UPLC INTACT MASS ANALYSIS APPLICATION KIT

I. UPLC INTACT MASS ANALYSIS APPLICATION KIT COMPONENTS

Description	P/N	Quantity
MassPREP™ Micro desalting column	186004032	1
Pre-Column Tubing	430001988	1
Tubing, Column to Desalting Valve (Tube, PEEK™, 0.062 o.d. x 0.004, 15.04″ long)	430001992	1
Tubing, Column to Desalting Valve (Tube, PEEK, 0.062 o.d. x 0.004, 22.20″ long)	430001993 (SQD System)	1
Tubing, Desalting Valve to MS (Tube, PEEK, 0.062 o.d. x 0.004, 8.5″ long)	430001991	1
Fittings (10-32)	410002276	4

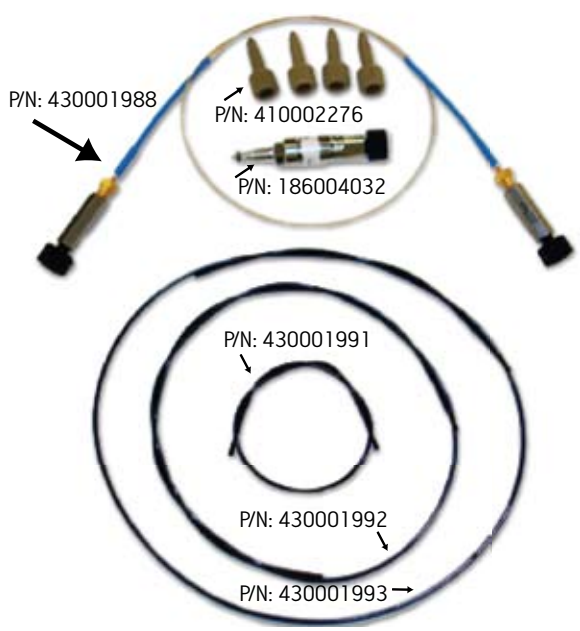

Figure 1: UPLC® Intact Mass Analysis Application Kit Components

CONTENTS

II. INSTALLING THE PRE-COLUMN TUBING INTO MassPREP MICRO DESALTING COLUMN

Required materials:

- 1/4-inch open-end wrench
- 5/16-inch open-end wrench

Figure 2: Installation of the pre-column tubing into the MassPREP Micro Desalting Column.

Installation Procedure

1. Place the column on a flat surface (O-ring end facing up).
2. Remove the O-ring from the column.
3. Secure the column (P/N 186004032) with a 5/16-inch wrench (bottom) as shown in Figure 2.
4. Tighten one end of the pre-column tubing (P/N 430001988) into the column with a 1/4-inch wrench (a full turn beyond hand-tight).
5. Connect the marked end (marked as solvent inlet) of the column tubing assembly to the solvent outlet of the ACQUITY UPLC® Column Heater (Figure 3).

   Note: Proceed from this point to either section III, IV, or V depending upon the type of mass spectrometer you have.

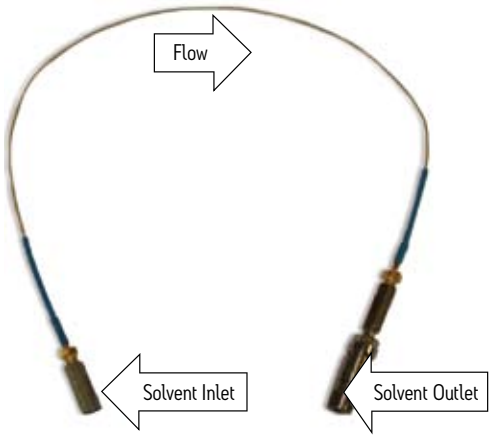

Figure 3: Column assembly after attaching the column to the pre-column tubing.

III. CONNECTING THE COLUMN TO LCT Premier MS

Installation Procedure

6. Connect one end of the 15.04-inch long PEEK tube (P/N 430001992) to the outlet of the column using a fitting (P/N 410002276).
7. Connect the other end of the tubing to the salt divert valve (Port 2) using another fitting, as shown in the Figure 4. The salt divert valve is located at the top-left corner of the LCT Premier front panel.
8. Place the column in the column heater as shown in the Figure 6.
9. Connect one end of the 8.5-inch long PEEK tube (P/N 430001991) to Port 1 of the divert valve using a fitting.
10. Connect the other end to the inlet of LCT Premier sample probe using another fitting (Figure 4).
11. Use a larger diameter PEEK tube (0.062 o.d. x 0.005 or 0.01-inch i.d.) for the waste line (not included in the kit).

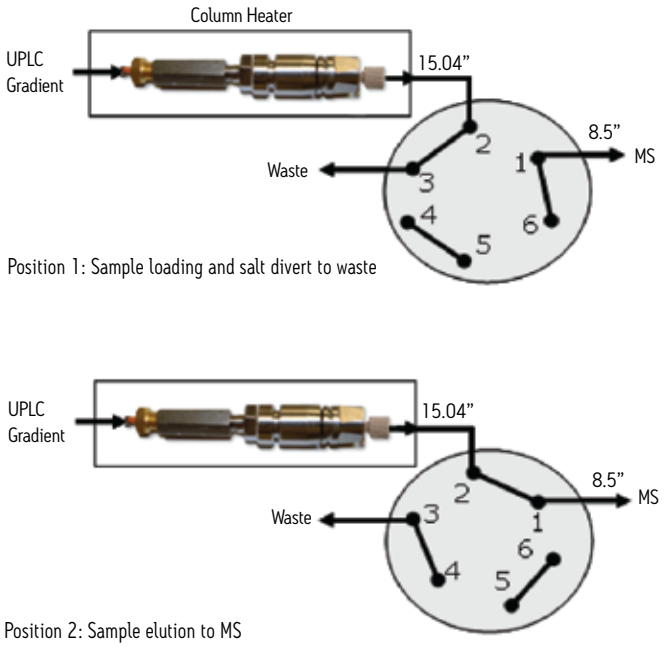

Figure 4: Fluidic configuration for LC/MS (UPLC/LCT Premier) analysis. A post-column salt diversion valve (top-left corner of the LCT Premier) can be utilized to divert buffers and nonvolatile salts to waste in the beginning of the LC/MS analysis.

The divert valve can be programmed from the ***MS Method Editor*** in MassLynx™. To set a salt divert time, open ***MS Method Editor***. Then select ***Option> Solvent Delay*** on the ***MS Method Editor*** to open the ***Solvent Delay*** dialog box. To enable the divert/injection valve to be used as divert valve, select ***Enable Divert Valve***. This diverts the flow of solvent during a solvent delay period (0.5 min or user defined value) either to, or away from, the source for the time period shown in the solvent delay timetable (Table 1).

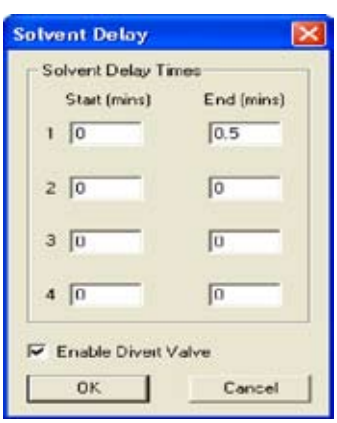

Table 1: Salt divert timetable

IV. CONNECTING THE COLUMN TO Q-TOF Premier MS, AND SYNAPT HDMS

Neither Q-Tof Premier MS nor Synapt HDMS has an integrated divert valve. However, an optional external 2 position valve (P/N 417000118) can be used with Q-Tof Premier and Synapt HDMS mass spectrometers to divert buffers and nonvolatile salts present in the sample to waste at the beginning of the LC/MS analysis.

Follow Steps 1-5 in Section II to attach the column to the pre column tubing. Then follow Steps 12-17 below to connect the column to the mass spectrometer.

12. Connect one end of the 15.04-inch long PEEK tube (P/N 430001992) to the outlet of the column using a fitting (P/N 410002276).
13. Connect the other end of the tubing to the external salt divert valve (Port 4) using another fitting, as shown in the Figure 5.
14. Connect one end of the 8.5-inch long PEEK tube (P/N 430001991) to the external divert valve (Port 5).
15. Connect the other end to the inlet of MS sample probe (Figure 5).
16. Place the column in the column heater as shown in the Figure 6.
17. Use a larger diameter PEEK tube (0.062 o.d. x 0.005 or 0.01-inch i.d.) for the waste line (not included in the kit).

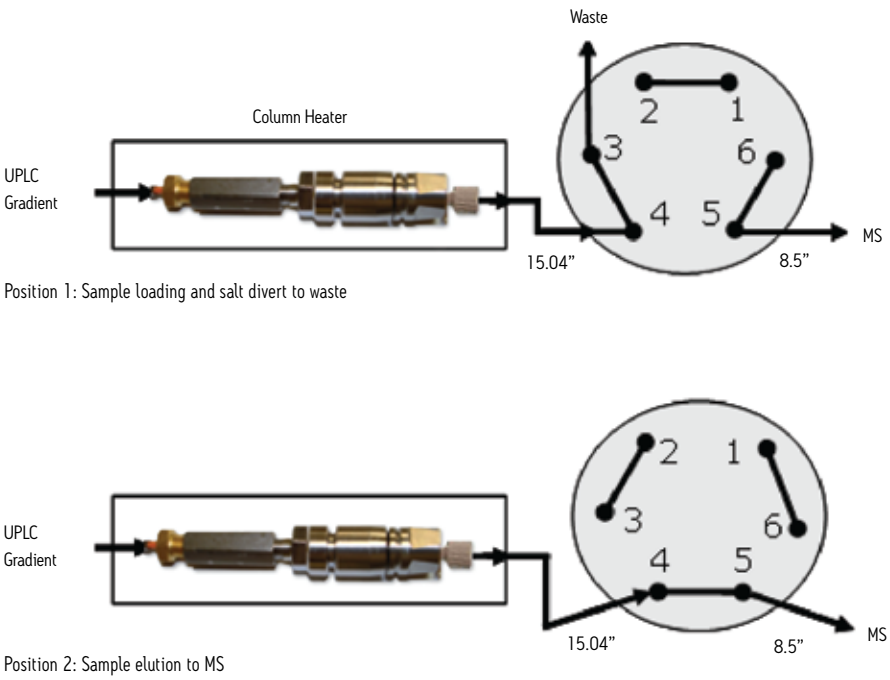

Figure 5: Fluidic configuration for LC/MS analysis using mass spectrometers with no integrated salt divert valve

Figure 6: Column placement in the column heater

Communications Connections for the External Divert Valve

The optional external divert valve (417000118) can be controlled via line control using the connecting wires with contact closures. Detailed instructions can be found in the valve operating manual.

1. Connect the red wire to pin # 4 and black wire to GND to the terminal block (wires and terminal block are included in the valve kit), as shown in the table below.

Pin #	1	2	3	4	5	6	GND
Wire	-	-	-	Red	-	-	Black

2. Plug the terminal block into terminal block I/O (terminal block I/O is located on the back panel of the external divert valve).

3. Connect the other side of the red wire to pin # 6 and black to the pin # 7 of a different terminal block as shown in table below

1 (+) 2 (-)	Gradient Start	In
3 (+) 4 (-)	Stop Flow	In
5	Ground	
6 (Red) 7 (Black)	Switch 2	Out
8 9	Switch 3	Out
10	Ground	Out
11 (+) 12 (-)	0-2 V Analog 2	Out

4. Plug the terminal block into Switch II (Switch II is located on the back panel of the ACQUITY UPLC® Binary Solvent Manager [BSM]).

The divert valve can be programmed from the ACQUITY UPLC ***inlet Method Editor*** in MassLynx. To set a salt divert time, open ***inlet Method Editor***. Then select ***Inlet› Events*** on the ACQUITY UPLC BSM Instrument Method to open the Events dialog box. To enable the external divert valve to be used as a divert valve, select ***Run Events*** and enter the events in the events table by clicking line 1 and 2. Table 2 depicts a salt divert event. When Switch 2 is in ON position at 0.01 min, the solvent flow is diverted to waste until 0.51 min, and then the valve is switched to allow the solvent flow to enter the MS.

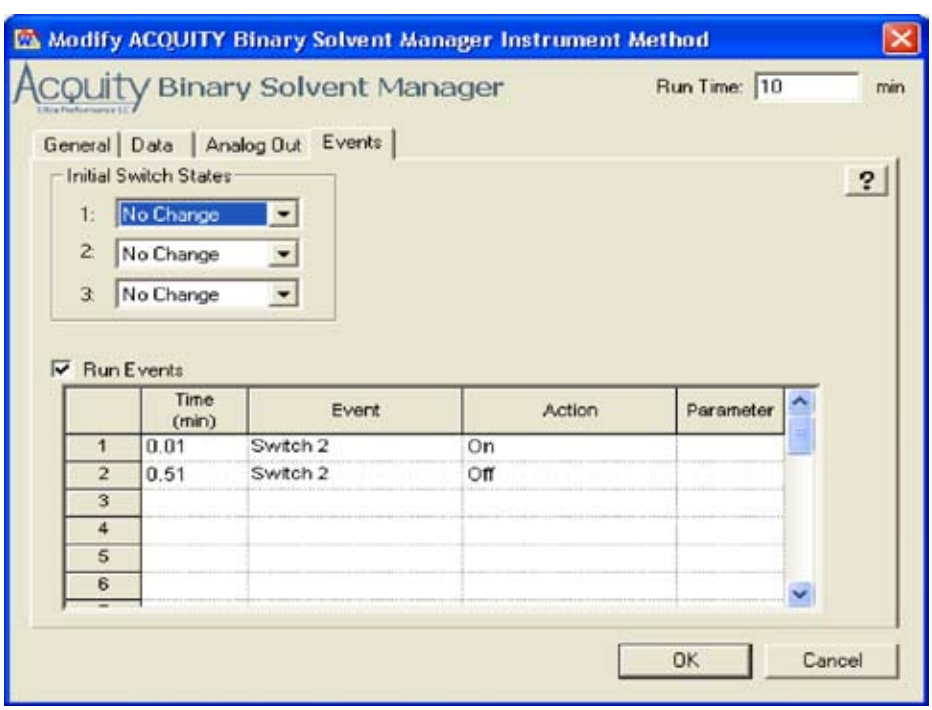

Table 2: ACQUITY UPLC BSM console of salt divert timetable/events

V. CONNECTING THE COLUMN TO WATERS SQ DETECTOR (SQD)

Follow steps 1-5 in Section II to attach the column to the pre column tubing. Then follow Steps 18-23 below to connect the column to the SQD.

18. Connect one end of the 22.20-inch long PEEK tube (P/N 430001993) to the outlet of the column using a fitting (P/N 410002276).
19. Connect the other end to the divert valve (Port 1) using another fitting, as shown in the Figure 7. The divert valve is located at the top-right corner of the SQD front panel.
20. Place the column in the column heater as shown in the Figure 6.
21. Connect one end of the 8.5-inch long PEEK tube (P/N 430001991) to Port 2 of the divert valve.
22. Connect the other end to the inlet of SQD sample probe (Figure 7).
23. Use a larger diameter PEEK tube (0.062 o.d. x 0.005 or 0.01-inch i.d.) for the waste line (not included in the kit)

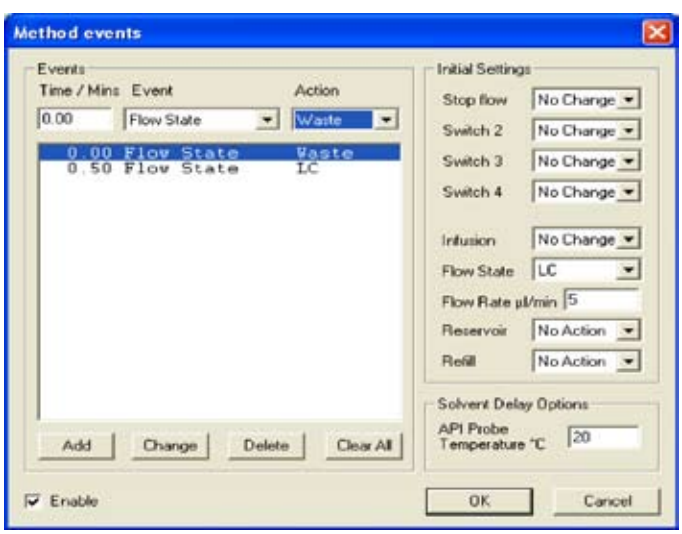

Table 3: Salt divert timetable for SQD.

The divert valve can be programmed from the ***MS Method editor*** in MassLynx. To set a salt divert time, open ***MS Method Editor.*** Then select ***Option> Method*** events on the ***MS Method Editor*** to open the Method events dialog box. To enable the valve to be used as divert valve, select ***Enable***. This diverts the flow of solvent to waste for the time period shown in the ***Method events*** timetable (0.5 min or user defined value) then the valve is switched allowing the flow to go to the MS (Table 3).

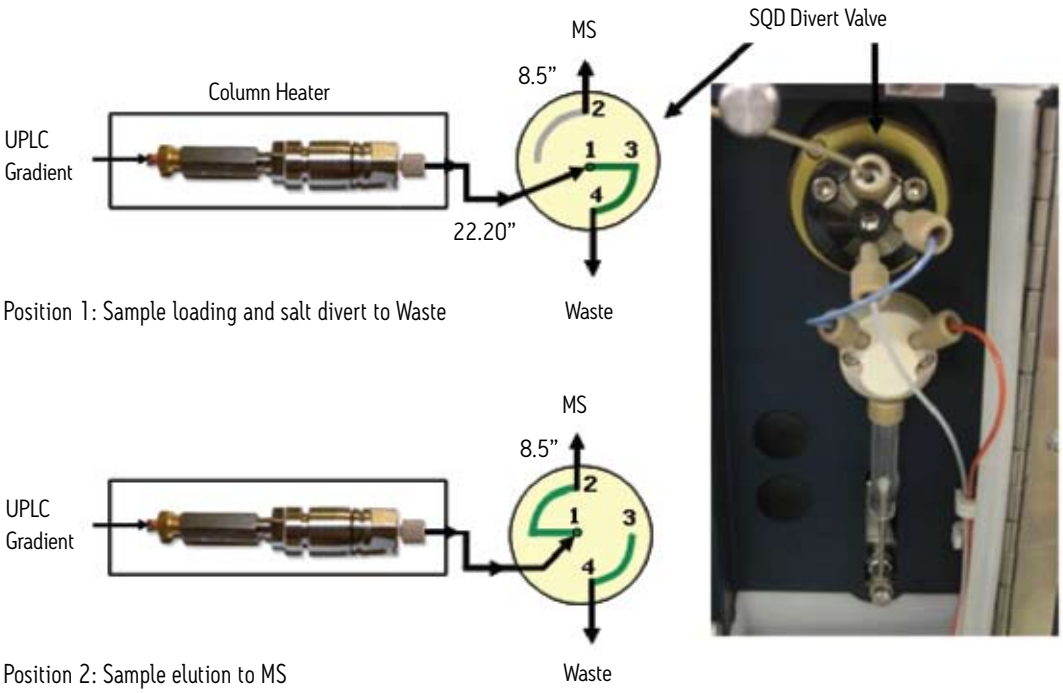

Figure 7: Fluidic configuration for LC/MS (UPLC/SQD) analysis. A post-column diversion valve located at the top right corner of the SQD (typically used to direct calibrant for use in automatic tuning), was utilized to divert buffers and non-volatile salts to waste at the beginning of the LC/MS analysis.

VI. SUGGESTED METHOD FOR MASS ANALYSIS USING UPLC INTACT MASS ANALYSIS APPLICATION KIT

Mass Spectrometry has become a powerful tool for therapeutic proteins analysis. However, most therapeutic proteins are stored in a matrix of biological buffers and non-volatile salts and stabilizers. Thus, one of the most significant challenges encountered during mass analysis of these proteins is processing of the sample to remove these agents, which often form non-covalent adducts that reduce MS response and further complicate the resulting mass spectral data. The following offers two rapid, sensitive and efficient desalting LC/MS methods that can be utilized as a starting point with the UPLC Intact Mass Analysis Application Kit for the characterization of an intact antibody and its variants, and, constituent heavy and light chain structures.

a. Intact Antibody Analysis

A fast (4-min cycle time) and efficient LC/ESI-MS method was used to profile multiple structural variants of an IgG. To minimize cycle time, and maximize system performance, higher flow rates (0.5 ml/min) were used for loading, desalting, and column regeneration.

A system controlled post-column valve was used for waste diversion of sample buffers and salts. Additional sawtooth (rapid) gradient cycles were applied to regenerate the column to pre-injection conditions as part of each analysis (Table 4). Overlaid TICs (y-axis linked) for this experiment and the associated summed mass spectra are shown in Figures 8 and 9, respectively.

Time (min)	%B	Flow (ml/min)	Curve	
0.00	5	0.5	Initial	Load/ Wash - Divert Flow-
0.50	5	0.5	6	
0.51	5	0.2	6	Gradient
2.00	90	0.2	6	
2.10	5	0.5	6	Column Washing and Regeneration
2.70	90	0.5	6	
2.80	5	0.5	6	
3.40	90	0.5	6	
3.50	5	0.5	6	
4.00	5	0.5	6	
A= 0.1 % Formic acid (Water)				
B= 0.1 % Formic acid (ACN) Column temperature: 80 °C				

Table 4: Gradient profile used for intact IgG1 analysis.

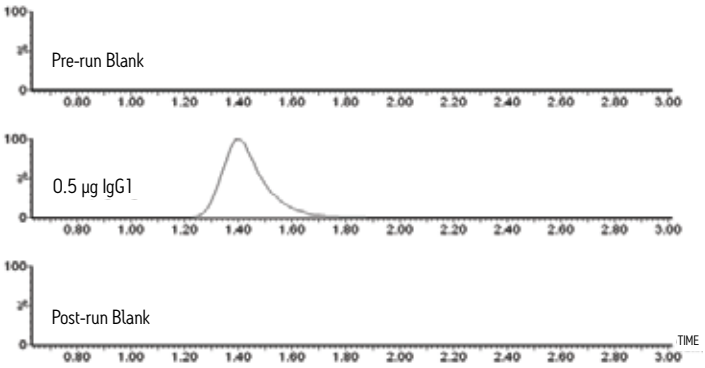

Figure 8: Total ion chromatograms (TICs) from Waters ACQUITY UPLC/LCT Premier ESI-TOF MS analyses of an intact IgG1, and pre and post blank runs. Column temperature was set to 80 °C for these analyses

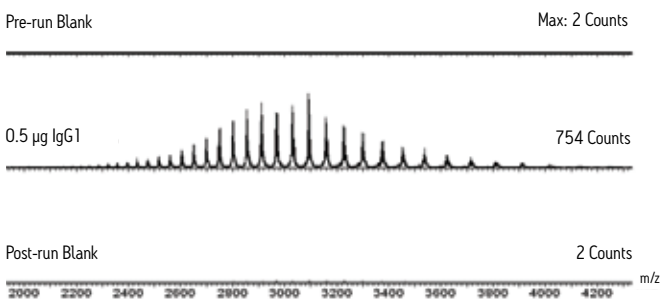

Figure 9: Combined ESI-TOF (ACQUITY UPLC/LCT Premier ESI-TOF MS) mass spectra of an intact IgG1 demonstrating regeneration to pre-injection conditions.

b. Reduced Antibody Analysis

A ten minutes LC/ESI-MS method was used to resolve and characterize IgG heavy and light chain subunits. For efficient sample desalting, a system controlled post-column valve was used for waste diversion of sample buffers and salts, prior to initiating the analysis gradient. Additional sawtooth gradient cycles were applied following the analysis gradient to regenerate the column back to pre-injection conditions (Table 5). To minimize run cycle time, and maximize system performance, higher flow rates (0.5 ml/min) were applied for column regeneration. The 10 min LC/MS run largely resolved the earlier eluting light chain from the later eluting glycosylated heavy chains (Figure 10).

Time (min)	%B	Flow (ml/min)	Curve	
0.00	5	0.2	Initial	Load/ Wash - Divert FLow-
0.50	5	0.2	6	
0.51	10	0.2	6	Gradient
7.61	50	0.2	6	
8.0	90	0.5	6	Column Washing and Regeneration
8.1	5	0.5	6	
8.6	90	0.5	6	
8.7	5	0.5	6	
9.2	90	0.5	6	
9.3	5	0.5	6	
9.8	5	0.5	6	
A= 0.1 % Formic acid (Water)				
B= 0.1 % Formic acid (ACN) Column temperature: 80 °C				

Table 5: Gradient profile used for reduced IgG1 analysis.

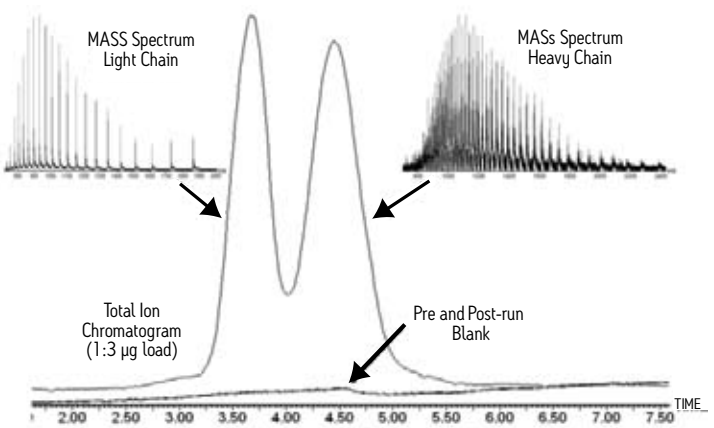

Figure 10: Total ion chromatograms (TICs) from LC/MS analyses of a reduced IgG1, and pre and post blank runs. Combined ESI-TOF mass spectra of light and heavy chains are shown in inset. Column temperature was set to 80 °C for these analyses.

MS Conditions

The MS parameters listed below can vary depending upon the physical characteristics of the analyzed proteins, flow rate, and MS probe position.

MS Conditions	LCT Premier MS	Synapt HDMS	SQD*
Ionization Mode	ESI Positive	ESI Positive	ESI Positive
Capillary Voltage	3-3.2 kV	2-3 kV	4.2-4.5 kV
Cone Voltage	40-50 V	35-37 V	39-45 V
Desolvation Temp	350-450 °C	350-450 °C	350-450 °C
Source Temp	120-150 ° C	120-150 °C	120-150 °C
Desolvation Gas	800 L/Hr	800 L/Hr	600-800 L/Hr
Acquisition range (m/z)	600-5000 (Intact)/ 600-3000 (Reduced)	600-5000 (Intact)/ 600-3500 (Reduced)	600-2000 (Reduced)
Ion Guide I	80- 100 V (Intact)/ 5 V (Reduced)		

Note: Desolvation temp, source temp, and desolvation gas values used in the above table are based on 0.5 ml/min flow rate.

** Waters SQD parameters can be optimized using IntelliSmart™ software.*

TIPS FOR SUCCESS

Results obtained with the UPLC Intact Mass Analysis Application Kit can vary depending upon the physical characteristics of the analyzed proteins as well as the performance characteristics of the LC/MS system, injector, and injection wash protocol used. Additional sawtooth gradient cycles can be applied following the analysis gradient to regenerate the column back to pre-injection conditions. Suggested needle wash solvents and column temperature for antibody LC/MS analysis are listed below:

- Needle Weak Wash Solution Composition: 0.1% Formic acid in Water
- Needle Strong Wash Solution Composition: 65% ACN/5% IPA/ 30% Water in 0.1% Formic acid
- Column temperature: 80 °C

Separation of light and heavy chains of an antibody can be obtained by optimizing gradient slope (gradient slope is measured as percent of organic such as acetonitrile per mobile phase volume passed through the column), flow rate, initial gradient strength and column temperature. Set the column temperature to 80 °C. Use the gradient method in Table 5 as a starting point for optimizing the separation of light and heavy chains of an antibody. Follow the steps below to further optimize the separation of light and heavy chains.

1. Find a gradient slope that provides an acceptable resolution of your reduced antibody.
2. Use the result from the initial separation to design a gradient (focus gradient slope) that improves the resolution between light and heavy chains by focusing the gradient (narrow gradient)
3. Then adjust the gradient initial strength (while keeping the gradient slope constant) to reduce the analysis time without sacrificing the resolution.

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