

Title: Five minute high-resolution metabolomics for human plasma and serum on Thermo Fusion Orbitrap

SOP: HRM_FusionMS_082016_01 Revision: 1

Date effective: 30 July 2016

Chemicals Needed:

- 5000 μ L LC-MS grade acetonitrile
- 125 μ L stable isotope internal standard solution containing: [$^{13}\text{C}_6$]-D-glucose, [^{15}N , $^{13}\text{C}_5$]-L-methionine, [$^{13}\text{C}_5$]-L-glutamic acid, [^{15}N]-L-tyrosine, [3,3- $^{13}\text{C}_2$]-cystine, [trimethyl- $^{13}\text{C}_3$]-caffeine, [U- $^{13}\text{C}_5$, U- $^{15}\text{N}_2$]-L-glutamine
- PierceTM LTQ ESI Positive Ion Calibration Solution, Product #88322
- PierceTM LTQ ESI Negative Ion Calibration Solution, Product #88324
- Positive ESI mobile phases: 1L LC-MS grade H₂O (Red-A); 1L LC-MS grade acetonitrile (Red-B); 1L 5% formic acid in LC-MS grade H₂O (Red-C)
- Negative ESI mobile phases: 1L LC-MS grade H₂O (Green-A); 1L LC-MS grade acetonitrile (Green-B); 1L 10mM ammonium acetate in LC-MS grade H₂O (Green-C)

Materials Needed

- 250 μ L q3June2014
- 50 μ L NIST SRM 1950
- 150 μ L conditioning plasma
- 40 study samples (≥ 50 μ L of sample required)
- Labeled 1.5mL microfuge tubes
- Calibrated P200 and P1000 Micropipettes with 200 μ L and 1000 μ L tips
- Refrigerated centrifuge at 4°C with speed $\geq 16,100 \times g$
- Vortexer
- Labeled, low-volume LC vials with snap caps,
- Higgins endcapped C18 stainless steel column. 2.1mm x 50mm x 3 μ m particle size, Product #TS-0521-C183
- Waters XBridge BEH Amide XP HILIC column. 2.1mm x 50mm x 2.5 μ m particle size. Product #186006089
- Thermo Accucore C18 guard column with holder, Product #17126-014005
- Thermo Accucore HILIC guard column with holder, Product # 17526-012105
- High-resolution Orbitrap mass spectrometer with ESI source
- Dual LC pumps with degasser, autosampler and switching valves
- Two cylinders ultra high-purity N₂

Instrumentation

- Centrifuge, Eppendorf 5430R, Room 225: Prior to starting sample preparation set speed to 16,100 $\times g$ and temperature to 4°C. Cool using “fast cool” option. When loading samples, makes sure samples are evenly distributed around the wheel.

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- HPLC, Thermo Scientific Dionex Ultimate 3000RSLCnano with refrigerated autosampler, dual channel pumps, 10-port and 6-port switching valves, with left pump set to control HILIC positive gradient and right pump set to control C18 negative gradient. Start pumps at 350 mL/min at initial conditions: Left pump 75% B and 2.5% C; Right pump 35% B and 5% C.
- High-resolution mass spectrometer, Thermo Scientific Orbitrap Fusion Tribrid Mass Spectrometer with Easy Max NG ESI source and Peak Scientific Genius NM32LA nitrogen generator system. Turn system on in Orbitrap Fusion Tune Application to allow for equilibration. If calibration is required, move source to closest position and load Cal_pos_03042016 in User Settings. If calibration is not required, ensure source is in position shown below and load Doug_metabolomics_26July2016 in User Settings.

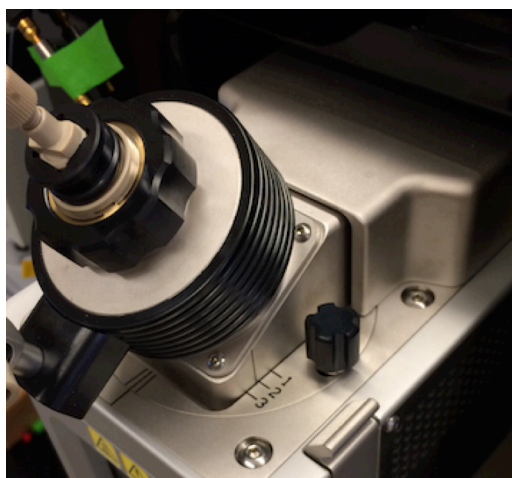


Figure 1: Source position for sample analysis by Easy Max NG ESI source

Mass Spectrometer Calibration

Mass calibration for both positive and negative mode is to be completed every 7 days (Monday) OR prior to the beginning of a new study. Other components, including the quadrupole, ion trap and predictive AGC is to be calibrated once every 30 days OR at the beginning of a large study. Calibration can be completed by trained staff only.

To calibrate in positive ion mode: Move source to closest position and load Cal_pos_03042016 in User Settings. Verify source settings match Table 1A. Fill positive calibration syringe with positive ion calibration solution. Place in syringe pump and attach to ion source using positive calibration peek line. In the Orbitrap Fusion Tune Application, select “Syringe” and set flow to 5 μ L/min. Click start. Monitor normalization level until intensity reaches $\sim 10^8$. When spray is stable, go to the “Calibration” pane and select appropriate tune procedures. Verify “Check tune”

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box is selected, and click start. If calibration fails, verify spray is stable and normalization level is still at $\sim 10^8$. Unselect “Check tune” and click start. When done, save .PDF and rinse syringe with methanol. Make sure to move source back to appropriate position for sample analysis (Figure 1).

To calibrate in negative ion mode: Move source to closest position and load neg_tune_VT_072016 in User Settings. Verify source settings match Table 1B. Fill negative calibration syringe with negative ion calibration solution. Place in syringe pump and attach to ion source using positive calibration peek line. In the Orbitrap Fusion Tune Application, select “Syringe” and set flow to 10 $\mu\text{L}/\text{min}$. Click start. Monitor normalization level until intensity reaches $\sim 10^8$. When spray is stable, go to the “Calibration” pane and select appropriate tune procedures. Verify “Check tune” box is selected, and click start. If calibration fails, verify spray is stable and normalization level is still at $\sim 10^8$. Unselect “Check tune” and click start. When done, save .PDF and rinse syringe with methanol. Make sure to move source back to appropriate position for sample analysis (Figure 1).

Table 2A: Positive calibration source parameters

Spray voltage	+4200
Sheath gas	10
Aux gas	0
Sweep gas	0
Ion transfer tube temp	320
Vaporizer temp	50
AGC	10^5
Scan Range	150-2000
Resolution	120,000
Max injection time	100
RF level	55

Table 2B: Negative calibration source parameters

Spray voltage	-3000
Sheath gas	8
Aux gas	0
Sweep gas	0
Ion transfer tube temp	320
Vaporizer temp	55
AGC	10^5
Scan Range	150-2000
Resolution	120,000
Max injection time	100
RF level	55

Sample preparation

Samples are to be prepared daily, and placed in the autosampler for analysis immediately upon completing sample preparation

1. Remove conditioning, QC and study samples from storage at -80°C and thaw on ice
2. Remove internal standard solution from storage at -80°C and thaw.
3. Label clean, microfuge tubes.
4. Add 125 μL of internal standard solution to 5000 μL acetonitrile, vortex and store on ice.

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5. Carefully pipette 50 μ L of thawed sample to appropriate microfuge tube. Ensure no air bubbles or clogs occur in pipette tip. Use a fresh tip for each sample
6. Carefully pipette 100 μ L of acetonitrile/internal standard solution into each tube and close snap top.
7. Vortex each tube for 10 sec.
8. Place tube on ice and allow to equilibrate for 30 min.
9. Return remaining samples to storage at -80°C .
10. Following equilibration period, centrifuge tubes at 4°C for 10 min at $16.1 \times g$.
11. Label clean, LC vials.
12. Carefully pipette 100 μ L of supernatant into corresponding LC vial.
13. Cap.
14. Load into autosampler racks based on predetermined run order.

Sequence Creation

All sequences should be created in Excel prior to completing sample preparation and saved as a .CSV file. Each sample is injected sequentially six times alternating between two different configurations, which includes three replicates per HILIC-positive configuration (odd numbered injections) and three replicates per C18-negative configuration (even numbered injections). Organization of samples into batches should be randomized by investigator providing the samples or with consultation by trained statistician. Sample run order is structured in the following order:

- Sample 1: NIST SRM 1950
- Sample 2: Qstd 1
- Sample 3: Qstd 2
- Sample 4-24: Study samples 1-20
- Sample 25: Qstd 3
- Sample 26: Qstd 4
- Sample 27-47: Study samples 21-40
- Sample 48: Qstd 5
- Sample 49: Qstd 6
- Sample 50: NIST SRM 1950

Each of the sequence file fields is to be completed using the following formatting:

- Sample type: Fill column with “Unknown”
- File Name: Filenames are to follow the format: VT_YYMMDD_StudyID_###, where VT are the initials of the individual preparing and running samples, YYMMDD is the date the samples were prepared and loaded onto the autosampler, StudyID is the

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designated Clinical Biomarkers Laboratory study identifier, and ### is batch injection order, starting at 001.

- **Sample ID:** Identifying number designated by the original study. Replicate injection order is designated by concatenating “_#”, starting at 1 and ending at 6. NIST samples are named “nist_batch#_#” and Qstd are labeled with “Qstdname_batch#_a-d_#” where batch# is the number of the batch and a-d is used to represent which Qstd in that batch (i.e. 1, 2, 3, 4 or 5).
 - **Path:** Local directory to store acquisition files. All files should be saved to the D:\Projects folder on the instrument control computer. Name master projected folders as “ProjectName_Investigator_DateStarted”. Each batch of samples is saved to a separate folder named “Batch_##”, starting at 01.
 - **Inst Meth:** Instrument control method.
- HILIC-positive:**
C:\Xcalibur\methods\Metabolomics_methods_HILICpos_C18neg\20160728_HILICpos_c18negwash_FullScan_5min
- c18-negative:**
C:\Xcalibur\methods\Metabolomics_methods_HILICpos_C18neg\20160728_c18neg_HILICposwash_FullScan_5min
- **Autosampler position:** Entered as plate color (green (G), red (R) or blue (B)) and position based on rows A-E and columns 1-8 (i.e. position 1 on green plate is GA1). Total number of vials per plate is 40.
 - **Inj Vol:** Fill column with 10
 - **Comment:** Enter batch number

Data Collection

All method and instrument parameters are to be used as designated in this document. Do not make any changes to the configuration without specific permission from Dr. Dean Jones, Vilinh Tran or Douglas Walker. If columns are new, prepare and analyze 20 conditioning samples (120 injections) prior to starting batch. If instrument has been idle prior to starting batch, prepare and analyze 5 conditioning samples (30 injections) prior to starting batch.

1. Verify all solvent and wash bottles are full. If not, add appropriate solvents.
2. Check N₂ generator and LC waste bottles. Empty/replace if needed.
3. Turn on HPLC and MS and set as specified in the Instrumentation section. Calibrate if needed according the Mass spectrometer calibration section.
4. Verify correct columns are installed.
5. Verify ion source is in correct position (Figure 1).
6. Verify samples are loaded into the autosampler in the correct positions.
7. In Xcalibur, go to Instrument Setup and verify methods match parameters given in Method Details section.

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8. Right click on Dionex Chromatography MS Link in the Xcalibur Status pane and select Turn Device On.
9. If columns are new,
10. In Xcalibur, select Sequence Setup.
11. Go to File dropdown menu and select Import Sequence. Load sequence created following protocol given in Sequence Creation section.
12. Navigate to D:/Projects folder, and create appropriate acquisition file folder.
13. Save sequence as “YYMMDD_StudyID_Batch#
14. Select all injections
15. Go to the Actions dropdown menu and select Run Sequence
16. Select “Standby” in After Sequence Set System box
17. Click OK
18. Verify batch starts by watching Real Time Plot TIC.

HILIC-positive Method

Location:

Metabolomics_methods_HILICpos_C18neg/20160728_HILICpos_c18negwash_FullScan_5min

LC settings

- Run length: 5.5 min
- Valve 1 position: 1_2 at 0 min; 10_1 at 5 min
- Valve 2 position: 1_2
- Column oven temperature: 40°C
- Pump left: A= Water; B= Acetonitrile, C=10mM ammonium acetate
- Pump right: A= Water; B= Acetonitrile, C=5% formic acid
- Sampler: Draw speed= 1000 nL/s; Draw delay= 3000 ms, Dispense speed= 5000 nL/s; Dispense delay= 500 ms; Dispense to waste= 4000 nL/s; Sample height= 4mm; Puncture depth= 8mm; Wash volume= 50 µL; Wash speed= 4000 nL/s; Loop wash factor= 2; Injection mode= Partial; Flush volume= 15 µL; Flush volume 2= 0 µL; Drawer temperature= 4°C

LC gradient and flow information:

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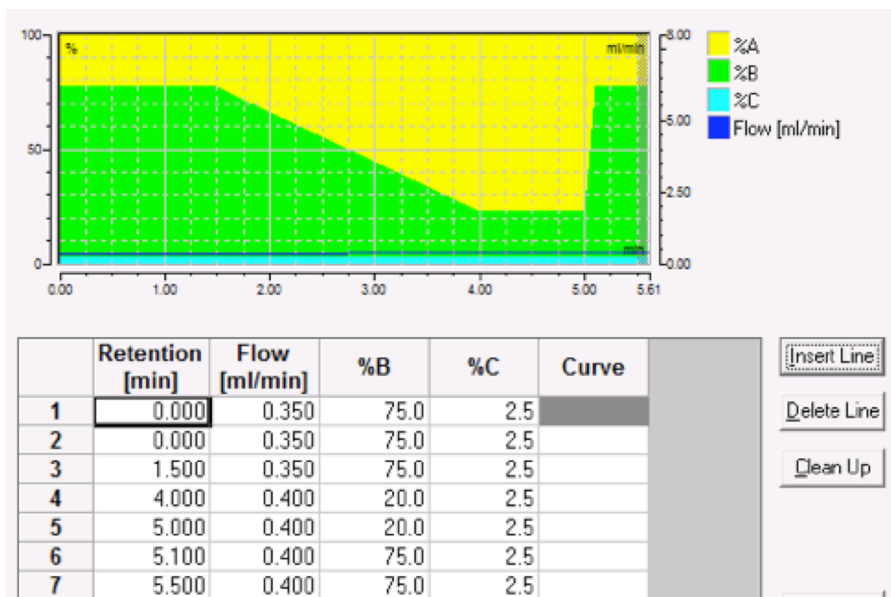


Figure 2A: Left pump mobile phase gradient and flow rate for HILIC-positive (HILIC analytical separation)

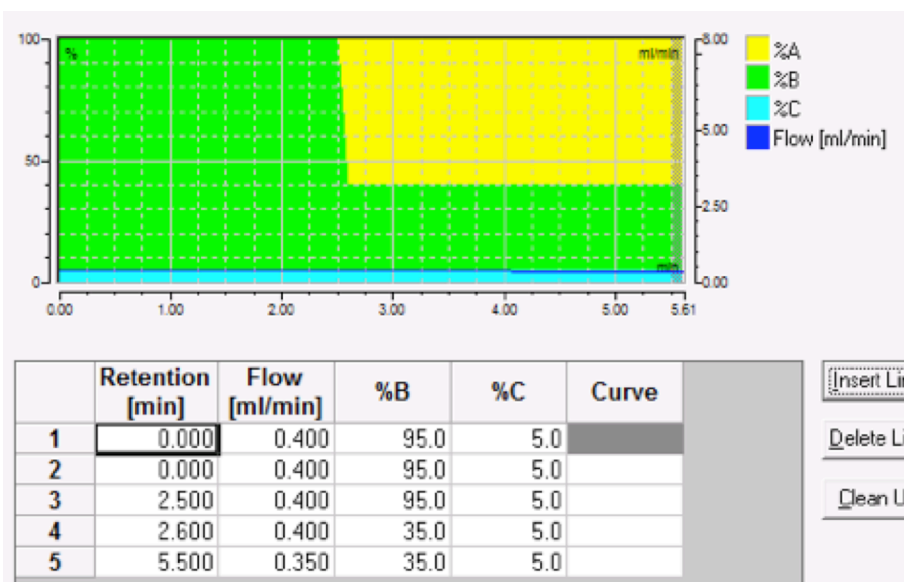


Figure 2B: Right pump mobile phase gradient and flow rate for HILIC-positive (washing C18)

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MS settings:

- Run length: 5 min
- Ion source type: HESI
- Detector type: Orbitrap
- Mass filter: Use quadrupole isolation
- Mass range: Normal
- Microscans: 1
- Data type: Profile

Table 2: Key MS settings for HILIC-positive

Spray voltage	+3500
Polarity	Positive
Sheath gas	45
Aux gas	25
Sweep gas	1
Ion transfer tube temp	300
Vaporizer temp	250
AGC	5.0e5
Resolution	60,000
Max injection time	118
S-Lens RF level	69
Scan range	85-1275

c18-negative Method

Location:

Metabolomics_methods_HILICpos_C18neg/20160728_c18neg_HILICposwash_FullScan_5min

LC settings

- Run length: 5.5 min
- Valve 1 position: 10_1 at 0 min; 1_2 at 5 min
- Valve 2 position: 1_6
- Column oven temperature: 40°C
- Pump left: A= Water; B= Acetonitrile, C=2% formic acid
- Pump right: A= Water; B= Acetonitrile, C=10mM ammonium acetate

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- Sampler: Draw speed= 1000 nL/s; Draw delay= 3000 ms, Dispense speed= 5000 nL/s; Dispense delay= 500 ms; Dispense to waste= 4000 nL/s; Sample height= 4mm; Puncture depth= 8mm; Wash volume= 50 μ L; Wash speed= 4000 nL/s; Loop wash factor= 2; Injection mode= Partial; Flush volume= 15 μ L; Flush volume 2= 0 μ L; Drawer temperature= 4°C

LC gradient and flow information:

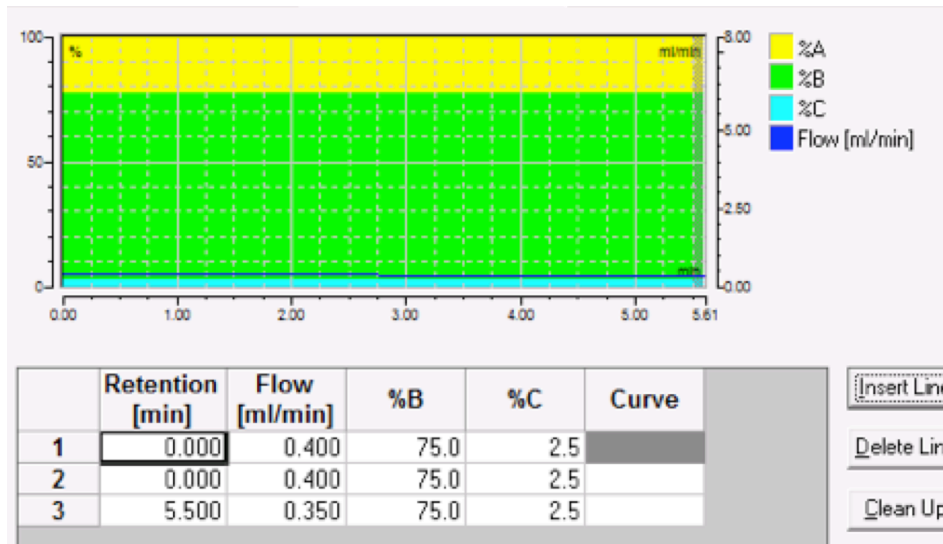


Figure 3A: Left pump mobile phase gradient and flow rate for C18-negative (washing HILIC)

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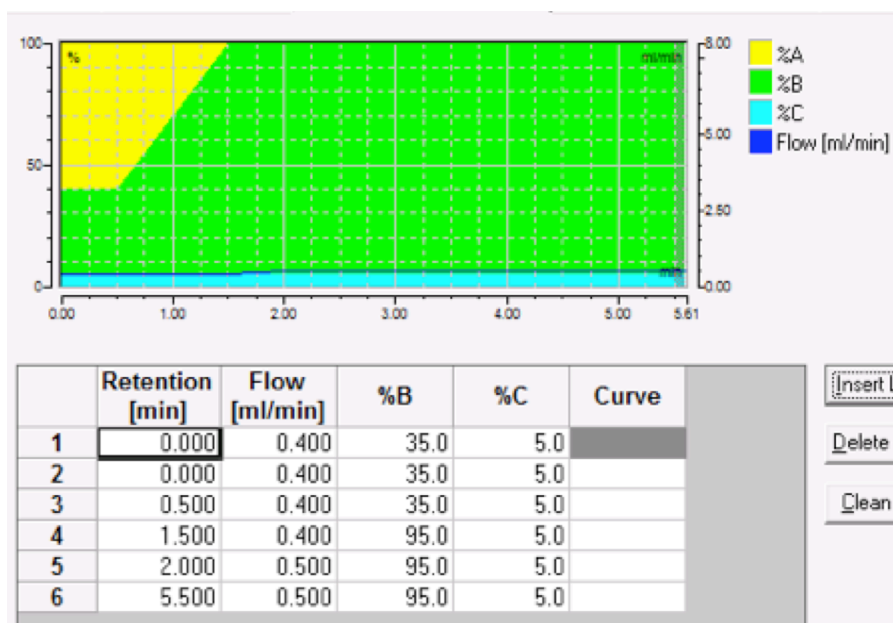


Figure 3B: Right pump mobile phase gradient and flow rate for C18-negative (C18 analytical separation)

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MS settings:

- Run length: 5 min
- Ion source type: HESI
- Detector type: Orbitrap
- Mass filter: Use quadrupole isolation
- Mass range: Normal
- Microscans: 1
- Data type: Profile

Table 3: Key MS settings for C18-negative

Spray voltage	-4000
Polarity	Negative
Sheath gas	45
Aux gas	5
Sweep gas	1
Ion transfer tube temp	300
Vaporizer temp	250
AGC	5.0e5
Resolution	60,000
Max injection time	118
S-Lens RF level	69
Scan range	85-1275

Spot Quality Control Checks

During the course of the run, spot quality control is to be completed to ensure proper instrument operation. The periodic checking is not intended to replace batch QC, but is meant to check status of the instrument while in operation. Spot QC is performed by checking peak shape, intensity, retention time and mass window isolation for selected targets and internal standards (Figure 4). Layout templates are saved in the following:

- **HILIC-positive:** C:\Xcalibur\methods\internal_standards_QC_template.lyt
- **C18-negative:** C:\Xcalibur\methods\c18_neg_internal_stds_QC_template.lyt

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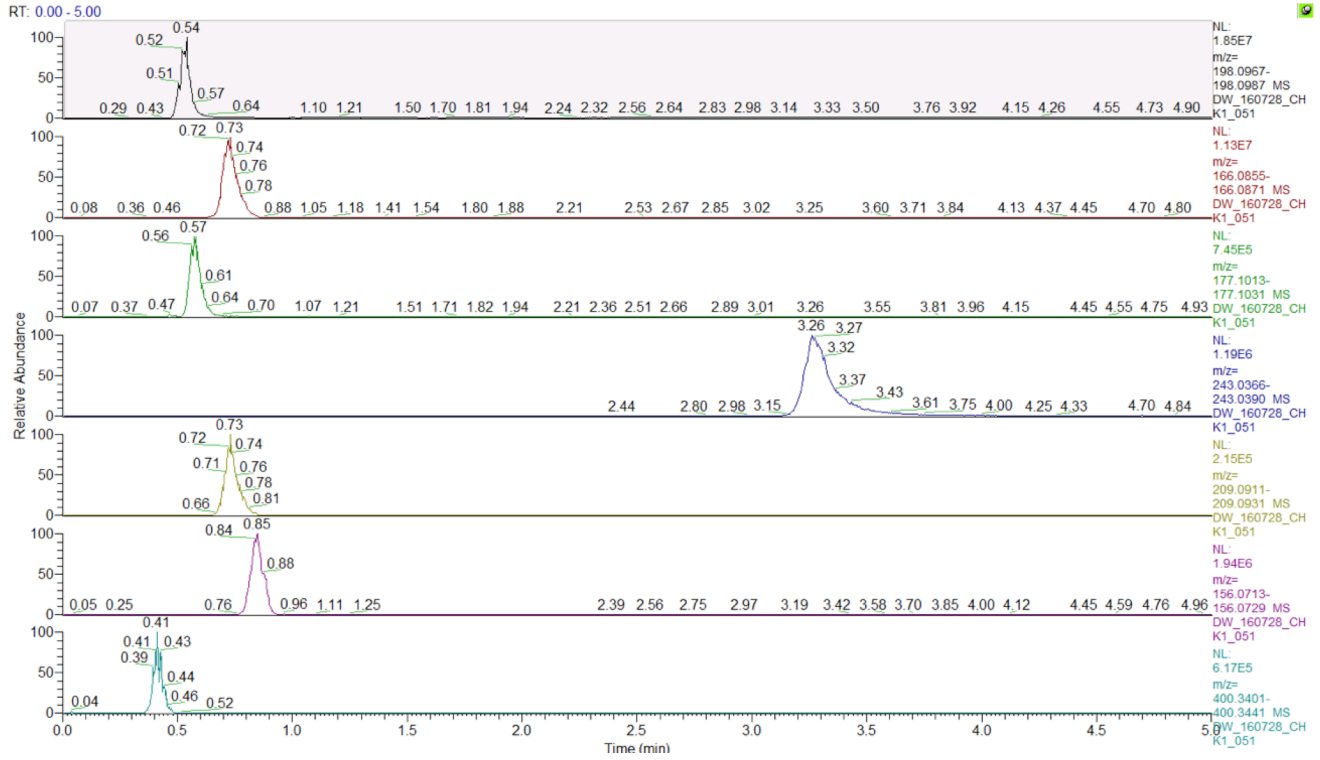


Figure 3A: EIC layout to check data quality for HILIC-positive

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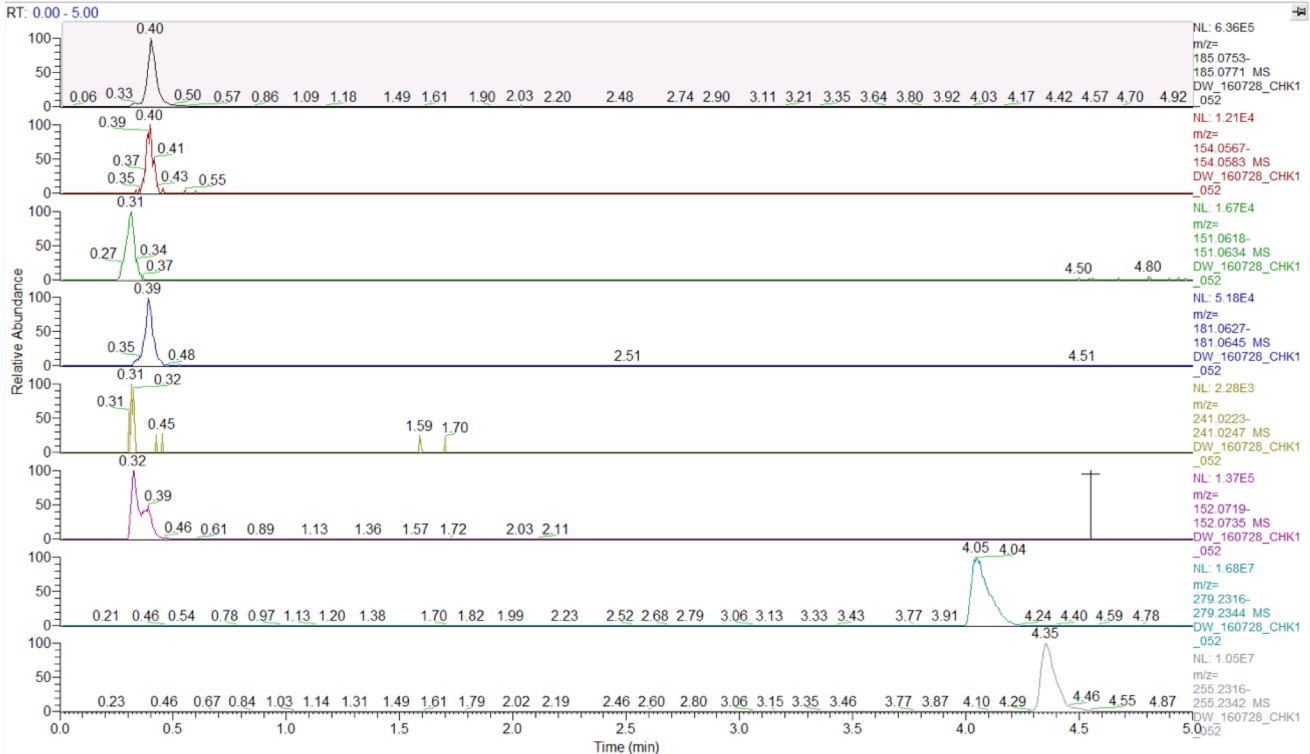


Figure 4B: EIC layout to check data quality for C18-negative

Instrument maintenance schedule

The following maintenance schedule is maintained to ensure the highest data quality possible. The time limits given should never be exceeded, however, it is permissible to perform maintenance early if coincides with the start of a new study. Maintenance is to be completed by trained staff only.

- Mass calibration: Once a week (Mondays)
- Ion trap, quadrupole, predictive AGC calibration (once a month)
- Capillary and sweep cone cleaning: Once a week (during mass calibration)
- Column lifespan: 3000 injections
- S-Lens: Clean once a month (during instrument calibration) OR prior to starting large study
- N₂ generator PM: Annually
- LC and MS PM: Annually



Department of Medicine

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SOP Details and Version Information

Created by: Douglas I. Walker	Date: 30 July 2016
Reviewed by: Vilinh Tran	Date: 02 August 2016
Approved by: Dean P. Jones	Date: 04 August 2016

Revision	Name	Reason	Effective date
01	Douglas I. Walker	Creation of SOP	30 July 2016