Therapeutic Monoclonal Antibodies from Non-Human Primates Using MSIA D.A.R.T.'S Technology

An Integral Part of Ligand Binding-Mass Spectrometric Immunoassay Workflow

Key Words

Q Exactive, HRAM, high resolution, accurate mass, MSIA, mass spectrometric immunoassay, high throughput, adalimumab, infliximab, denosumab, natalizumab, trastuzumab, Novus i

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Demonstrated here is the application of the developed LB-MSIA™ (Ligand Binding-Mass Spectrometric Immunoassay), for the targeted analysis of therapeutic mAbs of differing subclasses and allotypes from non-human primate plasma. LB-MSIA is a universal workflow that combines the robust nature of traditional ligand binding assays with HRAM (High Resolution/Accurate Mass) mass spectrometric detection of intact mAbs. Historically, ligand binding assays (LBAs) have represented the gold standard for protein quantitation. However, with the increase of biotherapeutic innovation and biological complexity, classic protein analytical techniques such as LBAs are not able to meet the data needs for pharmacokinetics, biotransformation assessment, and antibody functional determination studies. The development of novel antibody therapeutics requires structural information such as variants, sites of glycosylation, post-translation modifications (PTMs) necessary for drug safety, efficacy and stability. This hybrid bioanalytical workflow is specifically enabled by Streptavidin MSIA D.A.R.T.S; a proprietary product that contains molecular trapping micro



columns covalently derivatized with streptavidin embedded within a pipette tip housing. When the Streptavidin MSIA D.A.R.T.S are coupled with a high affinity reagent, such as biotinylated anti-human IgG Fc PK affinity ligands, the workflow is able to selectively analyze therapeutic antibodies within non-human primate plasma, tested here using human therapeutic mAbs of three different allotype subclasses; IgG1-kappa, IgG2-kappa and IgG4-kappa.

The qualitative bioanalysis of intact mAbs presented here utilized an LB-MSIA to provided percent coefficients of variation (% CV) between samples and sensitivity akin to a traditional ligand binding assay for adalimumab (Humira®), infliximab (Remicade®), denosumab (Prolia®), natalizumab (Tysabri®) and trastuzumab (Herceptin®). The detection of the therapeutic mAbs was achieved at concentrations as low as 78.125 ng/mL directly from monkey plasma.

Materials

- Thermo Scientific™ Streptavidin MSIA™ D.A.R.T.'S, PN: 991STR12
- Thermo Scientific™ Finnpiquette™ Novus i Multichannel Electronic Pipette, PN: 991SP12
- Thermo Scientific Finnpiquette F1 Adjustable-Volume Pipettes, PN: 4700850
- Life Sciences™ CaptureSelect™ Human IgG-Fc PK Biotin Conjugate, PN: 7103322100
- Abbvie™ Humira® (adalimumab)
- Janssen™ Remicade® (infliximab)
- Amgen® Prolia® (denosumab)
- Biogen® Tysabri® (natalizumab)
- Genentech™ Herceptin® (trastuzumab)
- Cynomolgus Monkey Plasma (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: 267245
- Thermo Scientific Nunc 1.3 and 2.0mL DeepWell Plates with Shared-Wall Technology, 96 DeepWell, Polypropylene, PN: 95040452

- 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 PK, an affinity ligand that specifically binds to the Fc portion of all four human IgG subclasses without cross-binding to rhesus and cynomolgus monkey IgG. The next step is to assay for the humanized, fully human and chimeric therapeutic monoclonal antibodies from non-human primate plasma samples by incubating the samples with the anti-IgG-Fc-PK-derivatized Streptavidin MSIA D.A.R.T.'S. The affinity retrieved mAbs are subsequently released from the micro-column by treatment with the elution buffer. The ensuing eluate containing the therapeutic mAb is then analyzed intact using LC-MS (HRAM). Utilizing Thermo Scientific 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.

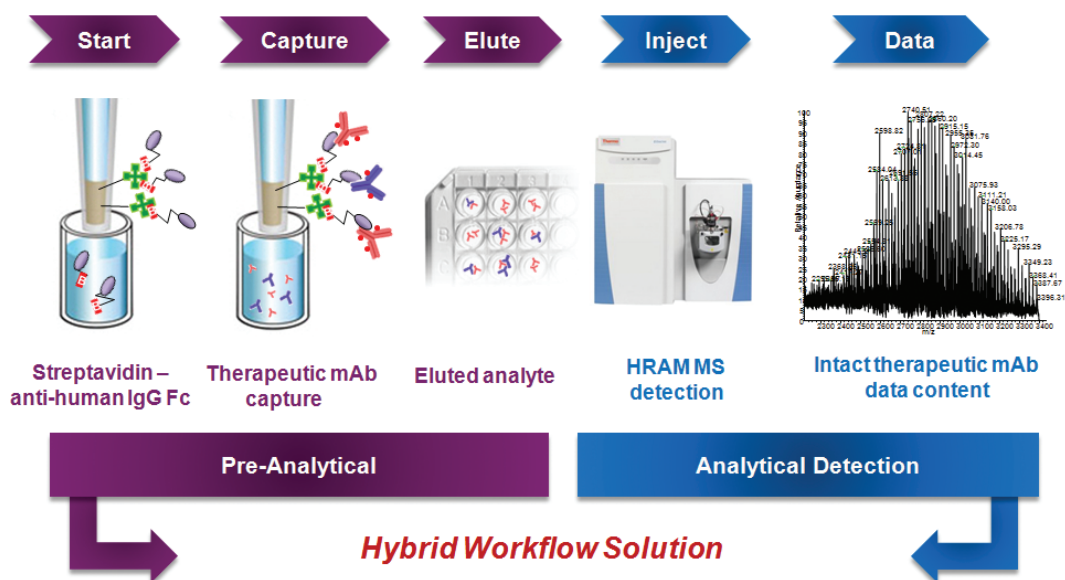


Figure 1: A schematic showing the five major steps of the LB-MSIA Workflow

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, fully human and chimeric mAbs, each of the streptavidin derivatized microcolumns were loaded with 125 μ L of 4 μ g/mL CaptureSelect™ Human IgG-Fc PK biotin conjugate (Life Technologies), a single domain antibody, 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.

Table 1: Derivatization of Streptavidin MSIA D.A.R.T.'S with anti-Human IgG-Fc PK biotin conjugate; Novus i Protocol in Descending Order

Assay Step	Assay Solution	Total Well Volume (μ L)	Asp/Disp Volume (μ L)	Asp/Disp Cycles	▲/▼ Speed	Time (mins)
Buffer Pre-Rinse	PBS	200	150	10x	4	0.5
Immobilization of anti-IgG-Fc	Human IgG-Fc PK biotin conjugate antibody	125	70	500x	1	22.5
Buffer Rinse	PBS	200	150	10x	4	0.5
Buffer Rinse	PBS	200	150	10x	4	0.5

Sample Preparation

All samples prepared consisted of 200 μ L of monkey (cynomolgus) 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.

Table 2: mAb Dosing Curve Ranges for LB-MSIA Sample Preparation

Therapeutic mAb	Dynamic Range (ng/mL)
Adalimumab (Humira)	78.125 - 20,000
Trastuzumab (Herceptin)	78.125 - 20,000
Infliximab (Remicade)	312.5 - 20,000
Denosumab (Prolia)	1250 - 40,000
Natalizumab (Tysabri)	78.125 - 20,000

Prior to incubation of the samples with the anti-IgG-Fc-PK-derivatized Streptavidin MSIA D.A.R.T.'S each sample was further diluted with 200 μ L PBS. Using the Novus i, the following steps outlined in Table 3 were performed to capture the therapeutic mAbs from the samples.

Table 3: Therapeutic mAb Capture; Novus i Protocol in Descending Order

Assay Step	Assay Solution	Total Well Volume (μ L)	Asp/Disp Volume (μ L)	Asp/Disp Cycles	▲/▼ Speed	Time (mins)
*Therapeutic mAb Capture	Sample Solution	400	300	500x	1	84
Buffer Rinse	PBS	200	150	10x	4	0.5
Buffer Rinse	PBS	200	150	10x	4	0.5
Water Rinse	Water	200	150	10x	4	0.5
Water Rinse	Water	200	150	10x	4	0.5

*Therapeutic mAb Capture performed by Anti-IgG-Fc PK MSIA D.A.R.T.'S

Sample Elution

Following the selective capture of the therapeutic mAb with the anti-IgG-Fc-PK-derivatized Streptavidin MSIA D.A.R.T.'S, each device was treated with 50 μ 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 D.A.R.T.'S. The intact mAb was then detected by LC-MS (HRAM).

Table 4 – Novus i Protocol for Eluting Affinity-Captured Therapeutic mAb from Anti-IgG-Fc-PK-Derivatized D.A.R.T.'S.

Assay Step	Assay Solution	Total Well Volume (μ L)	Asp/Disp Volume (μ L)	Asp/Disp Cycles	▲/▼ Speed	Time (mins)
Elution	Elution Buffer	50	30	20x	4	0.7

Analytical-Detection

Liquid Chromatography

The affinity-purified therapeutic mAb 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% - 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 for each intact mAb tested over the range of m/z 2000-3400 m/z 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 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 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 3.0 utilizing the ReSpect algorithm.

Results and Discussion

The MSIA workflow provides a unique push button and automated solution for therapeutic mAb bioanalytics for a broad variety of therapeutic mAbs with differing allotype subclasses present in non-human primate plasma. The allotype subclasses of the five therapeutic mAbs used in this study are presented in Table 5.

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

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

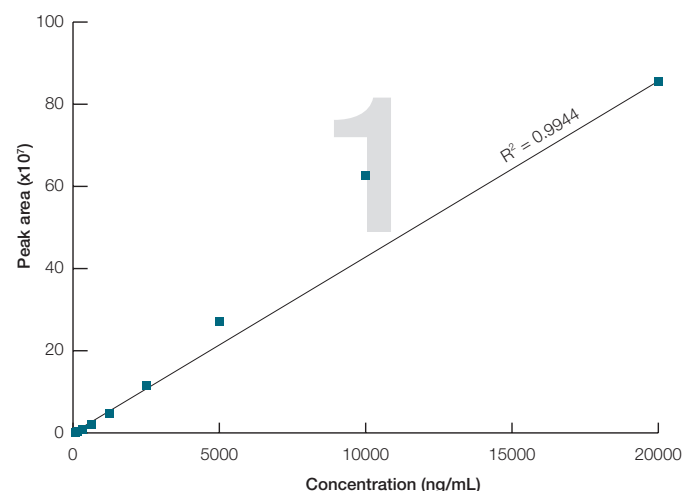
By combining the performance characteristics of traditional ligand binding assays with the benefits of HRAM MS detection, high value data content is produced that is highly sensitive, robust, and reproducible. The molecular trapping technology of the MSIA D.A.R.T.'S creates an ideal scenario to assay the different allotypes from a non-human primate plasma utilizing high affinity anti-IgG-Fc binders specific for fully human, humanized and chimeric mAbs. The integration of the Q Exactive 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 from Non-Human Primate Plasma

To test the sensitivity of the LB-MSIA workflow for the intact detection of each of the five therapeutic mAbs of differing allotypes from non-human primate 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 (Table 6). For each sample the AUC from the extracted ion chromatogram for the top five charge states for each intact mAb 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.

Table 6: Therapeutic mAb m/z values

mAb Analyzed	Deconvolved Mass (Da) ± 30ppm
Adalimumab (Humira)	148,081
Infliximab (Remicade)	148,514
Trastuzumab (Herceptin)	148,057
Denosumab (Prolia)	147,355
Natalizumab (Tysabri)	148,813



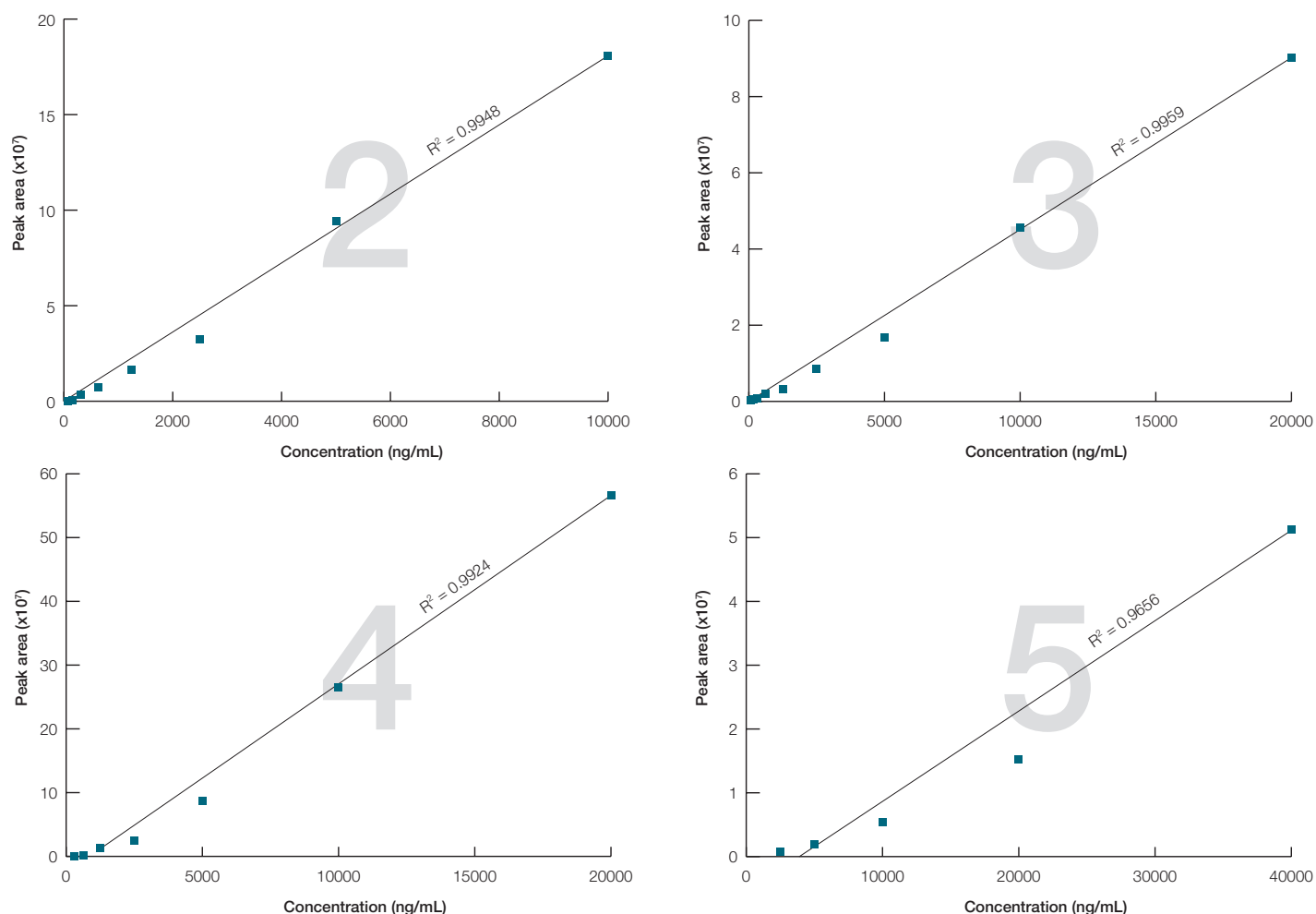


Figure 2: Dynamic range of five intact therapeutic mAb analyzed with LB-MSIA from non-human primate plasma: 1 - Dynamic range of natalizumab: 78.125-20,000 ng/mL. 2 - Dynamic range of adalimumab: 78.125-10,000 ng/mL. 3 - Dynamic range of trastuzumab: 78.125-20,000 ng/mL. 4 - Dynamic range of infliximab: 312.5-20,000 ng/mL. 5 - Dynamic range of denosumab: 1250-40,000 ng/mL

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

mAb Analyzed	Intact Dynamic Range (ng/mL)	Control Concentration (ng/mL)	Control CVs
Adalimumab (Humira)	78.125-10,000	4000	7.6%
Trastuzumab (Herceptin)	78.125-20,000	8000	0.49%
Infliximab (Remicade)	312.5-20,000	2500	5.38%
Denosumab (Prolia)	1250-40,000	9000	8.32%
Natalizumab (Tysabri)	78.125-20,000	3000	2.17%

Conclusion

- The LB-MSIA provided a highly sensitive and robust method that was able to successfully assay multiple intact therapeutic analogs of differing IgG subclasses and allotypes from non-human primate plasma.
- Each mAb was assayed over a wide dynamic range achieving at least two orders of magnitude or more while maintaining coefficients of variation of < 9 % for control samples.
- The CaptureSelect biotin anti-IgG-Fc PK conjugate combined with the molecular trapping technology of the

Streptavidin MSIA D.A.R.T.'S creates an ideal scenario to assay low abundant (ng/mL) intact human therapeutic mAbs from primate plasma.

- As a hybrid, immunoaffinity-LCMS approach, the LB-MSIA also resulted in no cross reactivity with interfering primate proteins.
- The use of a Q Exactive for HRAM detection allowed for intact analysis of each of the therapeutic mAbs, eliminating the need for a digestion protocol and providing additional analytical flexibility and data content over other methodologies reliant on peptide analysis.

Ordering Information

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

Description	Packaging	Cat. No.
300µl MSIA D.A.R.T.'S, Custom	Pack of 96 units	991CUS02
300µl MSIA D.A.R.T.'S, Protein A	Pack of 96 units	991PRT11
300µl MSIA D.A.R.T.'S, Protein A	Pack of 24 units	991PRT12
300µl MSIA D.A.R.T.'S, Protein G	Pack of 96 units	991PRT13
300µl MSIA D.A.R.T.'S, Protein G	Pack of 24 units	991PRT14
300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 96 units	991PRT15
300µl MSIA D.A.R.T.'S, Protein A/G	Pack of 24 units	991PRT16
300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 96 units	991STR11
300µl MSIA D.A.R.T.'S, Streptavidin	Pack of 24 units	991STR12
300µl MSIA D.A.R.T.'S, Insulin	Pack of 96 units	991001096
300µl MSIA D.A.R.T.'S, Insulin	Pack of 24 units	991001024
300 µL MSIA D.A.R.T.'S, Reloadable Rack	1 reloadable rack, D.A.R.T.'S are not included	991R

MSIA Streptavidin-EVO for Immunoaffinity Capture

Compatible with the Tecan™ Freedom EVO® 150 Platform equipped with a MCA96 liquid handling arm

Description	Packaging	Cat. No.
500µl MSIA Streptavidin EVO microcolumns	Pack of 96 units	992STR96

Automated Liquid Handling Platform

Description	Cat. No.
MSIA Versette Automated Liquid Handler	650-MSIA

Multichannel Pipettes and Pipette Stand

Description	Packaging	Cat. No.
Finnpiptette Novus i Adjustable Pipette Stand	1 pipette stand	991S
Finnpiptette Novus i Electronic 12-Channel Pipette, 30-300µl and Pipette Stand	1 pipette and 1 pipette stand	991SP12

Liquid Chromatography

Description	Cat. No.
Thermo Scientific Dionex UltiMate 3000 UHPLC System	
ProSwift RP-4H Monolith Column, 1.0 x 250 mm	066640

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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