EMORY UNIVERSITY SCHOOL OF MEDICINE Department of Medicine	**Clinical Biomarkers Laboratory** **Division of Pulmonary Allergy and Critical Care Medicine** **615 Michael St. Ste. 225, Atlanta GA, 30322**
Title: Five minute high-resolution metabolomics for human plasma and serum on Thermo Orbitrap Fusion Tribrid	
SOP: HRM_Fusion_092017_v1 Revision: 1	**Date effective:** 27 September 2017

Chemicals Needed:

- 5000 µL LC-MS grade acetonitrile
- 125 µL stable isotope internal standard solution containing: [$^{13}C_6$]-D-glucose, [^{15}N,$^{13}C_5$]-L-methionine, [$^{13}C_5$]-L-glutamic acid, [^{15}N]-L-tyrosine, [3,3-$^{13}C_2$]-cystine, [trimethyl-$^{13}C_3$]-caffeine, [U-$^{13}C_5$, U-$^{15}N_2$]-L-glutamine
- Pierce™ LTQ ESI Positive Ion Calibration Solution, Product #88322
- Pierce™ LTQ ESI Negative Ion Calibration Solution, Product #88324
- Positive ESI mobile phases: 1L LC-MS grade H_2O (Red-A); 1L LC-MS grade acetonitrile (Red-B); 1L 5% formic acid in LC-MS grade H_2O (Red-C)
- Negative ESI mobile phases: 1L LC-MS grade H_2O (Green-A); 1L LC-MS grade acetonitrile (Green-B); 1L 10mM ammonium acetate in LC-MS grade H_2O (Green-C)

Materials Needed

- 250 µL q3June2014
- 50 uL NIST SRM 1950
- 150 uL conditioning plasma
- 40 study samples (≥50 µL of sample required)
- Labeled 1.5mL microfuge tubes
- Calibrated P200 and P1000 Micropipettes with 200 µL and 1000 µL tips
- Refrigerated centrifuge at 4°C with speed ≥ 16,100 × *g*
- Vortexer
- Labeled, low-volume LC vials with snap caps,
- Higgins endcapped C18 stainless steel column. 2.1mm x 50mm x 3µm particle size, Product #TS-0521-C183
- Waters XBridge BEH Amide XP HILIC column. 2.1mm x 50mm x 2.5µm 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 N_2

Instrumentation

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

- HPLC, Thermo Scientific Dionex Ultimate 3000 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 0.350 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 ESI source and Peak Scientific Genius NM32LA nitrogen generator system. Turn system on in Thermo Orbitrap Fusion Tribrid Application to allow for equilibration.

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. **Calibration must be completed by trained staff only.**

To calibrate in positive ion mode:
Fill positive calibration syringe with positive ion calibration solution. On right panel, click on "Favorites" and click on "Cal_Pos_11292017". Place in syringe pump and attach to ion source using positive calibration peek line. In the Tune Application, select "Syringe" and set flow to 10 µL/min. Click on Syringe button to turn on. On left panel, select the Define Scan tab and make sure that the Detector Type is set to Orbitrap. Monitor normalization level until intensity reaches ~10^8. When spray is stable, " click on the "Calibration" tab on the left panel and select all of the Positive calibration. Click on "Start" button found on the bottom. Verify calibration is completed successfully.

To calibrate in negative ion mode:
Fill negative calibration syringe with negative ion calibration solution. On right panel, click on "Favorites" and click on "Cal_Neg_11292017". Place in syringe pump and attach to ion source using negative calibration peek line. In the Tune Application, select "Syringe" and set flow to 10 µL/min. Click on Syringe button to turn on. On left panel, select the Define Scan tab and make sure that the Detector Type is set to Orbitrap. Monitor normalization level until intensity reaches ~10^8. When spray is stable, " click on the "Calibration" tab on the left panel and select all of the Positive calibration. Click on "Start" button found on the bottom. Verify calibration is completed successfully.

Table 2A: Positive calibration source parameters

Spray voltage	+4000
Sheath gas	3
Aux gas	1.2
Sweep gas	0
Ion transfer tube temp	325
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	1
Aux gas	1
Sweep gas	0
Ion transfer tube temp	325
Vaporizer temp	50
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.
5. Carefully pipette 50 µL 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 µL 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 × *g*.
11. Label clean, LC vials.
12. Carefully pipette 100 µL 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: QC Sample 1
- Sample 3: QC Sample 2
- Sample 4-24: Study samples 1-20
- Sample 25: QC Sample 3
- Sample 26: QC Sample 4
- Sample 27-47: Study samples 21-40
- Sample 48: QC Sample 5
- Sample 49: QC Sample 6

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 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 QC are labeled with "QC_batch#_a-d_#" where batch# is the number of the batch and a-d is used to represent which QC 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\Clinical_Biomarkers_Lab_Metabolomics_Mthds/20160920_posHILIC120kres5min_ESI_c18negwash.meth
  c18-negative:

C:\Xcalibur\methods\Clinical_Biomarkers_Lab_Metabolomics_Mthds/20160920_negC18120kres5min_ESI_HILICposwash.meth

- 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 N_2 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.
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.

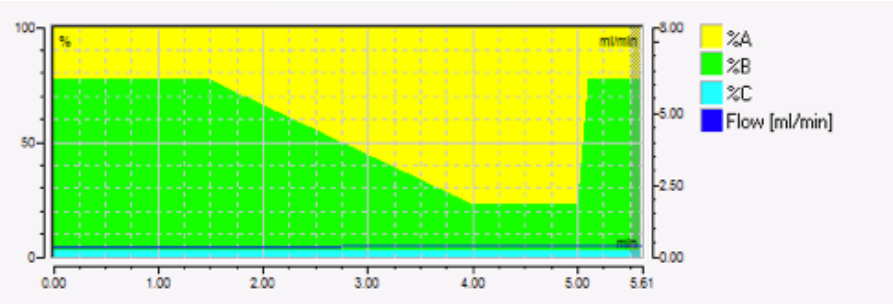
HILIC-positive Method

Location:
C:\Xcalibur\methods\Clinical_Biomarkers_Lab_Metabolomics_Mthds/20160920_posHILIC120kres5min_ESI_c18negwash.meth

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: 60°C
- Pump left: A= Water; B= Acetonitrile, C=2% formic acid in water
- Pump right: A= Water; B= Acetonitrile, C=10mM ammonium acetate in water
- Sampler: Draw speed= 2 µL/s; Draw delay= 1000 ms, Dispense speed= 25 µL/s; Dispense delay= 1000 ms; Dispense to waste= 32 µL/s; Sample height= 4mm; Inject wash= Both; Wash volume= 100 µL; Wash speed= 20 µL/s; Loop wash factor= 2; Injection mode= Normal; Drawer temperature= 8°C

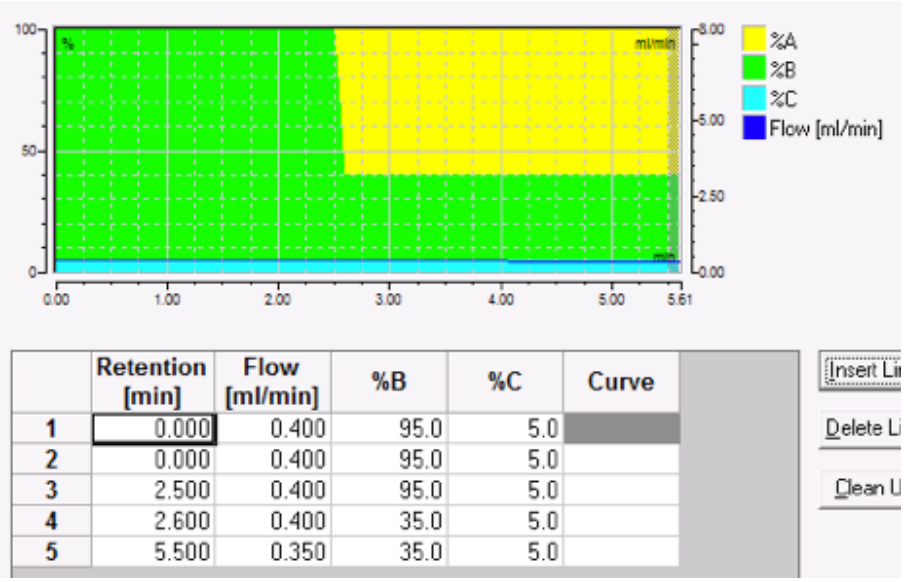
LC gradient and flow information:



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0.000	0.350	75.0	2.5	
2	0.000	0.350	75.0	2.5	
3	1.500	0.350	75.0	2.5	
4	4.000	0.400	20.0	2.5	
5	5.000	0.400	20.0	2.5	
6	5.100	0.400	75.0	2.5	
7	5.500	0.400	75.0	2.5	

Insert Line
Delete Line
Clean Up

Figure 1A: Left pump mobile phase gradient and flow rate for HILIC-positive (HILIC analytical separation)



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0.000	0.400	95.0	5.0	
2	0.000	0.400	95.0	5.0	
3	2.500	0.400	95.0	5.0	
4	2.600	0.400	35.0	5.0	
5	5.500	0.350	35.0	5.0	

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

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	+3.5
Polarity	Positive
Sheath gas	45
Aux gas	25
Sweep gas	1
Ion transfer tube temp	250
Vaporizer temp	150
AGC	1e6
Resolution	120,000
Max injection time	100
S-Lens RF level	55
Scan range	85-1275

c18-negative Method

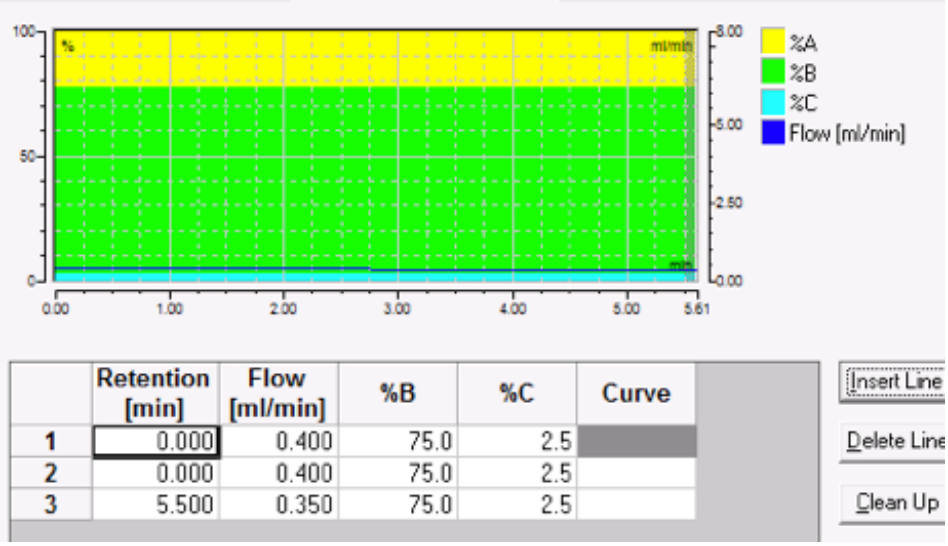
Location:

C:\Xcalibur\methods\Clinical_Biomarkers_Lab_Metabolomics_Mthds/20160920_negC18120kres5min_ESI_HILICposwash.meth

LC settings

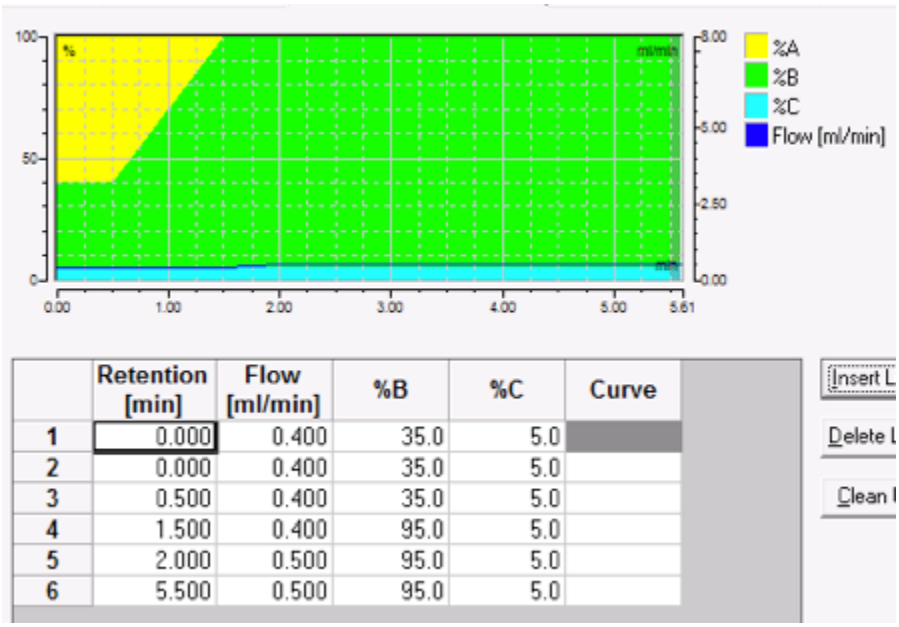
- Valve 1 position: 10_1 at 0 min
- Valve 2 position: 6_1
- Column oven temperature: 60°C
- Column oven temperature: 60°C
- Pump left: A= Water; B= Acetonitrile, C=2% formic acid
- Pump right: A= Water; B= Acetonitrile, C=10mM ammonium acetate
- Sampler: Draw speed= 2 µL/s; Draw delay= 1000 ms, Dispense speed= 25 µL/s; Dispense delay= 1000 ms; Dispense to waste= 32 µL/s; Sample height= 4mm; Inject wash= Both; Wash volume= 100 µL; Wash speed= 20 µL/s; Loop wash factor= 2; Injection mode= Normal; Drawer temperature= 8°C

LC gradient and flow information:



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0.000	0.400	75.0	2.5	
2	0.000	0.400	75.0	2.5	
3	5.500	0.350	75.0	2.5	

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



	Retention [min]	Flow [ml/min]	%B	%C	Curve
1	0.000	0.400	35.0	5.0	
2	0.000	0.400	35.0	5.0	
3	0.500	0.400	35.0	5.0	
4	1.500	0.400	95.0	5.0	
5	2.000	0.500	95.0	5.0	
6	5.500	0.500	95.0	5.0	

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

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	250
Vaporizer temp	150
AGC	1e6
Resolution	120,000
Max injection time	100
S-Lens RF level	55
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

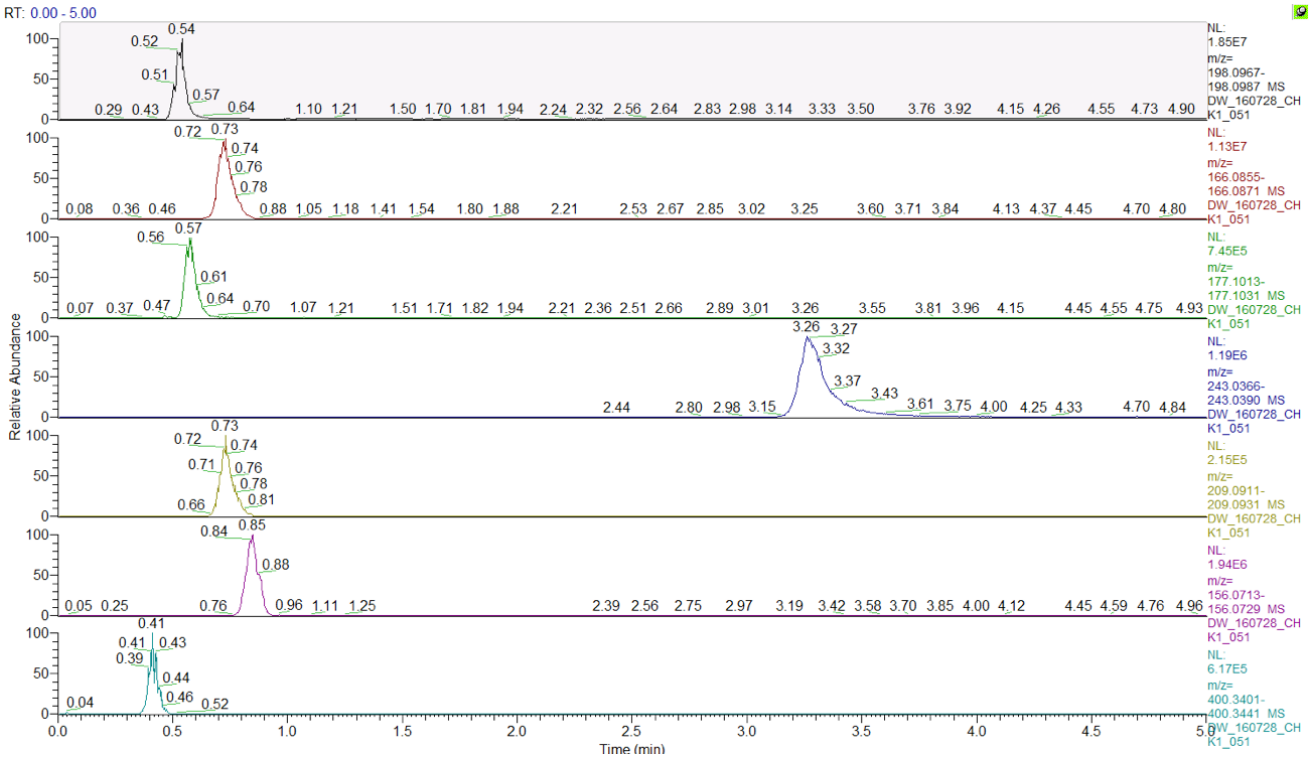


Figure 2A: EIC layout to check data quality for HILIC-positive

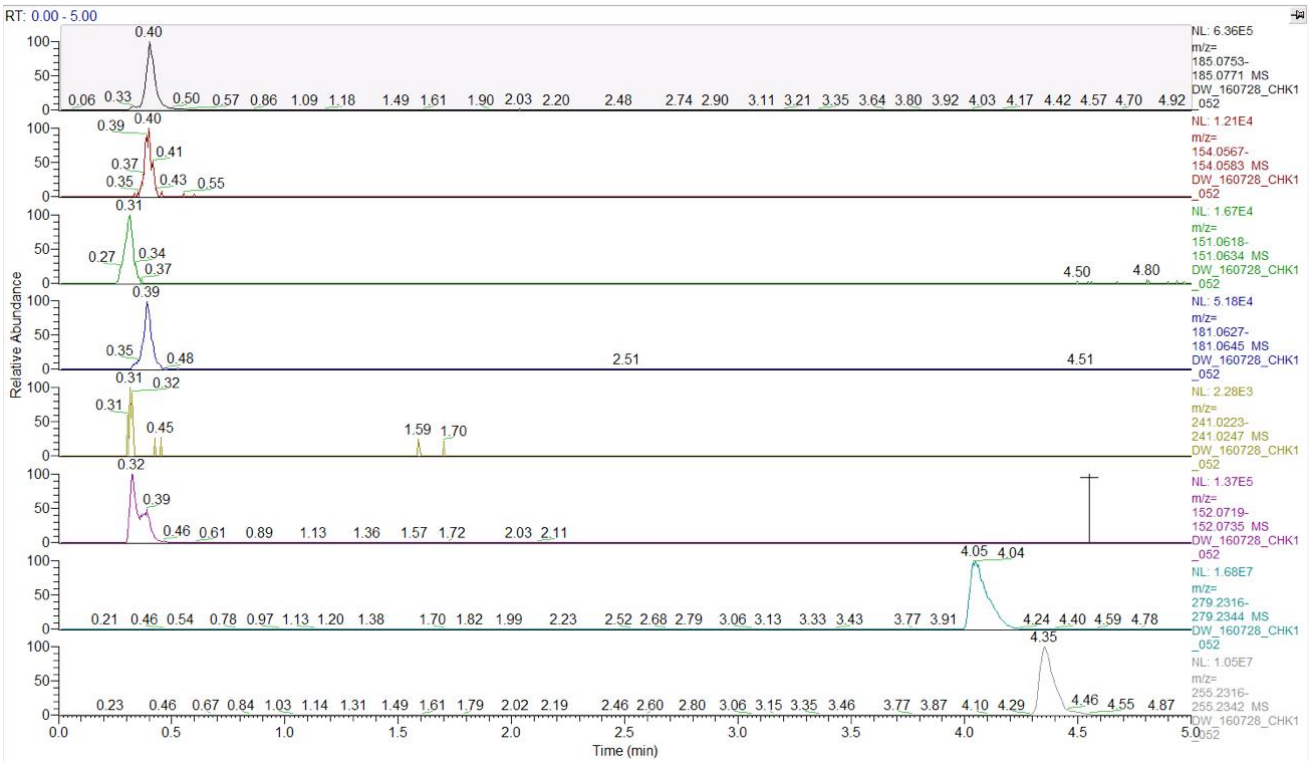


Figure 4B: EIC layout to check data quality for C18-negative

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
- N_2 generator PM: Annually
- LC and MS PM: Annually

SOP Details and Version Information

Created by: Douglas I. Walker, ViLinh Tran	**Date:** 27 September 2017
Reviewed by: Carolyn Accardi	**Date:** 27 September 2017
Approved by: Dean P. Jones	**Date:** 27 September 2017

Revision	Name	Reason	Effective date
01	Douglas I. Walker, ViLinh Tran	Creation of SOP	27 September 2017