

LC-MS setup

PPE (personal protective Equipment)

Do not use any of the equipment without the appropriate training. Always wear appropriate PPE when working in the lab, including goggles, lab coats and gloves. PPE is provided, ask if you cannot find the appropriate PPE or if PPE is missing. Always wear goggles when working with fused silica. Review MSDS and SOP's before working with chemicals. Before working with the gas cylinders make sure you complete the EH&S "Compressed Gas Safety" online training (<https://www.ehs.washington.edu/training/compressed-gas-safety-online>).

Before you connect your trap and column

You need to provide your own trap and column (Note our fittings accommodate trap and column made with fused silica 360 µm OD). Check our guide for packing traps and columns and list of consumables you should provide.

- [Packing capillary columns \(pdf\)](#)
- [LC-MS commonly used consumables \(xls\)](#)

Before you connect the column, check both the PEEK micro-tee and micro-cross for obstructions in the through whole. Remove all the fittings from both the tee and the cross and look at them under the microscope. The through wholes should be wide open. If not use air in the can and blow some air through them, and check again under the microscope. If you still see a blockage either grab a fresh tee or cross or remove the blockage; try sonication in 50% methanol and/or use a wire to poke through the whole to remove the debris.

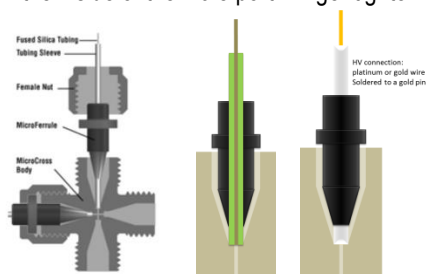
Connecting your trap and column

Most our mass specs are equipped with a nanoAcquity UPLC system. Although UPLC system is rated to 10000 psi, the fittings we use are only rated to max 4500 psi. If you wish to use higher backpressures you should provide your own fittings.

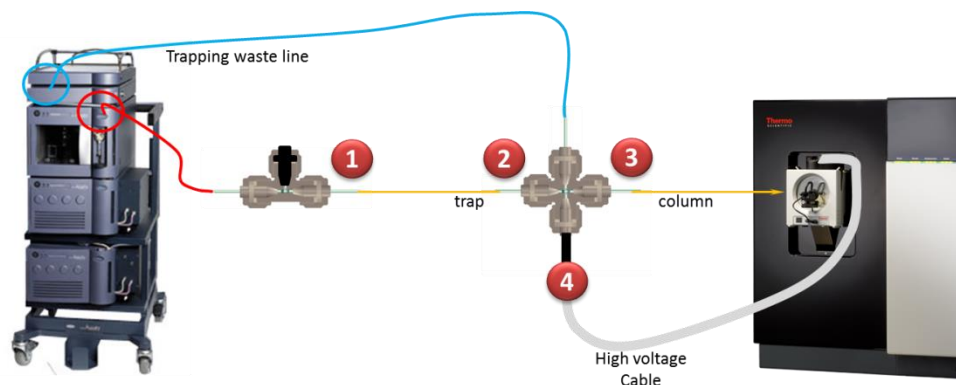
Micro-tee and cross use instructions

Warning: Please take care when tightening the Micro-Fingertight fittings, making sure to only tighten sufficiently to prevent leaks from occurring. Due to the nature of some fused silica tubing, it is possible to damage the tubing if over tightened.

1. Insert your tubing into green MicroTight tubing sleeve. Make sure your fused silica tubing is inserted far enough but does not extend past the end of the tubing sleeve.
2. Insert your sleeved tubing through the PEEK female nut and through the black Micro Ferrule, as shown in the picture.
3. Screw the assembled unit onto one of the available threaded ports on the MicroTee or MicroCross, making sure that the tubing is pushed firmly against the bottom ledge on the inside of the male port. Finger tighten securely.



The micro-tee should be connected to the fused silica line coming from the pump (on the right side of the system, red in the figure below),
The trapping waste line (upper module towards the left side, light blue in the figure below) should be connected to the micro-cross.



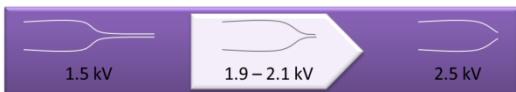
1. With the flow rate set to 0 $\mu\text{l}/\text{min}$ connect the open end of the trap to the micro-tee
Set the flow rate to 2 $\mu\text{l}/\text{min}$ wait for the back pressure to stabilize and note the back pressure (should not exceed 2000 psi)
(Use a solvent mix of 2-5% buffer B)
2. Connect the fritted end of the trap to the micro-cross
3. Set the flow rate to 0.1 $\mu\text{l}/\text{min}$
4. Connect the open end of the column to the micro-cross
Wait for the backpressure to stabilize, then increase the flow rate to 0.2 $\mu\text{l}/\text{min}$
Wait for the backpressure to stabilize, then increase the flow rate to 0.3 $\mu\text{l}/\text{min}$ etc. until the backpressure is 1000-2000 psi (depending on column length).
5. After about 20-30 min at stable backpressure, reduce the flow rate to 0 $\mu\text{l}/\text{min}$ and wait for the backpressure to reach 0 psi, remove the column and cut it to the desired length. Make sure there is no void volume at the end of the column, minimizing the void between the trap and the column will minimize peak broadening.
6. Reconnect the column to the micro-cross and set flowrate to 0.3 $\mu\text{l}/\text{min}$ (or whatever flow rate you determined above), wait for back pressure to stabilize, check for leaks.
7. Position the tip of the column in front of the ion transfer tube at about 3-5 mm distance. Be careful, the ion transfer tube is hot $>300^\circ\text{C}$.
8. Make sure the instrument is in standby (the blue scanning light is off). Connect the high voltage connector to the pin on the micro-cross.

Adjust your ESI spray

With your column connected and the flow set to the desired rate, target backpressure <2000 psi, from the tune window start scanning the instrument.

Monitor the live spectrum for consistency in intensity, no spectra dropping out, no sputtering at the tip. Adjust the tip position and spray voltage until you get a consistent spray.

Typical NSI spray voltage is ~ 2.1 kV



(The finer the spray tip the lower the voltage, as low as 1.5 kV. For blunt tips higher voltage may be needed, up to 2.4 kV)

Liquid droplet is forming at the tip:

If there was a liquid droplet at the tip of your column it should disappear as soon as you start scanning the instrument. If it doesn't, take a kimwipe, twist one corner and gently wipe the droplet off, without touching anything or breaking the tip of your column.

If the droplet forms again, check and make sure the high voltage cable is connected. If not, turn the instrument into standby and connect the high voltage cable and turn the instrument back on.

No peaks in the spectrum:

Check and make sure the high voltage cable is connected.

Check if there is a liquid droplet at the tip of your column.

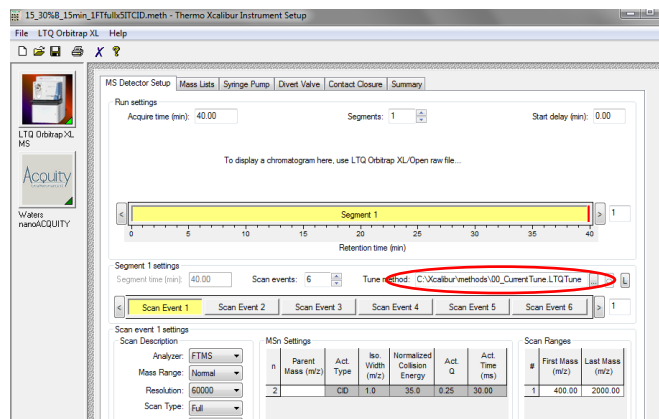
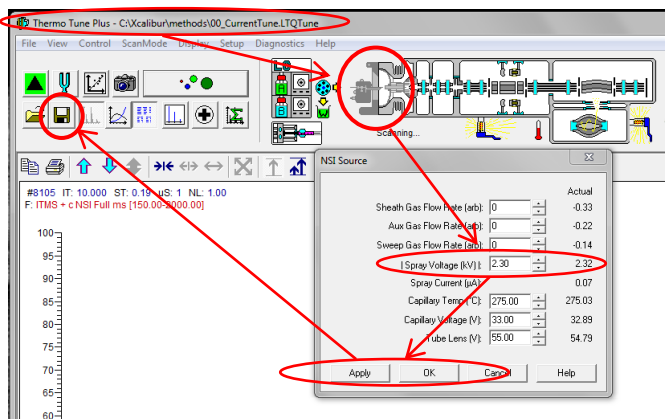
If not turn the instrument into standby and monitor the tip of your column you should see a liquid drop form, if not check for leaks.

Once you have a stable spray, depending on the instrument you use, either save the tune file (TSQ, QEplus, OTXL) or make sure the spray voltage in your method is set to the same voltage you just now determined to be best for your column (TSQ EZ, Fusion, Lumos method), now you are ready to get started.

Spray voltage on the LTQ-Orbitrap:

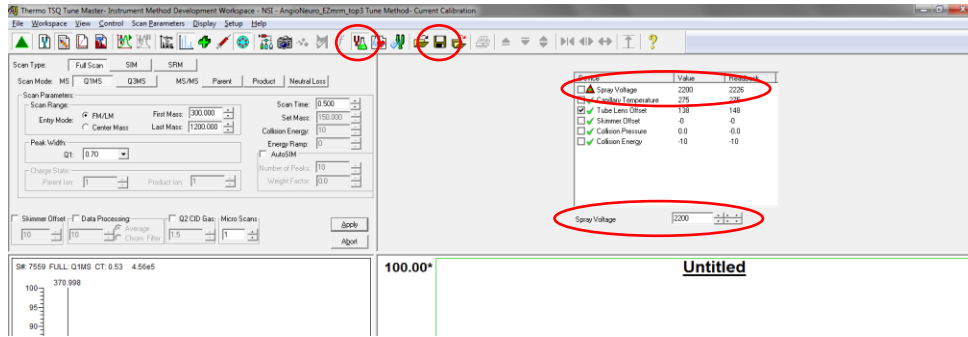
In Tune, adjust spray voltage and position the column tip until spray is stable (no drop outs, NL: stable), remember to save the tune file!

In the method select the correct tune file.



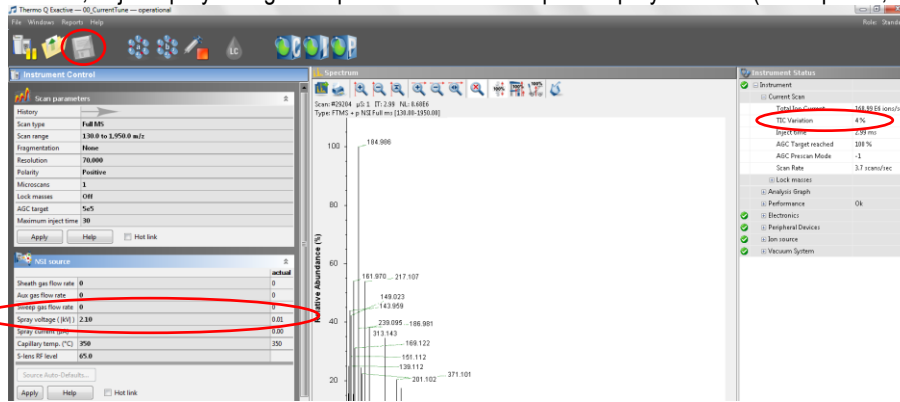
Spray voltage on the TSQ:

In Tune, adjust spray voltage and position the column tip until spray is stable (no drop outs, NL: stable), remember to save the tune file!
Remember to change the spray voltage in your method as well (EZ method only)



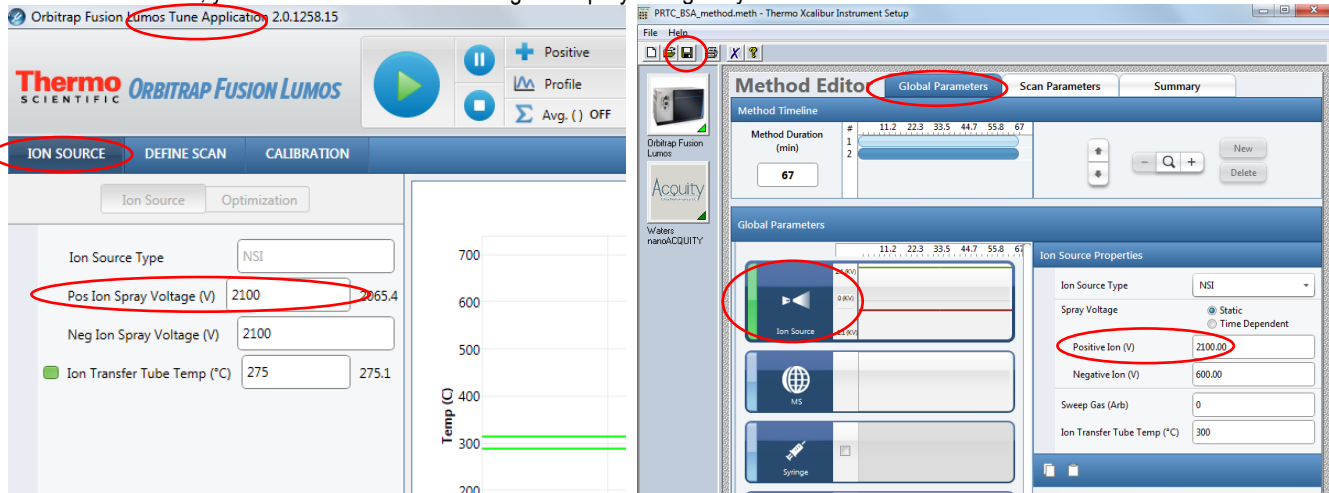
Spray voltage on the QExactive:

In Tune, adjust spray voltage and position the column tip until spray is stable (no drop outs, TIC variation <10%), remember to save the tune file!



Spray voltage on the Fusion/Lumos:

There is no tune file, you need to remember to change the spray voltage in your methods.



Start the first LC-MS run:

Always start by running a QC, you can choose your own QC or use the one provided by UWPR.

A QC assures that

1. The column gets conditioned
2. There are no leaks
3. Your column/trap setup is performing properly
4. The HPLC is performing properly (injection and gradient)
5. The mass spec is performing properly (mass accuracy and sensitivity)

In AS position 2:A,1 there should be a standard consisting of two peptides Angiotensin and Neurotensin, always check and make sure its there.

- [AngioNeuro a simple peptide standard \(xls\)](#)

Use the short gradient method provided by UWPR to run this standard.

After you start the run, monitor the actions taken by the instrument:

1. Both instruments should download the method, the HPLC will start running, and the mass spec should be "waiting for contact closure"
2. The autosampler should go through the sample injection process (~ 3mins) and finally start trapping (check the countdown timer for the correct trapping time, i.e. it should match what you have set in your method trapping)
3. After the trapping is complete the mass spec acquisition will start

The figure consists of three screenshots of the Thermo Xcalibur Sequence Setup software interface, showing the progression of a run. Each screenshot has a red circle highlighting a specific status or value.

- First Screenshot:** The 'Status' window shows 'Ready for Run' and 'Downloading'. The 'yBSMS Status' window shows 'Run Time: 0.0 min', 'Flow Rate: 0.300 uL/min', and 'System Pressure: 171 psi'. The 'ASMS Status' window shows 'Run Time: 0.0 min', 'Flow Rate: 0.000 mL/min', and 'System Pressure: -2 psi'. The 'ySMS Status' window shows 'Run Time: 0 min'.
- Second Screenshot:** The 'Status' window shows 'Running' and 'Waiting for Contact Closure'. The 'yBSMS Status' window shows 'Run Time: 0.0 min', 'Flow Rate: 0.000 uL/min', and 'System Pressure: 171 psi'. The 'ASMS Status' window shows 'Run Time: 0.0 min', 'Flow Rate: 0.000 mL/min', and 'System Pressure: -2 psi'. The 'ySMS Status' window shows 'Run Time: 0 min'. The 'nanoACQUITY Sample Manager' window shows 'Waiting for flow ramp (23 sec)'.
- Third Screenshot:** The 'Status' window shows 'Running' and 'Waiting for Contact Closure'. The 'yBSMS Status' window shows 'Run Time: 0.6 min', 'Flow Rate: 2.000 uL/min', and 'System Pressure: 1058 psi'. The 'ASMS Status' window shows 'Run Time: 0.0 min', 'Flow Rate: 0.000 mL/min', and 'System Pressure: -2 psi'. The 'ySMS Status' window shows 'Run Time: 0 min'. The 'nanoACQUITY Sample Manager' window shows 'Trapping (9.9 min)'.

After the run is complete check the peak shape, separation, and intensity, and mass accuracy. There should be a good/recent reference AngioNeuro.raw file on the desktop.

It is normal for a brand new column to show slightly reduced intensities, this is due to unspecific irreversible binding of the peptides to the column/trap (part of column conditioning). It is also normal to sometimes see some additional peaks, typically from contamination from the column packing process.

Peaks are not separated at all or are tailing

Check for obstructions/blockage in the flow path, micro-cross and micro-tee.

Check for void volume between trap and column.

Try a second QC run.

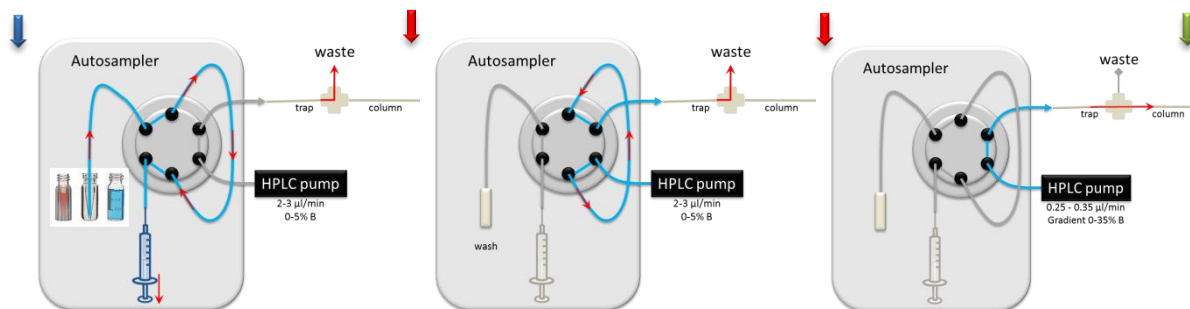
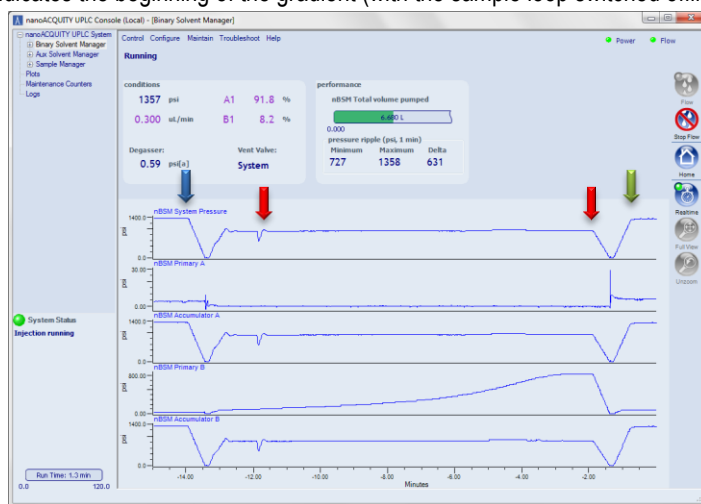
Try a new trap and column.

Run your first sample

If your QC is satisfactory, start your first sample. Monitor instrument actions as described above. In addition monitor the back pressure during the trapping and during the gradient.

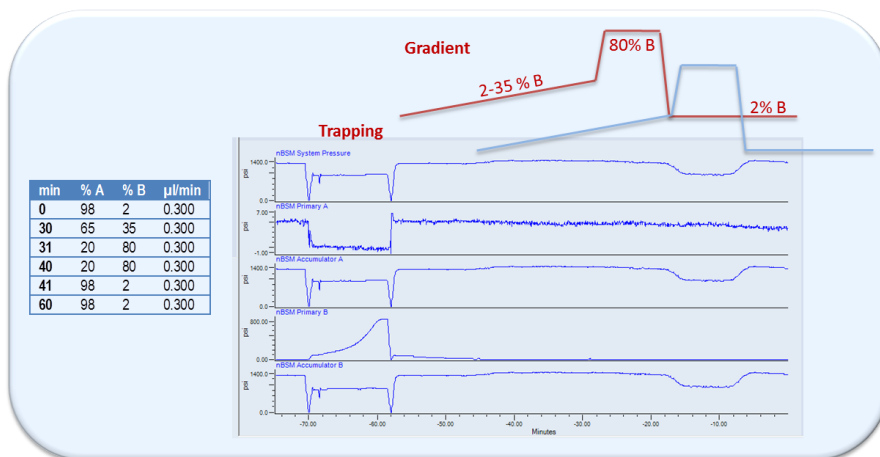
Trapping:

This is a screen shot of a typical trapping pressure trace with a 2.5cm trap C18 200Å 5µm at 2 µl/min (note the screen shot was taken after the gradient started). The blue arrow indicates the start of the injection (the autosampler places the sample in the sample loop), the two red arrows mark the start/end of the 10 min trapping (during this time the sample loop is in line with the flow of the pump, moving your sample towards the trap column) and the green arrow indicates the beginning of the gradient (with the sample loop switched offline again).



Gradient:

The figure below shows a typical pressure trace during a 60 min gradient, 2-35% B, 25 cm column C18 100Å 5µm at 0.300 µl/min, note there is a delay between the point when the pump mixes the gradient to when it is visible in the pressure trace. This is because the pressure will change once the gradient reaches the column. This delay is a result of the void volume between the pump and the column. On our systems the delay is about 15-20 min (the actual gradient is in red and the observed gradient/backpressure is light blue).



It is normal for the backpressure to drop at high % buffer B because of the viscosity of acetonitrile, but it should return back to the initial backpressure you established during the setup as the %B returns to initial condition.

If your first sample ran satisfactory and the back pressure trace is similar to the QC, you should be good to go. If all your samples were prepared identically, chances are good that the system will analyze all of them with no problems.

If your sample causes the system back pressure to rise much higher than during the QC run, the system may over pressure or start leaking. If this is the case running your samples in an automated fashion is risky as you do not know when a leak/over pressure may occur.

A small increase in backpressure is normal, but if the backpressure increases after every sample and does not recover down to initial conditions, eventually it will overpressure or start leaking.

QC runs and blanks

How many QC runs should you run?

Ultimately that is up to you. Good practice would be to run a QC every 5 – 10 runs, to monitor system performance over time.

I like to line them up such that there is a finished QC run for me to look at first thing in the morning when I come in, so I can take action if needed.

Should you run blanks?

This really depends on your project, but here are some hints to help you decide:

The typical carry over on our systems from one run to the next is less than 1%.

For MRM runs we see about 0.5-1% of the most abundant peptides in the next blank run.

And for DDA we got 18000 peptides in the sample run (complex peptide mix on the QE), and got ~50-100 peptides in a subsequent blank run.

If carry over is a critical concern for your project, test it with your own sample. Run a blank and look for carry over.

LC-MS setup checklist	Note	
Checked micro-cross and micro-tee through whole		
Connect trap and checked trapping backpressure	Backpressure Flow rate	psi µl/min
Connect column and established flow rate through column	Backpressure Flow rate	psi µl/min
Adjusted positioning and spray voltage to get stable spray and save the tune file		
Change spray voltage in method (if necessary)		
Change flow rate in methods		
QC run looks good		
First sample run looks good		