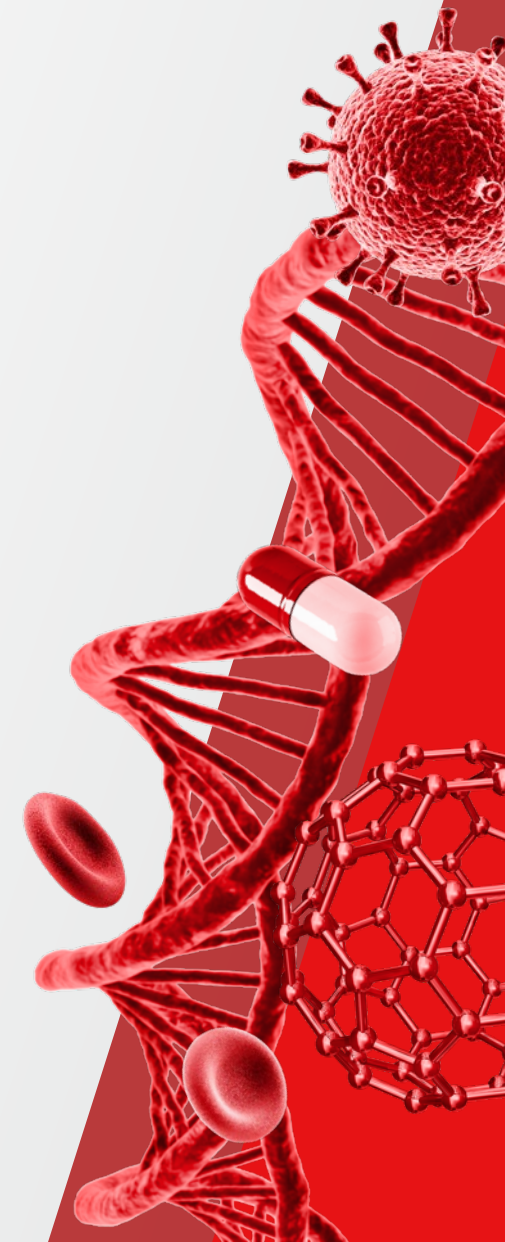


Method for Velocity DIA Workflows on Orbitrap Ascend Tribrid Mass Spectrometer

Kevin Yang

OMICS Applications

 The world leader in serving science



Velocity DIA workflow on Orbitrap Ascend MS

Data Independent Acquisition (DIA)



Thermo Scientific™ Vanquish™
NEO UHPLC System



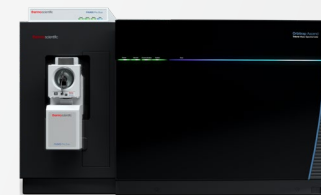
Thermo Scientific™ μPAC™
NEO 50cm Column



Thermo Scientific™ EASY-Spray™
Nano Source



Thermo Scientific™
FAIMS Pro Interface



Thermo Scientific™ Orbitrap Ascend™ Tribrid MS

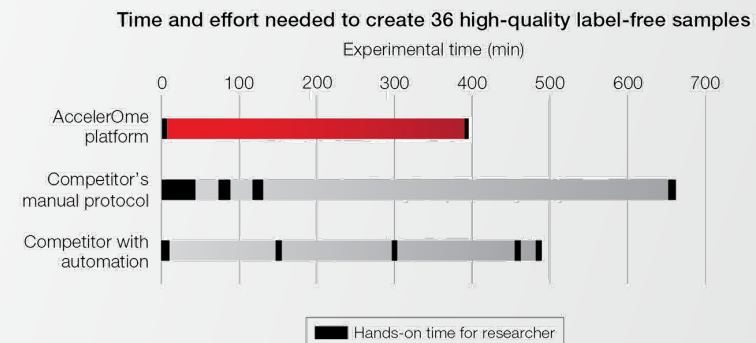


Spectronaut™ 18
DIA-NN 1.8.1



- The Thermo Scientific™ Velocity LFQ DIA workflow can also be coupled to Thermo Scientific™ Accelerome™ automated sample preparation platform to increase productivity.

Learn more at thermofisher.com/AccelerOme



Bundle Orbitrap mass spectrometer

Thermo Fisher Scientific

SKU	SKUName	Qty
	Orbitrap Ascend system with Easy IC	1
ES082	EASY-SPRAY SOURCE KIT (NG)	1
OPTON-31102	Thermo Scientific™ Proteome Discoverer™ 3.0 software with 1 Year CHIMERYS Full	1
FMS03-10001	FAIMS PRO Interface (NG SOURCE)	1
ES993	Nano EASY-Spray Emitter, Bullet Type without transfer line (pack of 2)	1
VN-S10-A-01	Vanquish Neo System	1
VN-C10-A-01	Column Compartment N	1
6036.1180	Vanquish Display	1
OPTON-30697	Kit which include 50um ID needle insert & nanoViper	1
ES75150PN	EASY-Spray PepMap Neo C18 2um 75umx150mm column	1
6250.1520	Valve 2p-6p, Low-Disp, 150 MPa, bio, VN-C	1
6250.1009	LOW DISPERSION Y-PIECE 50UMW. INSERT	1
COL-NANO050NEOB	uPAC NEO 50cm column	1
6000.1001	Power cord US version (4x)	4

Evosep

Cat No.	Name	Qty
EV1072	Thermo Scientific™ EASY-Spray™ Adapter	1
EV1111	Fused silica emitter (10µm)	1

Sample preparation



- Thermo Scientific™ Pierce™ HeLa Protein Digest Standard (#88328)
- Waters MassPREP E. coli Digest (#186003196)
- Promega Mass Spec Compatible Yeast Protein Extract Digest (#V7461)

Digest Stock

- HeLa: 650 ng/μL
- E. coli (E1): 500ng/μL
- E. coli (E2): 125 ng/μL
- Yeast (Y1): 500 ng/μL
- Yeast (Y2): 250 ng/μL

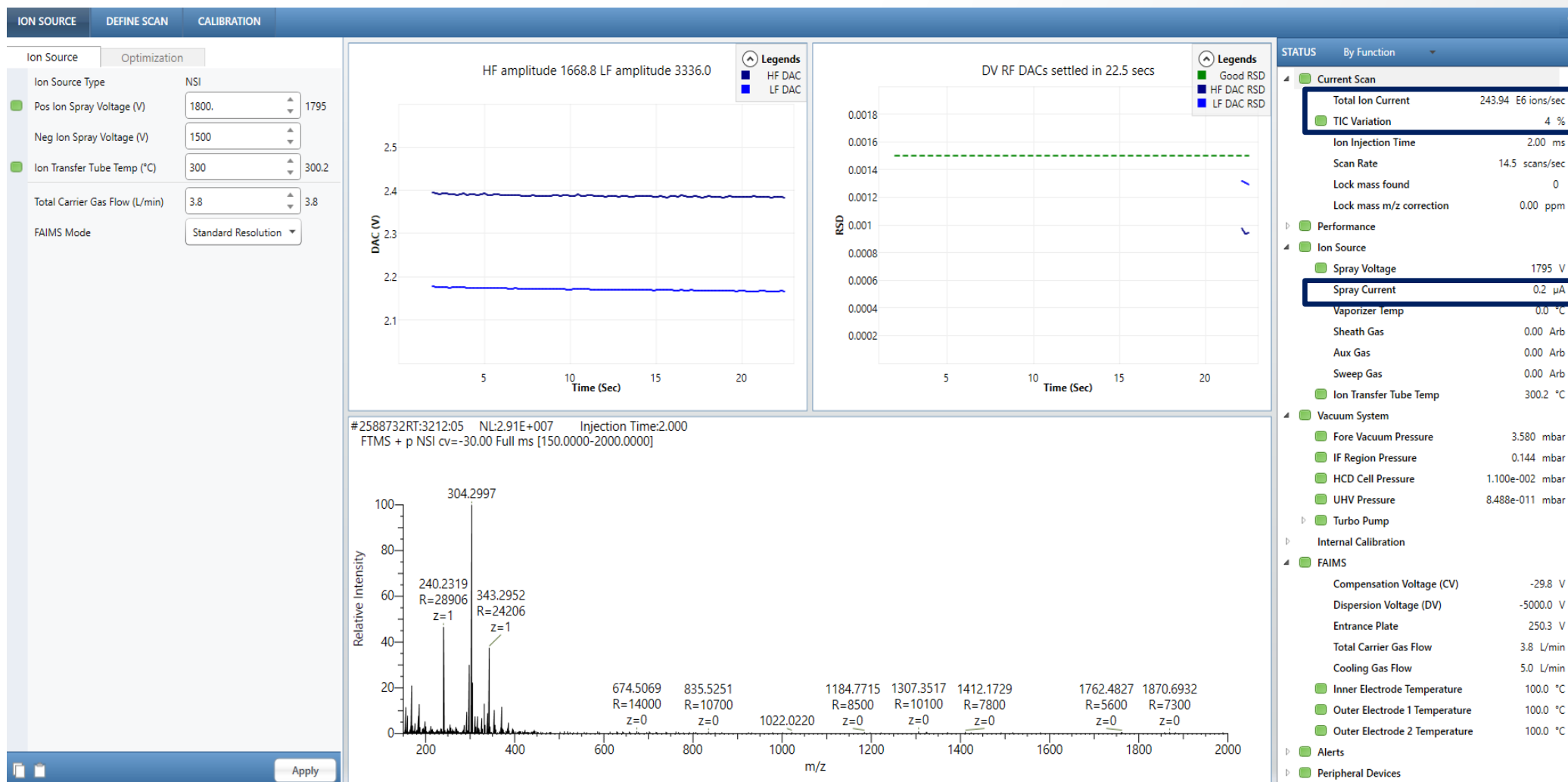
3 Proteome Mix

Samples	HeLa	E. coli	Yeast
E100Y75	5 μL	4 μL E1	6 μL Y2
E25Y150	5 μL	4 μL E2	6 μL Y1

Final concentration: 500 ng /μL

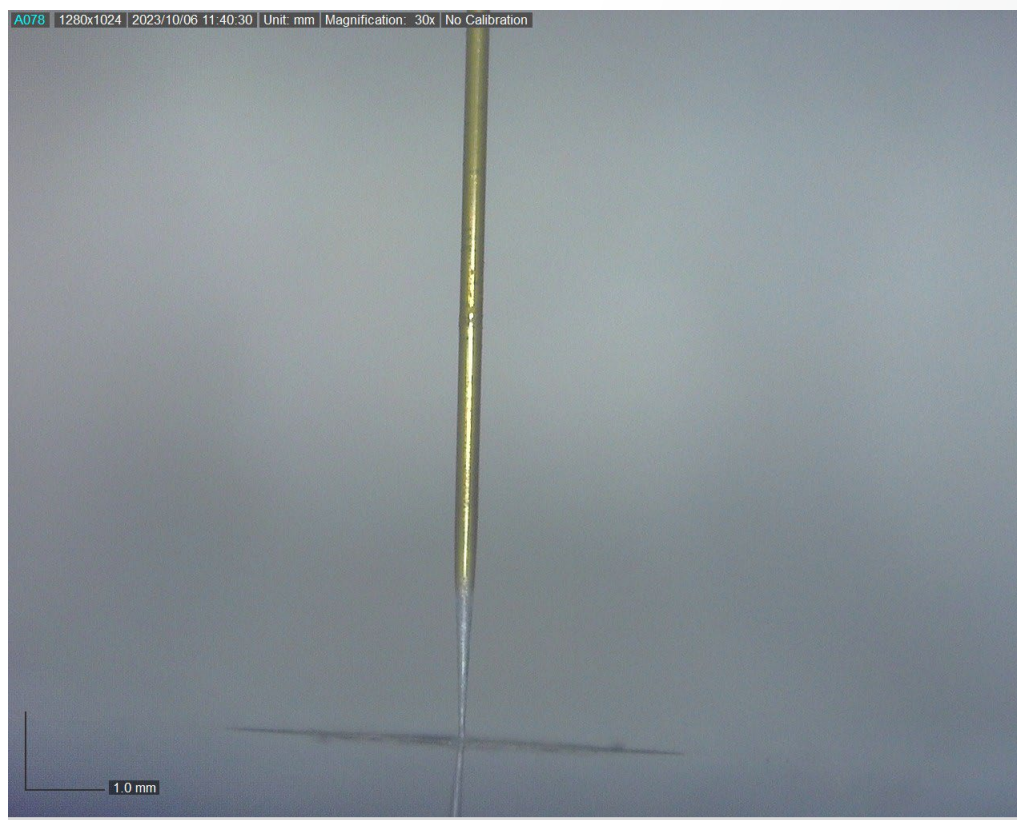
Positioning the emitter and spray stability

- To monitor the spray stability with FAIMS interface, please turn off the CV first and check the stability. The ion current should be around 0.1-0.2 μA , and the ion count ideally should be in the range of 100e6 at 100 nL/min with 4% B.



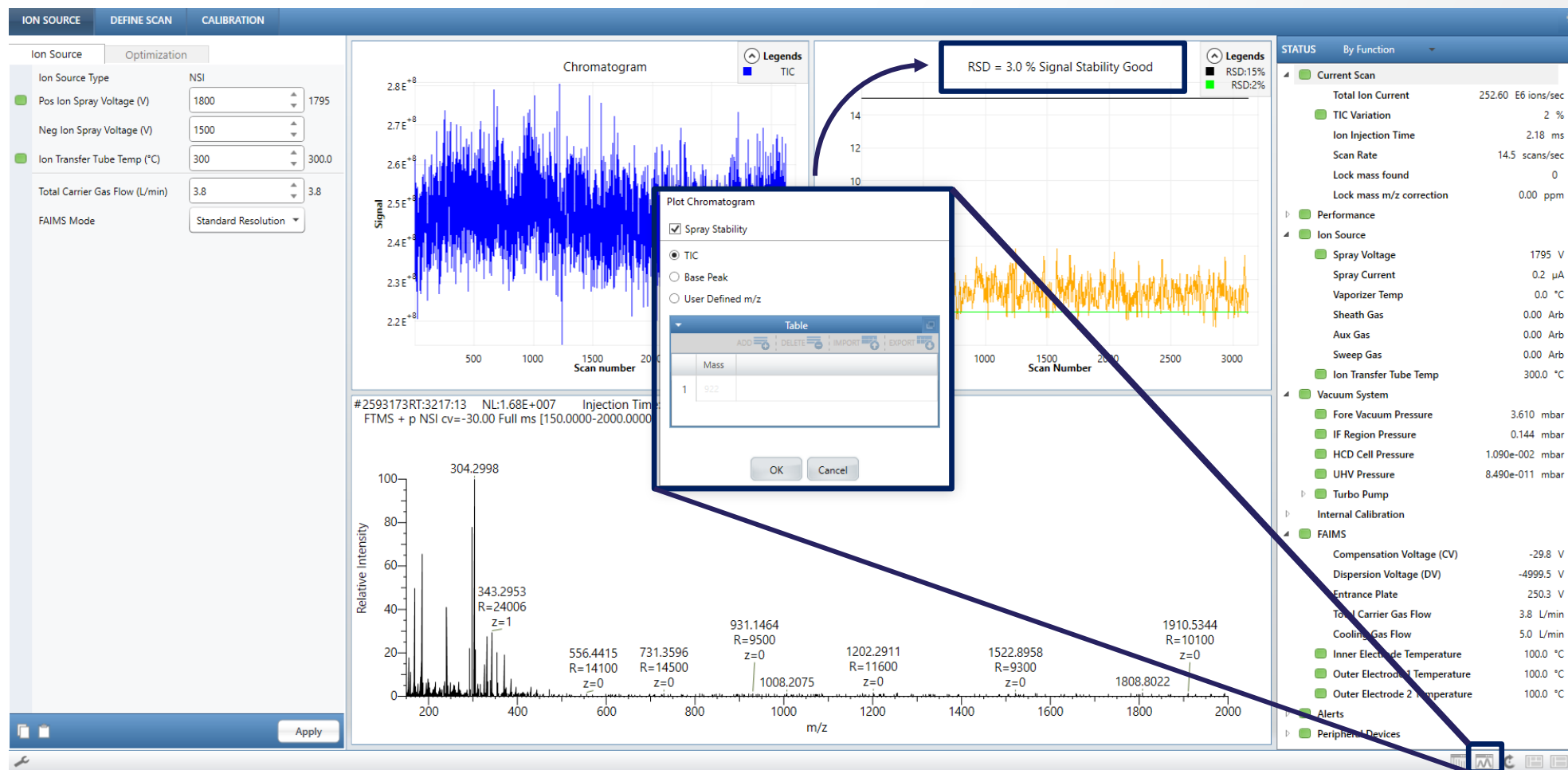
Positioning the emitter and spray stability

- The distance to the FAIMS interface orifice should be as close as possible but not in the orifice. Placing it where the tip meets the orifice cross-section border could be a good start. Then you can move the emitter from left to right, up and down, to search for the optimal position (look for best signal intensity and spray stability).



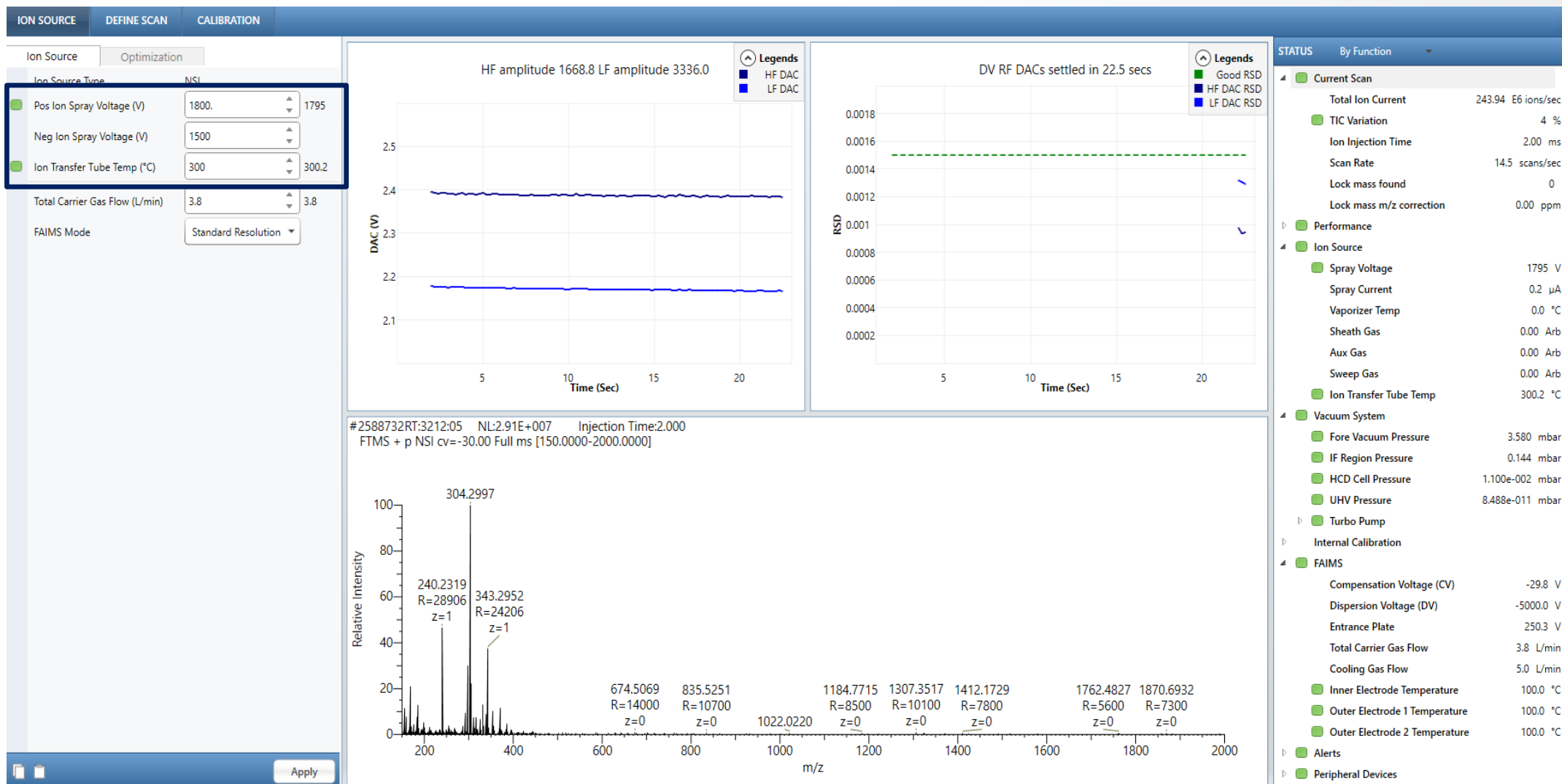
Positioning the emitter and spray stability

- At 100 nL/min with 4% B, monitor the spray stability. The RSD of TIC should be within 10%.



Positioning the emitter and spray stability

- At 100 Adjust the voltage and transfer tube temperature as needed to retrieve the best signal (typically 1800-2000 V and 275-300°C)



Fluidic setup

Separation Column(s) Specifications

Property	Value
Inner Diameter:	75 [μm]
Length:	50.0 [cm]
Void Volume:	1.480 [μl]
Maximum Pressure:	400 [bar]
Maximum Flow:	0.7 [μl/min]
Maximum Temperature:	60.0 [°C]
Maximum Pressure Change Up:	1000 [bar/min]
Maximum Pressure Change Down:	1000 [bar/min]

Load setting

☒ Fast Loading ⓘ

Mode: PressureControl ⓘ

Flow: ⓘ [0.000...0.700 μl/min]

Pressure: 350.0 ⓘ [20.0...400.0 bar]

Loading Volume: Automatic ⓘ [Automatic...1000.000 μl]

Wash and equilibration setting

Separation Column

☒ Fast Equilibration ⓘ

Mode: PressureControl ⓘ

Flow: ⓘ [0.000...0.700 μl/min]

Pressure: 350.0 ⓘ [0.0...400.0 bar]

Equilibration Factor: 2.0 ⓘ [0.0...1000.0]

Estimated Duration: n.a. [min]

Used Flow: n.a. [μl/min]

Used %B: 4.0 [%]

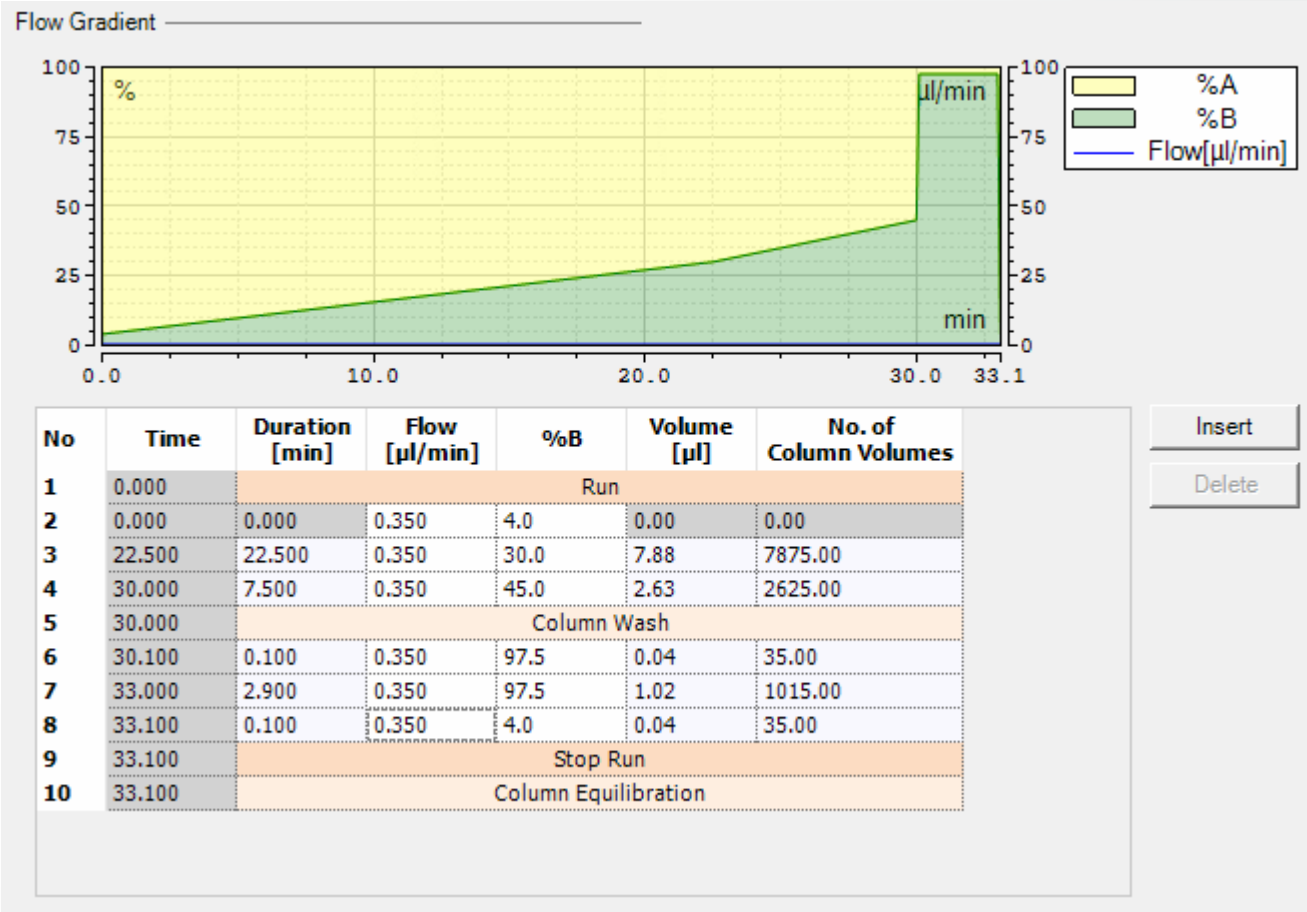
30 min (36 SPD)

Orbitrap Ascend MS + FAIMS Pro Interface

LC settings (30 min)

- Direct Injection setup
- Gradient optimized for 50cm μ PAC Neo column (COL-nano050NeoB)
- 36 SPD method (30 min active gradient)
- Column Temp: 50 °C

A	B
0.1% FA in water (Thermo Fisher LS118)	80% ACN/0.1%FA in water (Thermo Fisher LS122)



MS settings (30 min)

Method Summary

Method Settings

Global Parameters

Ion Source

MS Global Settings

Experiment #1 [MS (CV -45)]

Application Mode: **Peptide**
Method Duration (min): **33.1**

Use Ion Source Settings from Tune: **True**
FAIMS Mode: **Standard Resolution**
Total Carrier Gas Flow: **Static**
Total Carrier Gas Flow (L/min): **3.8**

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **10**
Advanced Peak Determination: **True**
Default Charge State: **2**
Enable Xcalibur AcquireX Ab method modifications: **False**
Internal Mass Calibration: **Off**

Start Time (min): **0**
End Time (min): **33.1**

Master Scan:

Full Scan

Orbitrap Resolution: **60000**
Scan Range (m/z): **400-900**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
RF Lens (%): **70**
AGC Target: **Custom**

Normalized AGC Target (%): **300**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment #2 [DIA Scan (CV -45)]

Start Time (min): **0**
End Time (min): **33.1**

Master Scan:

DIA

Precursor Mass Range (m/z): **400-900**
DIA Window Type: **Auto**
Multiplex Ions: **False**
Q1 Resolution (m/z): **12**
Window Overlap (m/z): **1**
Window Placement Optimization: **On**
Number Of Scan Events: **41**
DIA Window Mode: **m/z Range**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **30**
Orbitrap Resolution: **15000**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
Scan Range Mode: **Define m/z Range**
Scan Range (m/z): **145-1450**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Loop Control: **All**
Scan Description:

DIA m/z window

DIA m/z window

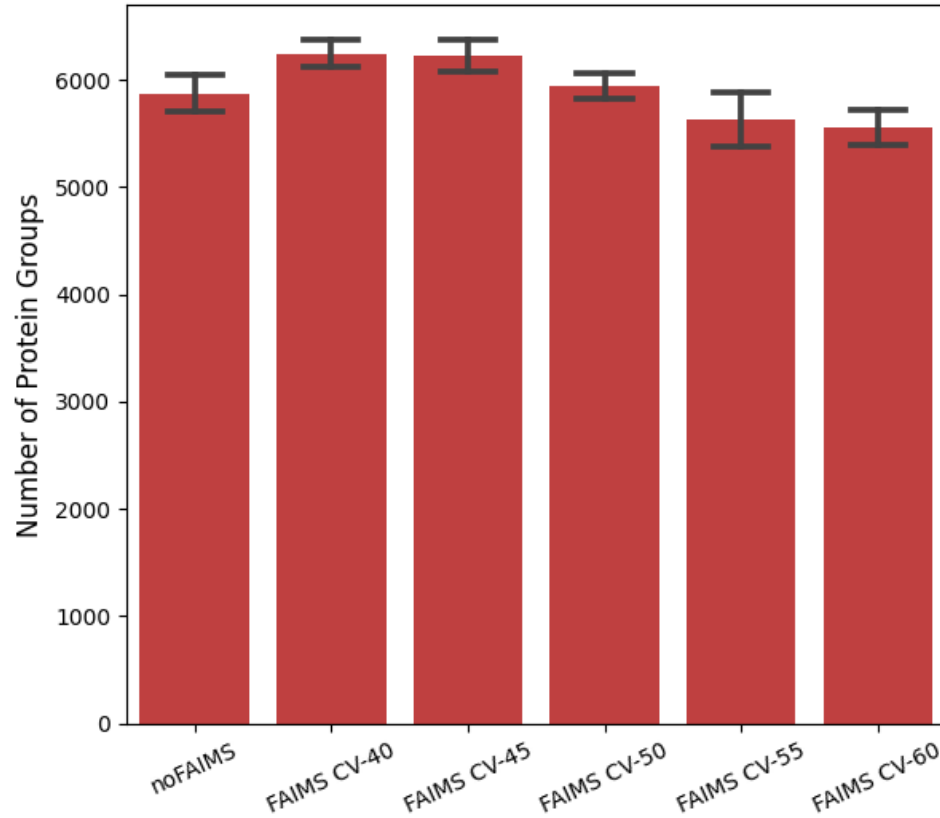
m/z range
399.43144525-412.437357
411.43690225-424.442814
423.44235925-436.448271
435.44781625-448.453728
447.45327325-460.459185
459.45873025-472.464642
471.46418725-484.470099
483.46964425-496.475556
495.47510125-508.481013
507.48055825-520.48647
519.48601525-532.491927
531.49147225-544.497384
543.49692925-556.502841
555.50238625-568.508298
567.50784325-580.513755
579.51330025-592.519212
591.51875725-604.524669
603.52421425-616.530126
615.52967125-628.535583
627.53512825-640.54104
639.54058525-652.546497
651.54604225-664.551954
663.55149925-676.557411
675.55695625-688.562868
687.56241325-700.568325
699.56787025-712.573782
711.57332725-724.579239

723.57878425-736.584696
735.58424125-748.590153
747.58969825-760.59561
759.59515525-772.601067
771.60061225-784.606524
783.60606925-796.611981
795.61152625-808.617438
807.61698325-820.622895
819.62244025-832.628352
831.62789725-844.633809
843.63335425-856.639266
855.63881125-868.644723
867.64426825-880.65018
879.64972525-892.655637
891.65518225-904.661094

- The method showing here includes FAIMS Pro interface. For method without FAIMS Pro interface, simply set “FAIMS Mode” as ‘Not Installed’ and keep the rest parameters identical.
- Parameters in Global Parameters such as voltage, FAIMS interface carrier gas flow should be optimized according to the instrument.
- Check the CV for each FAIMS device to decide the best CV(s) to use.

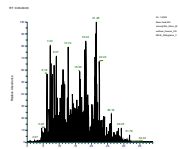
CV optimization

Identify the best CV for your FAIMS Pro interface



- Standard experiment: 200 ng HeLa digest / 30 min gradient
- Test different CVs to evaluate the best value for a given FAIMS device

Base Peak (30 min)

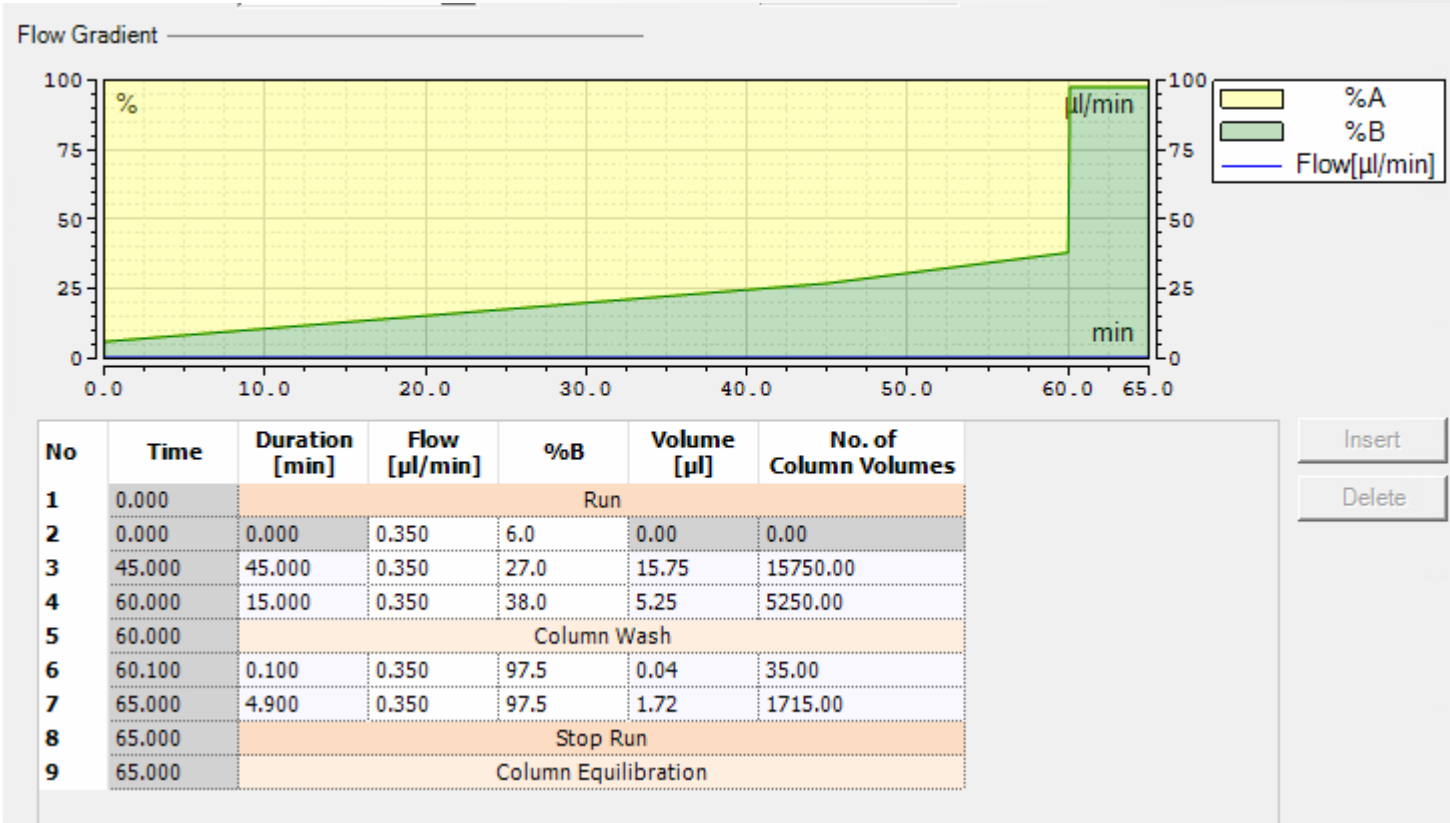


60 min (20 SPD)

LC settings (60 min)

- Direct Injection setup
- Gradient optimized for 50cm μ PAC Neo column (COL-nano050NeoB)
- 20 SPD method (60 min active gradient)
- Column Temp: 50 °C

A	B
0.1% FA in water (Thermo Fisher LS118)	80% ACN/0.1%FA in water (Thermo Fisher LS122)



MS settings (60 min)



Method Summary

Method Settings

Global Parameters

Ion Source

Ion Source Type: **NSI**
Spray Voltage: **Static**
Positive Ion (V): **2200**
Negative Ion (V): **1500**
Ion Transfer Tube Temp (°C): **300**
Use Ion Source Settings from Tune: **False**
FAIMS Mode: **Standard Resolution**
Total Carrier Gas Flow: **Static**
Total Carrier Gas Flow (L/min): **3.8**

MS Global Settings

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **10**
Advanced Peak Determination: **True**
Default Charge State: **2**
Enable Xcalibur AcquireX Ab method modifications: **False**
Internal Mass Calibration: **Off**

Experiment #1 [MS (CV -45)]

Start Time (min): **0**
End Time (min): **65**

Master Scan:

Full Scan

Orbitrap Resolution: **60000**

Scan Range (m/z): **400-900**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **300**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment #2 [DIA Scan (CV -45)]

Start Time (min): **0**
End Time (min): **65**

Master Scan:

DIA

Precursor Mass Range (m/z): **400-900**
DIA Window Type: **Auto**
Multiplex Ions: **False**
Q1 Resolution (m/z): **12**
Window Overlap (m/z): **1**
Window Placement Optimization: **On**
Number Of Scan Events: **41**
DIA Window Mode: **m/z Range**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **30**
Orbitrap Resolution: **15000**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
Scan Range Mode: **Define m/z Range**
Scan Range (m/z): **145-1450**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**

Source Fragmentation: **Disabled**
Loop Control: **All**
Scan Description:

DIA m/z window

DIA m/z window
m/z range
399.43144525-412.437357
411.43690225-424.442814
423.44235925-436.448271
435.44781625-448.453728
447.45327325-460.459185
459.45873025-472.464642
471.46418725-484.470099
483.46964425-496.475556
495.47510125-508.481013
507.48055825-520.48647
519.48601525-532.491927
531.49147225-544.497384
543.49692925-556.502841
555.50238625-568.508298
567.50784325-580.513755
579.51330025-592.519212
591.51875725-604.524669
603.52421425-616.530126
615.52967125-628.535583
627.53512825-640.54104
639.54058525-652.546497
651.54604225-664.551954
663.55149925-676.557411

675.55695625-688.562868
687.56241325-700.568325
699.56787025-712.573782
711.57332725-724.579239
723.57878425-736.584696
735.58424125-748.590153
747.58969825-760.59561
759.59515525-772.601067
771.60061225-784.606524
783.60606925-796.611981
795.61152625-808.617438
807.61698325-820.622895
819.62244025-832.628352
831.62789725-844.633809
843.63335425-856.639266
855.63881125-868.644723
867.64426825-880.65018
879.64972525-892.655637
891.65518225-904.661094

Experiment #3 [MS (CV -60)]

Start Time (min): **0**
End Time (min): **65**

Master Scan:

Full Scan

Orbitrap Resolution: **60000**
Scan Range (m/z): **400-900**
FAIMS Voltages: **On**
FAIMS CV (V): **-60**
RF Lens (%): **70**

AGC Target: **Custom**
Normalized AGC Target (%): **300**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment #4 [DIA Scan (CV -60)]

Start Time (min): **0**
End Time (min): **65**

Master Scan:

DIA

Precursor Mass Range (m/z): **400-900**
DIA Window Type: **Auto**
Multiplex Ions: **False**
Q1 Resolution (m/z): **12**
Window Overlap (m/z): **1**
Window Placement Optimization: **On**
Number Of Scan Events: **41**
DIA Window Mode: **m/z Range**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **30**
Orbitrap Resolution: **15000**
FAIMS Voltages: **On**
FAIMS CV (V): **-60**
Scan Range Mode: **Define m/z Range**
Scan Range (m/z): **145-1450**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Loop Control: **All**
Scan Description:

MS settings (60 min) continued

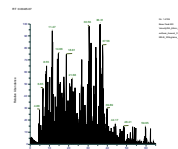
DIA m/z window

DIA m/z window
m/z range
399.43144525-412.437357
411.43690225-424.442814
423.44235925-436.448271
435.44781625-448.453728
447.45327325-460.459185
459.45873025-472.464642
471.46418725-484.470099
483.46964425-496.475556
495.47510125-508.481013
507.48055825-520.48647
519.48601525-532.491927
531.49147225-544.497384
543.49692925-556.502841
555.50238625-568.508298
567.50784325-580.513755
579.51330025-592.519212
591.51875725-604.524669
603.52421425-616.530126
615.52967125-628.535583
627.53512825-640.54104
639.54058525-652.546497
651.54604225-664.551954
663.55149925-676.557411
675.55695625-688.562868
687.56241325-700.568325
699.56787025-712.573782

711.57332725-724.579239
723.57878425-736.584696
735.58424125-748.590153
747.58969825-760.59561
759.59515525-772.601067
771.60061225-784.606524
783.60606925-796.611981
795.61152625-808.617438
807.61698325-820.622895
819.62244025-832.628352
831.62789725-844.633809
843.63335425-856.639266
855.63881125-868.644723
867.64426825-880.65018
879.64972525-892.655637
891.65518225-904.661094

- CVs are optimized according to FAIMS device.
- 2 CVs are used for 60 min gradient.
- The method showing here includes FAIMS Pro interface. For method without FAIMS interface, simply set “FAIMS Mode” as ‘Not Installed’ and keep the rest parameters identical.

Base Peak (60 min)



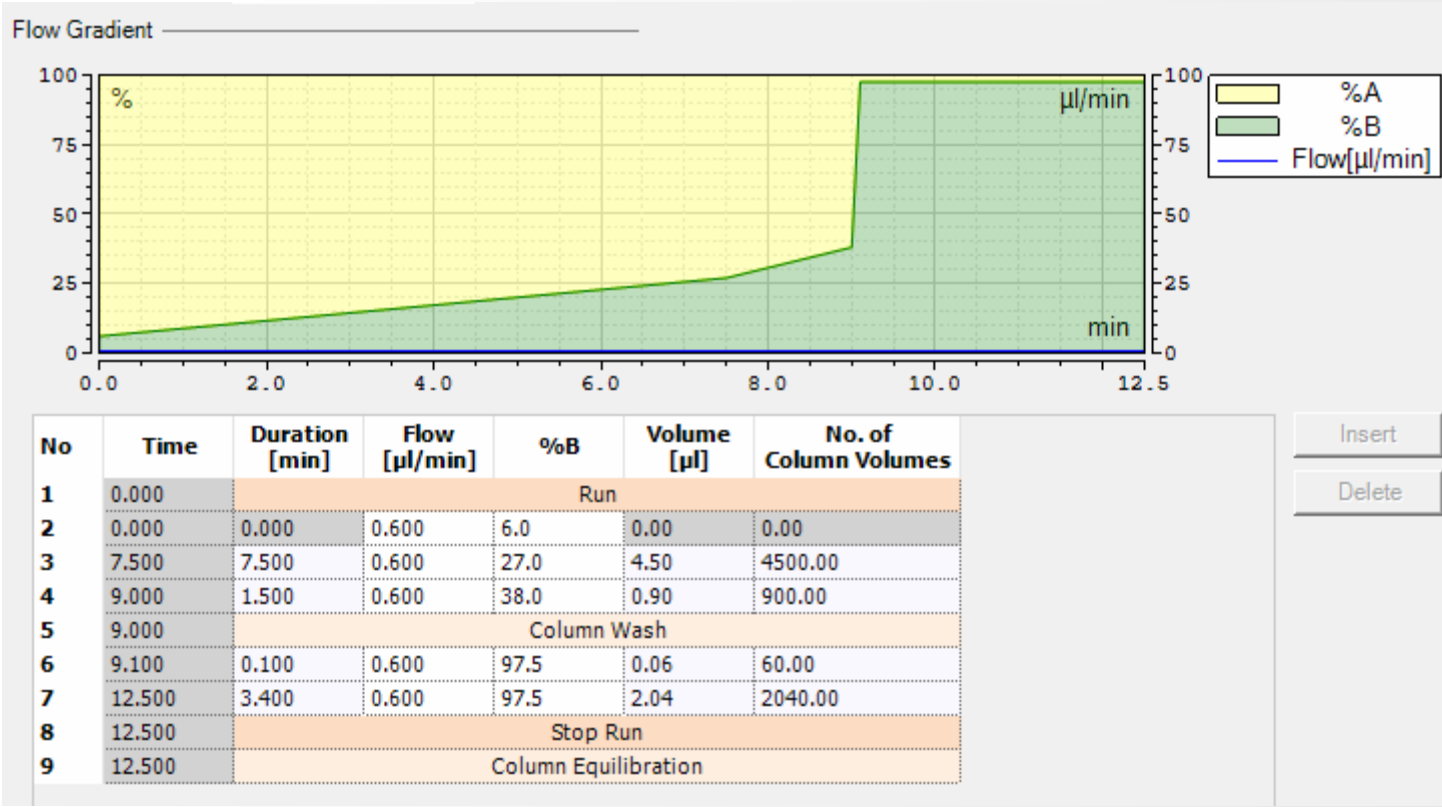
9 min (80 SPD)

Orbitrap Exploris 480 MS + FAIMS Pro Interface

LC settings (9 min)

- Direct Injection setup
- Gradient optimized for 50cm μ PAC Neo column (COL-nano050NeoB)
- 80 SPD method (9 min active gradient)
- Column Temp: 50 °C

A	B
0.1% FA in water (Thermo Fisher LS118)	80% ACN/0.1%FA in water (Thermo Fisher LS122)



MS settings (9 min, Max ID)



Method Summary

Method Settings

Global Parameters

Ion Source

MS Global Settings

Experiment #1 [MS (CV -45)]

Application Mode: **Peptide**
Method Duration (min): **12.5**

Ion Source Type: **NSI**
Spray Voltage: **Static**
Positive Ion (V): **2200**
Negative Ion (V): **1500**
Ion Transfer Tube Temp (°C): **300**
Use Ion Source Settings from Tune: **False**
FAIMS Mode: **Standard Resolution**
Total Carrier Gas Flow: **Static**
Total Carrier Gas Flow (L/min): **3.8**

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **10**
Advanced Peak Determination: **True**
Default Charge State: **2**
Enable Xcalibur AcquireX Ab method modifications: **False**
Internal Mass Calibration: **Off**

Start Time (min): **0**
End Time (min): **12.5**

Master Scan:

Full Scan

Orbitrap Resolution: **30000**

Scan Range (m/z): **400-800**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **300**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment #2 [DIA Scan (CV -45)]

Start Time (min): **0**
End Time (min): **12.5**

Master Scan:

DIA

Precursor Mass Range (m/z): **400-800**
DIA Window Type: **Auto**
Multiplex Ions: **False**
Q1 Resolution (m/z): **8**
Window Overlap (m/z): **1**
Window Placement Optimization: **On**
Number Of Scan Events: **49**
DIA Window Mode: **m/z Range**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **30**
Orbitrap Resolution: **30000**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
Scan Range Mode: **Define m/z Range**
Scan Range (m/z): **145-1450**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**

Source Fragmentation: **Disabled**
Loop Control: **All**
Scan Description:

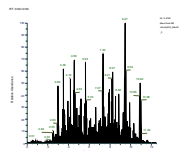
DIA m/z window

DIA m/z window
m/z range
399.43144525-408.435538
407.43508325-416.439176
415.43872125-424.442814
423.44235925-432.446452
431.44599725-440.45009
439.44963525-448.453728
447.45327325-456.457366
455.45691125-464.461004
463.46054925-472.464642
471.46418725-480.46828
479.46782525-488.471918
487.47146325-496.475556
495.47510125-504.479194
503.47873925-512.482832
511.48237725-520.48647
519.48601525-528.490108
527.48965325-536.493746
535.49329125-544.497384
543.49692925-552.501022
551.50056725-560.50466
559.50420525-568.508298
567.50784325-576.511936
575.51148125-584.515574

583.51511925-592.519212
591.51875725-600.52285
599.52239525-608.526488
607.52603325-616.530126
615.52967125-624.533764
623.53330925-632.537402
631.53694725-640.54104
639.54058525-648.544678
647.54422325-656.548316
655.54786125-664.551954
663.55149925-672.555592
671.55513725-680.55923
679.55877525-688.562868
687.56241325-696.566506
695.56605125-704.570144
703.56968925-712.573782
711.57332725-720.57742
719.57696525-728.581058
727.58060325-736.584696
735.58424125-744.588334
743.58787925-752.591972
751.59151725-760.59561
759.59515525-768.599248
767.59879325-776.602886
775.60243125-784.606524
783.60606925-792.610162
791.60970725-800.6138

- The method showing here includes FAIMS Pro interface. For method without FAIMS interface, simply set “FAIMS Mode” as ‘Not Installed’ and keep the rest parameters identical.
- Parameters in Global Parameters such as voltage, FAIMS carrier gas flow should be optimized according to the instrument.
- Check the CV for each FAIMS device to decide the best CV(s) to use.

Base Peak (9 min, Max ID)



MS settings (9 min, Max Quantitation)

Method Summary

Method Settings

Global Parameters

Ion Source

MS Global Settings

Experiment #1 [MS (CV -45)]

Application Mode: **Peptide**
Method Duration (min): **12.5**

Ion Source Type: **NSI**
Spray Voltage: **Static**
Positive Ion (V): **2200**
Negative Ion (V): **1500**
Ion Transfer Tube Temp (°C): **300**
Use Ion Source Settings from Tune: **False**
FAIMS Mode: **Standard Resolution**
Total Carrier Gas Flow: **Static**
Total Carrier Gas Flow (L/min): **3.8**

Infusion Mode: **Liquid Chromatography**
Expected LC Peak Width (s): **10**
Advanced Peak Determination: **True**
Default Charge State: **2**
Enable Xcalibur AcquireX Ab method modifications: **False**
Internal Mass Calibration: **Off**

Start Time (min): **0**
End Time (min): **12.5**

Master Scan:

Full Scan

Orbitrap Resolution: **30000**

Scan Range (m/z): **650-770**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **300**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**
Source Fragmentation: **Disabled**
Scan Description:

Experiment #2 [DIA Scan (CV -45)]

Start Time (min): **0**
End Time (min): **12.5**

Master Scan:

DIA

Precursor Mass Range (m/z): **650-770**
DIA Window Type: **Auto**
Multiplex Ions: **False**
Q1 Resolution (m/z): **8**
Window Overlap (m/z): **1**
Window Placement Optimization: **On**
Number Of Scan Events: **14**
DIA Window Mode: **m/z Range**
Collision Energy Type: **Normalized**
HCD Collision Energy (%): **30**
Orbitrap Resolution: **15000**
FAIMS Voltages: **On**
FAIMS CV (V): **-45**
Scan Range Mode: **Define m/z Range**
Scan Range (m/z): **145-1450**
RF Lens (%): **70**
AGC Target: **Custom**
Normalized AGC Target (%): **800**
Maximum Injection Time Mode: **Auto**
Microscans: **1**
Data Type: **Profile**
Polarity: **Positive**

Source Fragmentation: **Disabled**
Loop Control: **All**
Scan Description:

DIA m/z window

m/z range
649.54513275-658.5492255
657.54877075-666.5528635
665.55240875-674.5565015
673.55604675-682.5601395
681.55968475-690.5637775
689.56332275-698.5674155
697.56696075-706.5710535
705.57059875-714.5746915
713.57423675-722.5783295
721.57787475-730.5819675
729.58151275-738.5856055
737.58515075-746.5892435
745.58878875-754.5928815
753.59242675-762.5965195
761.59606475-770.6001575

- The method showing here includes FAIMS Pro interface. For method without FAIMS interface, simply set “FAIMS Mode” as ‘Not Installed’ and keep the rest parameters identical.
- Parameters in Global Parameters such as voltage, FAIMS interface carrier gas flow should be optimized according to the instrument.
- Check the CV for each FAIMS device to decide the best CV(s) to use.

Base Peak (9 min, Max Quantitation)

