

## Data Acquisition & QTOF Methods

### Standard Operating Procedure

Document ID: ACHBC00012

Faculty/ Department or Institute/Centre	Cumming School of Medicine, Department of Pediatrics, Neurocritical Care Program, ACH BioCORE	Date Created:	January 7 2019
Location	<b>ACH BO-103</b>	Created By:	Matthew Rosin
Supervisor	Dr. Michael Esser & Dr. David Sinasac	Revision #:	1.0

### Purpose

This document details how to create QTOF data acquisition methods and navigate the Data Acquisition MassHunter software for the Agilent QTOF Mass Spectrometer.

### Applicability & Authorization

BioCORE staff and BioCORE users must be trained on the Agilent 1290 LC and Agilent 6550 QTOF MS instrument, and on the Data Acquisition software.

### Instrumentation & Software

Agilent 1290 UHPLC multi-sampler liquid chromatography system linked to the Agilent 6550 Quadrupole Time-Of-Flight (QTOF) mass spectrometer. MassHunter Data Acquisition (DA) software is used to control settings in both the LC and the QTOF for data collection.

### Reference SOPs

Users should be trained as above and be familiar with the following SOPs:

- QTOF Tuning & Calibration\_ACHBC00008
- LC-QTOF General Maintenance\_ACHBC00010

### Additional Resources

Contact Agilent Technical Support: 1-800-227-9770

### QTOF Instrument Status & Log Updating

When: -Daily -Before every new worklist, if less than daily

1. Update the maintenance tab in the excel file logbook: DATA (D:)> 1290II-6550 LC-QTOF> 1290II-6550 LC-QTOF log> 1290II-6550 logbook.xlsx
2. Record the Date, Initials of the user, description (only if something is wrong or maintenance is done).
3. Record the Rough, Quad, and the TOF vacuum values as found in the Actuals section of DA software.
4. Record the Nitrogen gas pressure (psi). Value must be between 90-100 psi for reliable MS function.
  - a. Note 1: If psi is <90 when the QTOF is on standby, care should be taken when running samples to ensure the psi does not drop during a run to be close to 80 (see note 2).

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- b. Note 2: If psi goes below 80 for >30 seconds, the MS instrument will automatically turn off. The vacuum will slowly be lost over 2-3 days, and post-venting procedures will need to be followed to return the instrument to vacuum (this takes up to 1 week).
- 5. Perform a Mass Calibration/Check Tune (or other if appropriate) and record initials after it completes.

### QTOF Operation Log Updating

When: -Any use of the QTOF instrument by any user

1. Update the operation log tab in the excel file logbook: DATA (D:)> 1290II-6550 LC-QTOF> 1290II-6550 LC-QTOF log> 1290II-6550 logbook.xlsx
2. Record the date, users initials, worklist name, data folder, # runs (injections), method name, and column used.

### LC Column Log Updating

When: -Column is ordered    -Column is installed    -Column is un-installed

1. Update the LC column log tab in the excel file logbook: DATA (D:)> 1290II-6550 LC-QTOF> 1290II-6550 LC-QTOF log> 1290II-6550 logbook.xlsx
2. When a new column is ordered and received, record the column type and name, product number, serial number, lot number, and the date it was received.
3. Record the date a column is installed, or un-installed, in the LC column compartment.

### Before Method Development

- Users must fill out the LC-QTOF maintenance log, instrument operation log, LC column log (if applicable) before developing any methods in DA or running samples.
- Users must tune the instrument (see the QTOF Tuning & Calibration SOP) as appropriate before running any samples. The instrument tune settings must match the DA method for collecting accurate mass data.

### Window Layout in DA

- Instrument status window: changes here apply to the system, not samples. This area details the ready state of each piece of equipment involved in the LC-QTOF system. Green means Ready, Red means not

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ready. The Multi-Sampler block details the injection volume of a run and the temperature of the sample chamber. The Binary Pump 2 block will detail the current solvent ratio and, if the pump is running, will show the flow rate and pressure. Right clicking in this block gives options for adjusting bottle fillings and performing pump purge, prime, and conditioning (see LC-QTOF General Maintenance SOP). The Column Compartment block details the current left-side and right-side column temperatures. Right clicking in this block allows for changing column assignments in the DA software. The DAD block will indicate if a UV trace is being collected. The QTOF block will detail the injection system (should always be Dual AJS ESI), the current instrument state (for example: Standard (3200) 2GHz Dynamic Range), if the Reference Mix bottle A or the Tuning Mix bottle B is in use, if it is collecting spectra, and if it is collecting data from 1 or >1 samples. Note that if Bottle A will be highlighted if it is in use, it will be green if both ref. masses (121 and 922 m/z, always want >500 m/z separation in ref. masses) are found, yellow if only 1 ref. mass is found and red if none are found. If <5 scans failed to find ref. masses out of a 10 min run, the run is probably 100% fine. Note that the user always wants to have ref. mass abundances >150,000 counts each, because the Lc and matrix will suppress the ref. mass abundances close to the 5000 count threshold. Right clicking in the QTOF block allows the user to perform venting of the instrument if certain maintenance is required.

- Actuals window: this configurable window displays the details status of vacuums, equipment ready states, and more. Right clicking in this window will allow the user to add or modify the actuals.
- Chromatogram window: This allows for various instrument parameters to be displayed simultaneously in real time. Binary Pump 2: Pressure, Solvent Ratio B (%B), DAD: Signal A (UV trace), and Total Ion Chromatogram (TIC) should always be included.
- Profile & Centroid windows: These windows can be displayed and configured to show the instantaneous MS spectrum (m/z vs. Abundance) in Profile and Centroid formats.
- Method Editor: This window contains 3 tabs and is where the user creates Worklists, runs Individual samples (Sample Run), and creates LC-QTOF DA methods (Method Editor). Method development is very detailed and will be outlined below.

#### Method Editor

- Properties tab: Will show the method file path, estimated run time, allows for inclusion of pre- or post-run scripts (not advised except under special circumstances, please discuss with BioCORE technician), and method Description.
- Data Analysis tab: User can associate a Qual. and/or Quant. analysis method with a DA method such that they automatically initiate after the run is complete. This is often not necessary if running more

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than one sample, runs the risk of overloading the PC causing the Worklist to fail. It is advised not to link Qual. or Quant. analysis directly to data acquisition.

- **Multisampler tab:** User can set Injection volume, Needle Wash type, Stoptime (As Pump), Posttime (Off), Sampling speed (should be based on viscosity of sample: do technical replicates to check reliability; default is 50-60 uL/min for Draw and Eject with a 2.0 sec wait time after draw), Needle Height (Use Well Bottom Sensing, which results in injection 2.0 mm from bottom, with 0.0 offset; an offset of -1.0 means needle is 1 mm above bottom), High Throughput (flush out factor of 10 is default; the other settings are to specify settings for overlapped injections which is not recommended due to carry over), and Injection Path Cleaning (Multi-wash is the best and allows a 3 step washing; each wash step should have the Seat Back Flush and Needle Wash boxes checked to make sure needle and injection path is very clean; it is best to wash with a low organic (S1: H2O), then a high organic (S2: CAN), then an aggressive wash (S3: 1:1:1:1, IPA:MeOH:H2O:ACN), followed by ending wash in the same solvent as Solvent A to prepare for the next run). Note that all the multi-wash times are added to the LC run time, making the run longer than just the LC gradient.
- **Multisampler Pretreatment tab:** User can modify settings here but it is only useful if user is injecting multiple times in a single run.
- **Binary Pump 2 tab:** User can set Flow rate (0.5 – 0.6 mL/min is standard), Solvent A and B (user can select one of the defaults, but can modify the percentage by right clicking, and can also add notes about additives), Pressure Limits (always set max pressure >50 bar below the column max pressure limit), Stoptime (user should set stop time at the end of the LC gradient), Posttime (1-1.5 minutes is common to condition column), Advanced settings (do not change Minimum Stroke, enable Use Solvent Types for Compressibility, do not change max flow gradient ramp up/down, do not change mixer), and design the LC gradient using the Timetable and Adding rows where the % B is specified at each time (the flow rate and pressure limits will be the same as row 1, and many rows can be added for complicated chromatography).
- **Column Comp. tab:** User can set the column temperatures by setting Left and Right temperatures, choose the column (column position: right side column = position 2) to be used in the method, Advanced settings including the temperature tolerance for Ready State (1.0 °C is default) and Do not switch the valve position, and the timetable does not need to be included in most methods. Increasing temperature lowers viscosity and results in compounds eluting faster and sharper, but users must make sure the column can handle the temperature chosen.
- **DAD tab:** User can include signals from various wavelengths such as Signal A @ 272 nm (a UV trace) with a 360 nm reference wavelength (each with 100 nm bandwidth), a peakwidth of >0.1 min, and setting Stoptime and Posttime As Pump and Off, respectively.

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- **QTOF tab:** This tab contains all settings related to the QTOF instrument and how the user designs a method must be informed by the goals of the research sample. Only an overview of all options will be given. Please discuss your research goals with a BioCORE technician before designing a method.

- On the left side of the QTOF tab the user can select the ion source (Dual AJS ESI), Stop Time (As Pump), and Time Segments and Experiments. Time segments are used to change QTOF parameters at set times during a run, and the only thing that cannot be changed during a time segment is temperatures (because the instrument takes time to change temperatures). Experiments apply only to changing voltage settings (funnel, fragmentor, skimmer, cap, nozzle) sequentially for each scan. Up to four (4) experiments can be associated with each time segment. A minimum of two Time Segments is very common. At T=0, LC stream should go to Waste, and at T=0.5 min, LC stream should go to MS. However, usually additional time segments and experiments are often not needed in a method. The remaining QTOF settings should all be set while the T=0.5 min time segment is active.
- **General tab:** User sets Ion Polarity (Positive is common; Fast Polarity Switching is hard on the instrument and is not advised), Data Storage (Both Centroid and Profile is best), LC Stream (set to MS), Absolute threshold (counts) for MS (200 counts default) and MS/MS (5 counts default), and relative threshold (0.01% default).
- **Source tab:** User sets gas temperatures, gas flow rates, and voltages for the source and the TOF fragmentor. For the 6550 QTOF, generally lower temperatures are best, while very high gas flow rates are essential for consistency. Defaults for the Agilent 6550: Nebulizer 35 psi, Drying Gas 17 LPM, Drying Gas Temp. 175 °C, Sheath Flow 11 LPM, Sheath Gas Temp. 250 °C, Voltage Cap 3500 V, Voltage Nozzle: Lipids in (+) mode 1000V, very polar in (+) mode 0-500 V, or 1500-2000 V in (-) mode.
- **Ref Mass tab:** User should Enable Reference Mass correction and Use Bottle A with the reference masses of 121.05083 and 922.009798 selected. These two masses have >500 m/z separation and allow for calibration at the low and high end of m/z, which is important for looking at small molecules and metabolites. The Recalibration Reference Mass Detection window is the window around the specified mass to look for the reference peak. The highest peak in this window is taken as the reference peak. Be careful to set this window small enough so that a peak in the sample is not mistaken for the reference peak. Default is 50 ppm, but can go from 1-999. The Minimum Height (counts) is the minimum abundance in counts that a peak must have in order to be considered for further processing. The default is 500 counts. Additional masses can be added to the list and used for reference mass correction if desired.
- **MS Mode (Acquisition tab):** After user selects MS Mode, user can specify the Mass Range of data that will be saved in the data file. Note: the mass spectra will collect all data in the Tune

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range (50 – 1700 m/z), but only the range specified above will be saved. User will specify the Acquisition Rate/Time, which specifies how much time it will spend to collect a single spectrum, how many transients are summed to get each spectrum (more transients means more signal), and the acquisition rate is the number of spectra per second. The number of transients per spectrum depends on the mass range of data being collected (specified by the tune, 50 – 1700 m/z), the acquisition rate, and the flight tube length. In general, Quant. requires a minimum of 10-12 scans across a peak, while clinical guides may require 15-20 scans across a peak. Note that increasing the number of scans per second will reduce the sensitivity (signal abundance). Note that the transient pulsor rate is ~30% faster in the 1700 m/z range vs. the 3200 m/z range, resulting in 30% more transients and therefore 30% more signal. In MS only mode, the cycle time is equal to the acquisition rate (this is not true for MS/MS). Users will need to perform multiple runs to determine the balance between peak coverage and sensitivity.

- Auto MS/MS (Acquisition tab): This mode is used for qualitative analysis only, and cannot be used to compare one sample to another. This mode allows the user to set an include and exclude list, set the charge states expected, and set intensity thresholding. This mode works by acquiring MS1 level (TOF) data, sorting the mass list, applying user thresholds, excluding masses, selecting top ions (limited by max precursors ions per cycle), prioritizes preferred ions to the top of the list, then sorts the remaining list by charge priority. User should understand that running auto MS/MS will always miss a lot of information.
  - Spectral Parameters tab: User sets the Mass Ranges for MS1 and MS/MS data to save, the acquisition rates, and for MS/MS the Isolation Width (Medium ~4 m/z is default). In Auto MS/MS, the acquisition rate equals [MS rate + N(MS/MS rate)], where N is the number of precursors, set in the Precursor selection I tab. The goal of Auto MS/MS setup is to match the chromatographic peak width and complexity. User should keep MS1 scan time low because MS will always have more signal than MS/MS. The maximum precursors is 20, but it is best practice to never go below 10. The number of precursors should be matched to sample complexity (more chromatographic peaks requires more precursors). An isolation width window of Medium is default, but Narrow is better for library building while Wide is almost never useful. A Medium window has more signal than Narrow. These windows are not symmetrical but skew to the right. User should understand that a Narrow window will exclude isotope peaks and close contaminants, which is why it is useful for libraries.
  - Collision Energy tab: User can set Fixed Collision Energies, Use Table, or Use Formula. All Agilent spectral libraries are collected at three fixed CE: 10, 20, 40V, and this is the suggested best practice. User should understand that if multiple CE are used, then one

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MS/MS scan is done at each CE, meaning the signal intensity will be lower because now the MS/MS acquisition rate is being split among 3 CE.

- Precursor Selection I tab: User can choose the max number of precursor ions to fragment per cycle (10-20 is recommended), the precursor threshold (do not change defaults of 2000 counts and 0.01% rel. abundance), Active Exclusion (when Enabled using the default settings, if a precursor is observed in 2 consecutive spectra, it will be removed from the following scans for the next 0.5 minutes, and then released to be included again in the next scan; this allows the mass spec to search for other compounds that might be eluting at the same time, as well pick up isomers that might elute later in the run), and Static Exclusion (where the user can select a static range of m/z to exclude from the Auto MS/MS precursor list; if the user knows that a certain range can be excluded, this will make the Auto MS/MS run more efficient).
- Precursor Selection II tab: User designates the Isotope Model (expected isotope pattern of target molecules; default for metabolomics work is Common Organic Molecules, however, low molecular weight compounds with poor M1 peaks may be overlooked, so in these cases the isotope model can be set to OFF), Precursor Charge-State Selection and Preference (higher in Active list is higher priority; default for metabolomics is 1, or 1 then 2), option to Sort Precursors by Charge State then Abundance OR by Abundance only (user preference), vary scan speed based on precursor abundance (this sets the minimum counts per MS/MS spectra and automatically adjusts data accumulation time for precursors, with the option to choose to limit the accumulation time for lower abundance precursors to within the limits determined by the MS/MS acquisition rates [so that the instrument doesn't waste time with low abundance precursors], with an option to reject precursors that cannot reach the target counts), and set Purity stringency and cutoff (setting purity values helps user avoid precursors that produce mixed/chimeric product ion spectra; Stringency is the weight that purity has in determining when an ion is selected and 70-100% is default, while Cutoff is the minimum % total abundance an ion must meet to be selected and 30% is default; setting both to zero (0%) disables purity).
- Preferred/Exclude tab: User can set preferred ions, which are always collected but still have to meet all thresholding for data collection. User can set ions to exclude also. User has option to collect only the preferred ion list. User can set a mass window (ppm) or RT window (min) around the ions in the preferred/exclude list for data collection.
  - When performing qualitative targeted analysis on selected ions it is better to use Auto MS/MS with the Use Preferred ion list only option, rather than Targeted

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MS/MS. This is because during Auto MS/MS an MS1 scan is obtained ensuring detection of accurate precursor masses, while in Targeted the user enter the precursor mass into a Table and no MS1 measurement is taken (it only looks for that precursor mass).

- Targeted MS/MS (Acquisition tab): This mode allows the user to collect all data as set out in a precursor table, but does not undergo any filtering (a disadvantage compared to Auto MS/MS).
  - Spectral Parameters tab: User sets the mass ranges in MS1 and MS/MS to collect, the acquisition rates, and the max time between MS1 spectra (5 seconds is default). The Targeted MS/MS acquisition rate/time is the same as for Auto MS/MS: Rate equals [MS1 rate + N(MS/MS rate)]. User should keep MS1 time low because the MS1 signal is always much higher than MS/MS signal.
  - Collision Energy tab: User sets the CE as fixed (at multiple CE: 10, 20, and 40V if building a library), using a table, or using a formula. If multiple fixed CE are used, the signal intensity at each CE will be proportionally lower as data at each CE is collected within each MS/MS acquisition cycle.
  - Targeted List tab: User can specify targets by m/z (to at least four (4) decimal places, with an isolation window width [see Auto MS/MS section for details]), charge, and retention time (with a retention time window for MS/MS collection). Optional is to include a CE and acquisition time on a precursor ion basis (not recommended). The Default Values will apply for each new line added to the table. It is best practice to create a target list in excel and use this import into the DA method. The column headers must match exactly to the Targeted Table.
    - Understanding Targeted MS/MS Acquisition: In order to get peak coverage, it is important to have MS/MS data collected evenly across the peak. Due to the nature of the QTOF MS/MS method, at a given RT within the RT window for a precursor in the table, the instrument will collect one (1) MS1 scan (at the exact precursor m/z), then switch to collect one (1) MS/MS scan (split among X number of CE), then search the Targeted List for any other precursors at the exact same RT. If only 1 precursor at say 300 m/z is in the table at that RT, the instrument will collect MS1, MS/MS, MS1, MS/MS, MS1, MS/MS (the TIC will look like spikes due to this pattern and the fact that MS1 signal is always much higher abundance than MS/MS signal). This pattern does not result in good MS/MS coverage across a chromatographic peak, and 50% of the information is missing due to wasted time on MS1 scans.

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- Tricking the system: The trick to fixing this is to repeat each precursor target 20-40 times in the target list in excel then importing into the Targeted Table. This will instruct the QTOF to perform MS1 followed by n(MS/MS), where n is the number of rows of the same precursor at the same RT. The same precursor must show up sequentially in rows because a different precursor will trigger an MS1 scan.
- The maximum number of co-eluting precursors is 4-5 (at the same RT) to get good sampling over the chromatographic peak(s). This Targeted List has to be designed carefully to get proper coverage across the peak for each precursor, and the user might consider altering LC conditions to allow better RT separation.
- User may have to increase the acquisition rate so that the data points (# rows in precursor table for each ion) can be collected during the elution time of the peak.

➤ **Cycle Time:** Cycle = 1 scan of each ‘thing’. Affected differently in each mode:

- MS1 mode: -experiments, time segments, acquisition rate
- Auto MS/MS mode: MS1 rate + n(MS/MS rate), n = # precursor ions based on thresholds
- Targeted MS/MS mode: MS1 rate + n(MS/MS rate), n = # precursors in Table
  - If >1 target at RT, best to input into Table as ABC, ABC, ABC for even peak coverage.
  - Minimum MS1 scan time = 50 msec/spectrum
  - Minimum MS/MS scan time = 25 msec/spectrum\*
  - \*For good MS/MS data, use minimum of 75 msec/spectrum
- **Cycle time = Peak Width in msec / # cycles required**
  - Example: A user is performing Targeted MS/MS on a compound that elutes in a chromatographic peak that is 12,000 msec wide and they want to get good MS/MS peak coverage (for quantitation, typically a minimum of 20 points across the peak is desired). How many rows of this precursor ion are needed in the Targeted Table?
    - First, we understand that if a minimum of 20 points across the peak are needed, this mean that a minimum of 20 rows of this precursor must be entered into the Targeted Table. The next question is what is the MS/MS acquisition time?
    - MS/MS Acquisition time is calculated using the cycle time formula above:
      - $12,000 \text{ msec} / (1*\text{MS1} + 20*\text{MS/MS} = 21) = 571 \text{ msec/spectrum (max.)}$
    - So 571 msec/spectrum is the maximum acquisition time needed in MS/MS to collect 1 MS1 scan and 20 MS/MS scans over a time of 12 seconds.
    - Note that in Targeted MS/MS there is a setting for the max time between MS1 scans, which is by default 5 seconds (5000 msec). It should be obvious that this

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would interfere with the above calculations, where after 9-10 MS/MS scans, this setting would force a new MS1 scan followed by MS/MS scans. This could be a big problem if the MS1 scan happened to be triggered at the apex of the chromatographic peak, having a large influence on the resulting MS/MS peak shape and quantitation. The suggested solution to this is to decrease the MS/MS acquisition time (571 msec/spectra is quite long in fact) and add the corresponding number of rows to the Targeted Table. This will result in lower signal per spectrum, but more points across the chromatographic peak and therefore a smaller influence of the any triggered MS1 scans within the chromatographic peak. Also keep in mind that the MS1 scan can be very short (as low as 50 msec/spectra) as the signal is much higher than MS/MS, which can also help minimize the influence of lost data when an MS1 scan is triggered within a chromatographic peak.

### Optimizing Mass Accuracy in MS Only Mode

- Calibrate the QTOF
- Increase Acquisition Rate to increase points across peak
- Optimize cycle time – reduce max mass to 1700 m/z
- Optimize source temps, gas flows, voltages to maximize signal

### Optimizing Accuracy in MS/MS modes

- Use Narrow Isolation Width when building libraries (less ‘junk’ is fragmented into MS/MS)
- Targeted MS/MS: Increase number of spectra across chromatographic peaks

### Optimizing Sensitivity in MS (Limit: 1 pg @ 1:1000 SNR) or MS/MS (Limit: 1 pg @ 1:3000 SNR)

- Increase MS1 and/or MS/MS Acquisition Time (msec/spectra)
- Change Slicer to High Sensitivity (20 – 30% increase at the expense of resolution (Tuning))
- Tuning instrument in 50 – 1700 m/z range (20 – 30% increase over 3200 m/z)
- Fewer Collision Energies (MS/MS only)

### Worklist

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- > User can fill out the worklist details manually, or by pasting in data from excel.
- > User can right click> Fill down... OR Fill down with increment to quickly fill values down columns.
- > Fill down is useful for the Method, while Fill down with increment is useful for Data File.
- > User can also right click> Add multiple samples> to specify the number of samples, the method, the data file naming, automatically adding increments, and more details.
- > Injection volume default is 'As Method', but can be altered here on an individual sample basis.
- > Sample Type is a very useful column to fill as these values (calibrants, controls, samples, blanks) can be imported into Quantitative Analysis software for quantitative method creation and sample quant.
- > This table can be configured to contain various columns, including fragmentor voltage (for MS/MS).
- > User must be aware that any changes made in the Worklist table will OVER RIDE the method.
- > Worklists can and should be saved with a data format and column abbreviation and placed in a folder based on month. For example: 180107\_worklist\_rp.wkl inside the 1801 folder.

#### Sample Run

- > User can run an individual sample using this tab.
- > User gives the run a Name, Position (typically 1-5 in the autosampler), Injection Volume, Data File Name, and Data File Path.
- > User can modify run parameters using the Additional Information panel on the right side of the tab. These will override the DA method.

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