Analysis of an Antibody Drug Conjugate using MSIA D.A.R.T.'S. Technology, an Integral Part of Ligand Binding Mass Spectrometric Immunoassay (LB-MSIA) Workflow

Application Note

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Key Words
Q Exactive™ Plus, Antibody-Drug Conjugate (ADC), Deconvolution, HRAM, High Resolution, Accurate Mass, MSIA, Mass Spectrometric Immunoassay, High Throughput, Versette™, Streptavidin MSIA™ D.A.R.T.'S

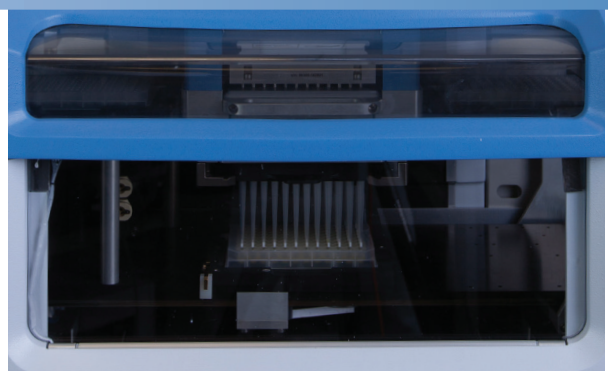
Goal

To illustrate the qualitative analyses of an ADC using Thermo Scientific™ MSIA™ D.A.R.T.'S with the LB-MSIA™ workflow; a pre-clinical bioanalytical solution, based on mass spectrometric detection of an intact biotherapeutic.

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 solution for targeted pre-clinical analysis of biotherapeutics, such as ADCs, that combines the robust nature of traditional ligand binding assays with HRAM (High Resolution/Accurate Mass) mass spectrometric detection. By focusing on the enablement of preclinical discovery and development research the resultant automated and high throughput LB-MSIA provides characterization data necessary to keep pace with new biotherapeutic innovation and increased biological complexity. This hybrid bioanalytical workflow is specifically enabled by Streptavidin MSIA D.A.R.T.'S; a unique pipette tip that contains molecular trapping microcolumns, covalently derivatized with streptavidin. By mounting the Streptavidin MSIA D.A.R.T.'S onto a Thermo Scientific™ Versette™ automated liquid handler or a Thermo Scientific Finnpipette® Novus i Multichannel Electronic Pipette, the LB-MSIA workflow may be applied to high throughput sample processing for standard routine applications. The functional design of the Streptavidin MSIA D.A.R.T.'S combined with the consistency of the Versette automated



liquid handler or the Thermo Scientific Finnpipette® Novus i Multichannel Electronic Pipette, provide easy-to-use and reproducible approach that is not provided by beads-based methodologies. When the Streptavidin MSIA D.A.R.T.'S are paired with a high affinity reagent, such as biotinylated anti-human IgG Fc affinity ligands, the workflow is able to selectively analyze for mAbs of a human IgG subclass.

Presented here is a qualitative analysis that utilized LB-MSIA to characterize an ADC spiked into rodent plasma. The MSIA workflow for biotherapeutics offers several advantages over traditional ligand binding assays and MS based methods. While this study focuses on applications of intact analysis of an ADC, the LB-MSIA workflow has the potential to perform bottom up, middle down as well as intact analyses for comprehensive characterization of a biotherapeutic as referenced in the application note, "MSIA Workflow for Therapeutic Antibodies: Qualitative, Quantitative, and Functional Verification Data from HR/AM Detection of Intact, Reduced, and Peptide-level Forms of Adalimumab". The combined benefits of the unique affinity purification by Streptavidin MSIA D.A.R.T.'S and the high specificity of MS detection enable the characterization of multiple drug-antibody ratio (DAR) species of the ADC over a wide dynamic range (2.5-320 µg/mL) and identified the induced modification of the ADC when the spiked rodent plasma samples were incubated at 37 °C over a 24 hour period.

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Materials

- Thermo Scientific™ Streptavidin MSIA™ D.A.R.T.'S, PN: 991STR11; 991STR12
- Thermo Scientific™ Versette™ Automated Liquid Handler
- Thermo Scientific™ Finnpipette™ F1 Adjustable-Volume Pipettes, PN: 4700850
- CaptureSelect™ Biotin Anti-IgG-Fc (Human) Conjugate, PN: 7103262100
- Antibody-Drug Conjugate (ADC) (Custom Made)
- 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 an ADC may be broken down into five major steps as illustrated in Figure 1. A Thermo Scientific™ Versette™ Automated Liquid Handler was used to provide the repetitive bi-directional pipetting (aspirating and dispensing cycles) necessary to pass solutions through the microcolumn housed within each of the Streptavidin MSIA D.A.R.T.'S. The Streptavidin MSIA D.A.R.T.'S are first derivatized with a biotin-conjugated anti-human 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 ADC from rodent plasma samples by incubating the samples with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S. The affinity bound ADC is subsequently released from the D.A.R.T.'S by treatment with the elution buffer. The ensuing eluate containing the ADC is then analyzed using LC-MS (HRAM). Utilizing Thermo Scientific's™ Xcalibur™ (Version 2.2) and Protein Deconvolution (Version 4.0) Software the resulting raw HRAM MS data is processed to provide high content qualitative data.

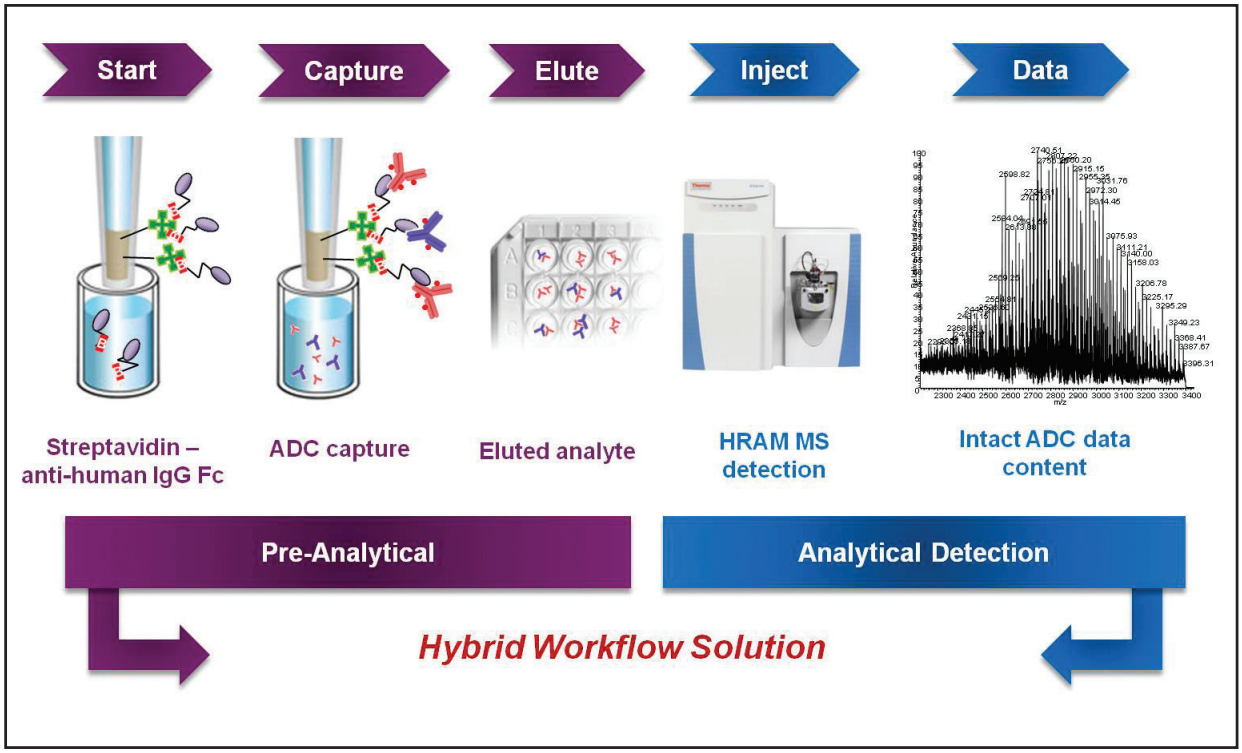

Figure 1. A schematic showing the five major steps of the LB-MSIA Workflow for ADC Analysis

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 the Fc region of human IgG subclasses, each of the streptavidin derivatized microcolumns were loaded with 125 µL of 4 µg/mL CaptureSelect™ biotin anti-IgG-Fc (Human) conjugate, a single domain antibody (Thermo Scientific), prepared in PBS (BupH™ Modified Dulbecco's PBS). This was accomplished by following the steps provided in Table 1 utilizing a Thermo Scientific™ Versette™ Automated Liquid Handler equipped with Streptavidin MSIA D.A.R.T.'S.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	Buffer Pre-Rinse	PBS	200	150	10x	100
2	Immobilization of anti-human IgG-Fc	Biotin anti-IgG Fc conjugate antibody	125	70	500x	100
3	Buffer Rinse	PBS	200	150	10x	100
4	Buffer Rinse	PBS	200	150	10x	100

Table 1 – Derivatization of Streptavidin MSIA D.A.R.T.'S with biotinylated anti-human IgG Fc; Versette protocol in descending order

Sample Preparation

All samples prepared consisted of 20 µL of mouse plasma supplemented with 50 to 6400 ng of ADC resulting in a concentration range of 2.5 to 320 µg/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 80 µL of PBS supplemented with 0.7 µg of SILu™Lite SigmaMAb. Using the Versette Liquid Handler, the following steps outlined in Table 2 were performed to capture the ADC from the samples.

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	ADC Capture*	Sample Solution	100	70	700x	50
2	Buffer Rinse	PBS	200	150	10x	100
3	Buffer Rinse	PBS	200	150	10x	100
4	Water Rinse	Water	200	150	10x	100
5	Water Rinse	Water	200	150	10x	100

**ADC Capture performed using Anti-human IgG-FC MSIA D.A.R.T.'S*

Table 2 – ADC Capture; Versette protocol in descending order

Sample Elution

Following the selective capture of the ADC with the anti-IgG-Fc-derivatized Streptavidin MSIA D.A.R.T.'S, each device was treated with 100 µL of the MSIA™ Elution Buffer liberating the ADC. Reference Table 3 for the specifics of the repetitive pipetting used to elute the captured ADC from the D.A.R.T.'S. The intact ADC was then detected by LC-MS (HRAM).

	Assay Step	Assay Solution	Total Well Volume (µL)	Asp/Disp Volume (µL)	Asp/Disp Cycles	Syringe Speed (%)
1	Elution	Elution Buffer	100	30	20x	100

Table 3 – Versette protocol for elution of affinity-captured ADC from anti-IgG-Fc-derivatized D.A.R.T.'S.

Analytical-Detection

Liquid Chromatography

The affinity-purified ADC 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 were collected using Thermo Scientific's Xcalibur™ Software, Version 3.0. From the raw MS data an extracted ion chromatogram was generated for the three most abundant charge states (XIC Method) of each intact ADC species, which were then integrated to obtain the AUC (Area Under the Curve) value for each sample analyzed.

Further characterization of the ADC was obtained from processing the MS raw data using Thermo Scientific's™ Protein Deconvolution™ Software Version 4.0 utilizing the ReSpect™ algorithm.

Results and Discussion

Identification of Individual DAR Species

The biological complexity of an ADC is partly due to the heterogeneous mixture of drug conjugates bound to the antibody carrier. In-depth characterization of the ADC must be performed in order to calculate the drug-to-antibody ratio (DAR) by first identifying the different DAR species present. The molecular weight of the antibody carrier (DAR0 species) for the ADC used in this study was determined to be 148,081 Da ± 30ppm prior to the binding of the drug conjugate. The LB-MSIA workflow was performed and a total of nine DAR species were identified from one ADC sample represented in Figure 2. The ADC MS data was initially deconvolved (Figure 2.C) in order to simplify the data allowing for the identification of the nine DAR species. The deconvolution software also provided individual intensities for each DAR species that were summed resulting in the total intensity generated by the ADC. The percentage of each DAR species was then calculated based off of the total ADC (Figure 2.C).

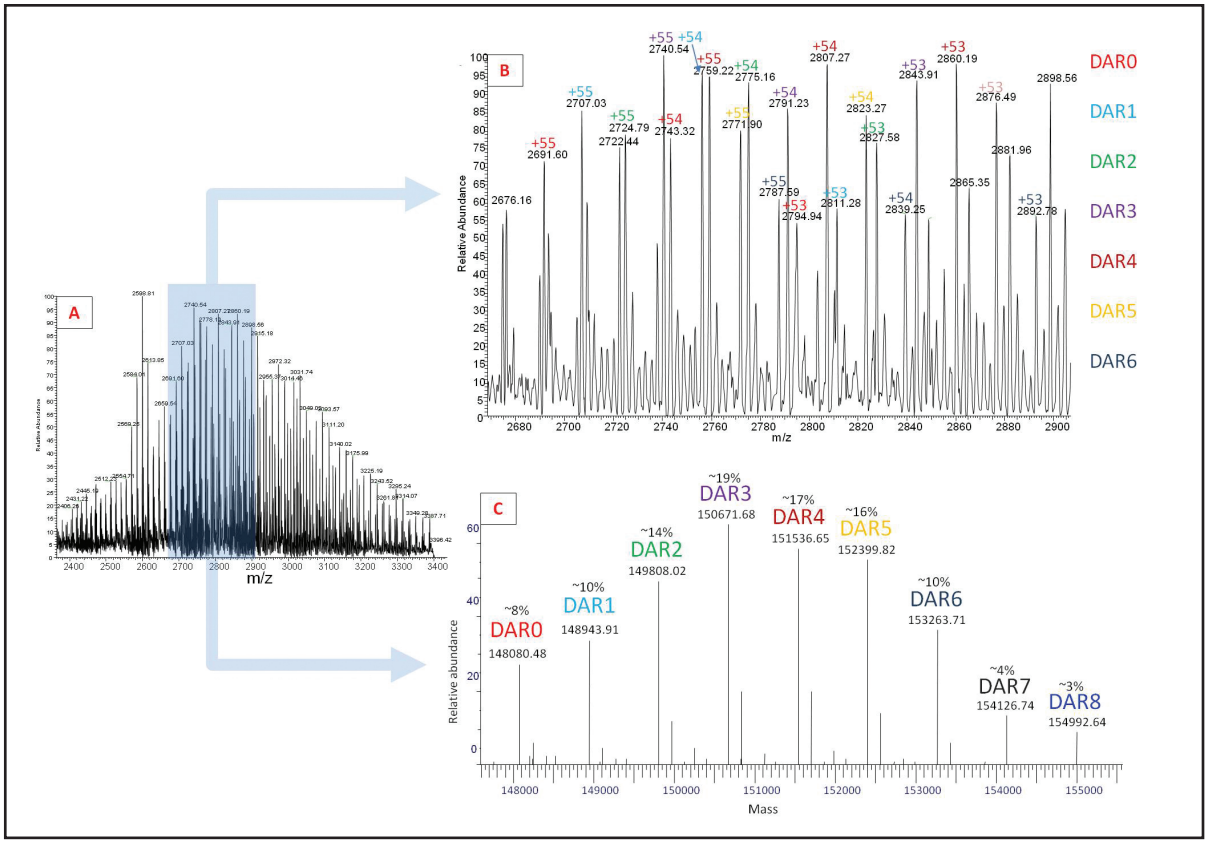

Figure 2. The results of the LB-MSIA workflow performed on a 20µL sample containing 53.5 µg/mL of the ADC from rodent plasma. A-B) Ion Chromatogram showing multiple charge states of seven DAR species from the ADC sample. Each DAR species represents a different number of attached drug conjugates to the carrier antibody. C) Deconvolution of the MS data from the same ADC sample resulting in a reduction in data complexity. The deconvolved data was able to identify a total of 9 DAR species. The intensities of all the DAR species were summed and the percentage of each DAR species was calculated based off of the total.

Identification of Biotransformation and Stability of ADC

In addition to PK studies, it is also necessary to identify any biotransformation occurring to the ADC that may result in changes in the DAR over time. The LB-MSIA was used to analyze in vitro induced biotransformations generated upon incubating rodent plasma spiked with the ADC at 37 °C for 24 hours. Figure 3 shows changes in the DAR that become evident as the apex of the DAR distribution shifts from the peak at 151535 Da at zero hours to the peak at 150672 Da after four hours and then again to 148080 Da after 24 hours as the cytotoxic drug decouples from the antibody. Additional peaks were also observed represented by the green peaks in Figure 3. Identification of these additional peaks requires further investigation.

Analysis of the resulting MS data was performed using the XIC method; for each sample the AUC from the extracted ion chromatogram for the top three charge states of each DAR species were summed and separately the top five charge states of the SILuLite IRS were summed. Then the AUC of the ADC was divided by the AUC of the IRS to give the Area Ratio. The average of replicate samples (n=5) was used to generate the plots in Figure 4. The assay achieved a linear dynamic range for the ADC analyzed with a linear regression of R^2 = 0.9978.

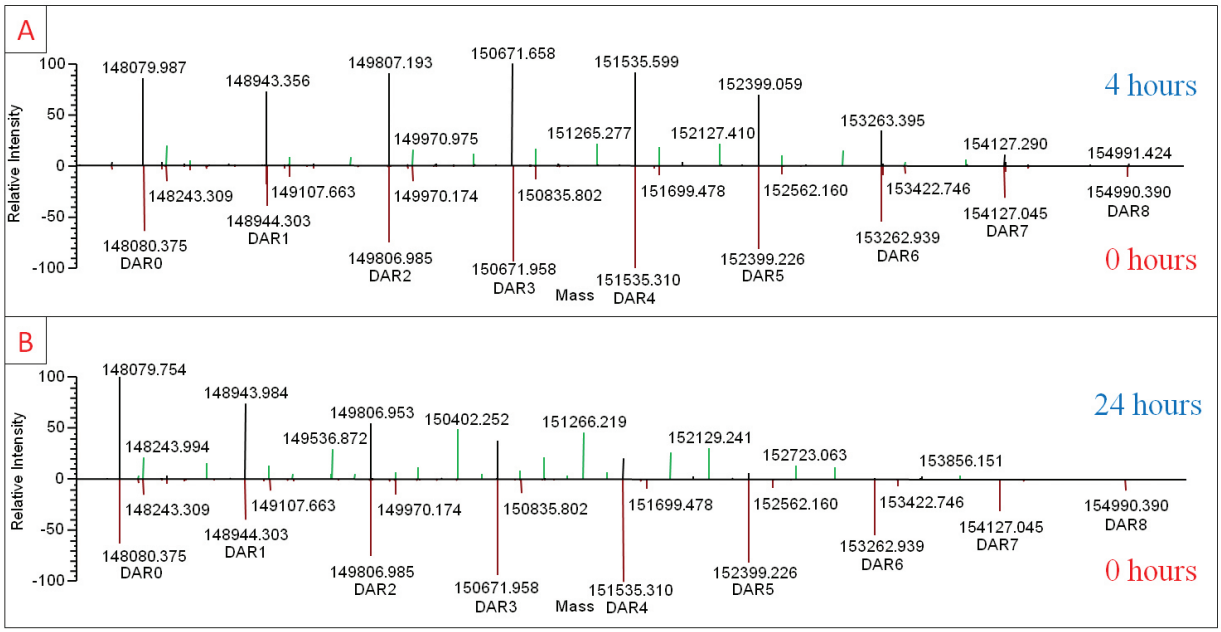

Figure 3. Qualitative data that shows biotransformation occurring in the ADC in rodent plasma over time; 50 µg/mL of the ADC was spiked into 20uL of mouse plasma and incubated at 37 °C for 24 hours. A) A comparison of the samples at 4 hours and 0 hours. B) A comparison of the samples at 24 hours and 0 hours. The green peaks represent peaks requiring further investigation.

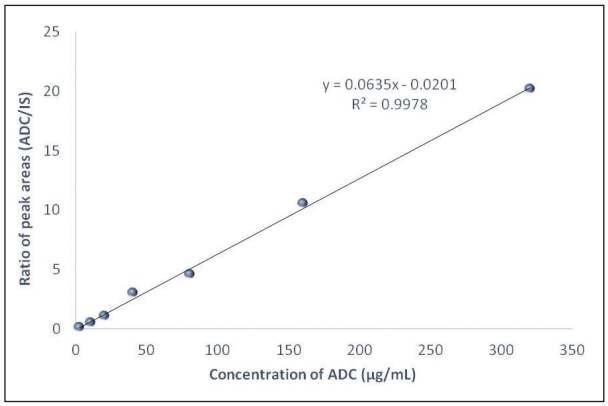

Figure 4. Dynamic range of the ADC analyzed with LB-MSIA: 2.5-320 µg/mL. Data analysis was performed directly from the ion chromatogram using the XIC method.

Dynamic Range of ADC

To test the sensitivity of the LB-MSIA workflow for the ADC from rodent plasma, samples were run producing a plot akin to a dosing curve represented in Figure 4.

Conclusion

The demonstrated universal LB-MSIA workflow utilizing Streptavidin MSIA D.A.R.T.'S with biotin-conjugated anti-human IgG Fc provided an unmatched, sensitive, robust and reproducible method for the generation of high value data content for the bioanalysis of an ADC. 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 created an ideal scenario for assaying a low abundant (µg/mL) intact ADC from rodent plasma. Furthermore, the workflow supports a high throughput application by performing the pre-analytical steps on an automated liquid handler; the Thermo Scientific™ Versette™. As a hybrid approach, the use of the Q Exactive for HRAM detection helped provide additional analytical flexibility and data content over other developing triple quadrupole methods that are reliant on peptide analysis. This LB-MSIA demonstrated the identification of nine DAR species within each ADC sample. The sensitivity of this LB-MSIA was tested achieving a dosing curve dynamic range of 2.5-320 µg/mL of the ADC with a linear regression of R^2 = 0.9978. This method enabled the identification of biotransformation occurring to this ADC while the ADC was subjected to an in vitro stability study conducted in rodent plasma at 37 °C for 24 hours.

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

MSIA Streptavidin-EVO for Immunoaffinity Capture		
Compatible with the Tecan™ Freedom EVO® Liquid Handling Robotic Platform equipped with a MCA96 option		
Cat. No.	**Description**	**Packaging**
992STR96	500µl MSIA Streptavidin EVO microcolumns	Pack of 96 units

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
	Thermo Scientific™ Vanquish™ 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™ Q Exactive™ Plus 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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