Agilent Technologies

Protocol for RapidFire Post-Installation Quality Control

A. Prepare the quality control (QC) plate

IMPORTANT! The performance evaluation plate MUST be prepared the same day as the test is performed.

Required parts

- HPLC Peptide Standard Mixture (p/n Sigma-Aldrich H2016-1VL)
- 20 mL of LCMS grade water
- One polypropylene 96-well plate (p/n VWR 62408-948)
- One 1 mL single channel pipette (p/n VWR 89079-974) (pipette A)
- One 200 µL single channel pipette (p/n VWR 89079-970) (pipette B)
- One lab vortex mixer

Standard operating procedure

1. Add 1 mL of LCMS grade water into the HPLC Peptide Standard Mixture vial using pipette A and mix well by vortexing the vial 10 seconds to make the 0.5 mg/mL intermediate stock solution (solution S).
   Note: solution S can be stored at room temperature in the supplied glass vial.
2. Add 5 mL of LCMS grade water into the RapidFire matrix bottle 1 (MAT1) using pipette A.
3. Using pipette B, add 25 µL of solution S (from step 1) into MAT1 (from step 2).
   Vortex for 10 seconds to make a solution of 2500 ng/mL (solution W).
4. Add 4 mL of water into each the other three matrix bottles (MAT2, MAT3, and MAT4).
5. Using pipette A, transfer 1 mL of solution W (from step 3) from MAT1 to MAT2.
   Mix up and down at least three times with pipette A to make the 500 ng/mL standard (solution X).
6. Using pipette A, transfer 1 mL of solution X (from step 5) from MAT2 to MAT3.
   Mix up and down at least three times with pipette A to make the 100 ng/mL standard (solution Y).
7. Using pipette A, transfer 1 mL of solution Y (from step 6) from MAT3 to MAT4.
   Mix up and down at least three times with pipette A to make the 20 ng/mL standard (solution Z).

8. Using pipette B, transfer 150 µL of solution W (2500 ng/mL from MAT1) into each well of rows G and H, columns 1 through 12 of the 96-well plate (24 wells in total).
9. Using pipette B, transfer 150 µL of solution X (500 ng/mL from MAT2) into each well of rows E and F, columns 1 through 12 of the 96-well plate (24 wells in total).
10. Using pipette B, transfer 150 µL of solution Y (100 ng/mL from MAT3) into each well of rows C and D, columns 1 through 12 of the 96-well plate (24 wells in total).
11. Using pipette B, transfer 150 µL of solution Z (200 ng/mL from MAT4) into each well of rows A and B, columns 1 through 12 of the 96-well plate (24 wells in total).
12. The 96-well plate now contains the 4 concentrations of peptide mixture, 20, 100, 500, and 2500 ng/mL (solutions Z, Y, X, and W) in rows A & B, C & D, E & F, and G & H, respectively.

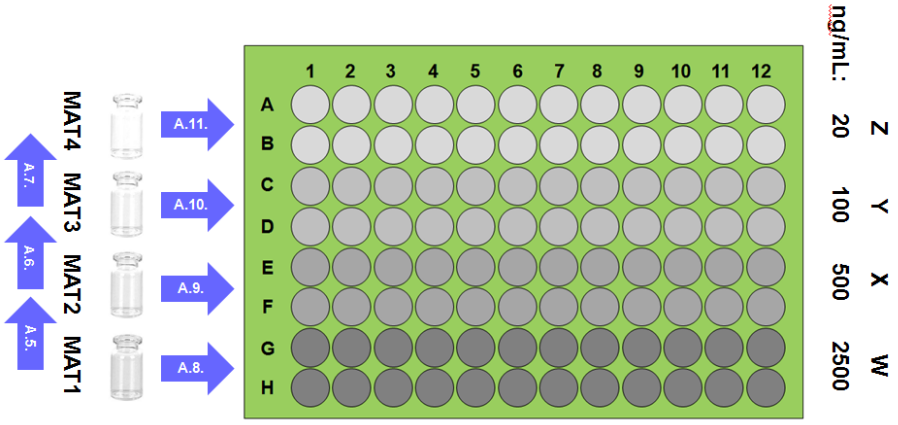


Figure 1

B. Prepare the RapidFire system

Required assay buffers, solvents, and cartridge

- One new RapidFire cartridge "A" (C4) (p/n G9203-80103)
- 2.5 liters of LCMS grade water
- 2 liters of LCMS grade acetonitrile
- 2 mL of formic acid (FA)
- 1 mL of trifluoroacetic acid (TFA)
- Four 2-liter clean glass bottles
- One 1 mL single channel pipette (pipette A)

1. Pour 1 L of LCMS grade water into one glass bottle, label as *aqueous wash*, and insert the aqueous wash line into this bottle (blue cap, connected to peristaltic pump).
2. Pour 1 L of LCMS grade acetonitrile into one glass bottle, label as *organic wash*, and insert the organic wash line into this bottle (red cap, connected to peristaltic pump).

3. Pour 1 L of LCMS grade water into one glass bottle, and using pipette A, add 0.9 mL of FA and 0.1 mL of TFA into the water, mix well, label as *aqueous assay buffer*, and insert the tubing connected to degasser A, then pump 1, into this bottle.
4. Pour 0.8 L of LCMS grade acetonitrile and 0.2 L of LCMS grade water into one glass bottle, add 0.9 mL of FA and 0.1 mL of TFA into the acetonitrile, mix well, label as *organic assay buffer*, insert the tubing connected to degasser C and D, then pump 2 and pump 3, into this bottle.

Set up the RapidFire hardware and software

1. Launch the RapidFire software.
2. Home the cartridge changer.
3. Place the cartridge at position 1 on the cartridge changer, update its info (*Type = A*), and have the cartridge changer *Go To* position 1.
4. Home the X, Y and Z stages (through the *Sipper Configuration Wizard*).
5. Home all three nanovalves and toggle V1, V2 and V3 several times to ensure no loud noise occurs during valve turning.
6. Open the purge valves of all three isocratic pumps and turn on P1, P2 and P3 at flow rates of 5 mL/min to purge the system for at least 5 minutes.
7. Enter flow rates of 1.5 mL/min for P1, and 1.25 mL/min for P2 and P3, and then close the pumps' purge valves.
8. Verify the absence of leaks and overpressures, with all three nanovalves in (a) *blue/inject*, then in (b) *green/load*.
9. Press the *Flush Now* button.

C. Prepare the mass spectrometer (MS)

C. 1. Agilent MS

1. Copy the *.m* data acquisition method specified in *Table 1* for the MS model at hand.
2. Launch the MassHunter data acquisition software (MHAcq).
   Note: You can also create from scratch the method(s) listed in *Table 1* (and detailed in Appendix) directly within MHAcq.
3. Open and apply the *RapidFire QC.m* method.
   The MS source will thus warm up for at least 20 minutes.
4. Double-click the *RapidFire communicator* shortcut on the MS computer desktop and check that two RapidFire executables are effectively listed in the *Windows Task Manager* processes.

C. 2. AB Sciex MS

1. Copy the "Agilent Tests" folder into D:\Analyst Data\Projects\ (see *Table 1*).
2. Launch the Analyst software.
   Note: You can also create from scratch the *.dam* methods and *.dab* batch listed in *Table 1* (and detailed in Appendix) directly within Analyst.
3. In Analyst, select "Agilent Tests" as the current *project.*
4. Open, convert, and save the *Template1.dab* file.
5. Open and apply the *Equilibrate.dam* method.
   The MS source will thus warm up for at least 20 minutes.
5. Double-click the *RapidFire communicator* shortcut on the MS computer desktop and check that two RapidFire executables are effectively listed in the *Windows Task Manager* processes.

Table 1: MS methods and locations

If you have…	Copy	From (RF computer)	To (MS computer)
Agilent 6460 QQQ	RapidFire QC.m	C:\Program Files\ Agilent\ RapidFire\QC\ Agilent QQQ\ MS \ JetStream\	D:\MassHunter\Methods\ RapidFire\
Agilent 6490 QQQ	RapidFire QC.m	C:\Program Files\ Agilent\ RapidFire\QC\ Agilent QQQ\ MS \ iFunnel\	D:\MassHunter\Methods\ RapidFire\
Agilent 6530 QTOF	RapidFire QC.m	C:\Program Files\ Agilent\ RapidFire\QC\ Agilent QTOF\ MS \ JetStream\	D:\MassHunter\Methods\ RapidFire\
AB Sciex 4000 QQQ/QTRAP	"Agilent Tests" folder*	C:\Program Files\ Agilent\ RapidFire\QC\ AB Sciex QQQ\	D:\Analyst Data\Projects\
AB Sciex 5500 QQQ/QTRAP	"Agilent Tests" folder*	C:\Program Files\ Agilent\ RapidFire\QC\ AB Sciex QQQ\	D:\Analyst Data\Projects\

* includes *Equilibrate.dam, Template1.dam, RapidFire QC.dam* (all three identical in contents, differing in title names only) and *Template1.dab*.

D. Ascertain a sample concentration adequate for the QC test

D. 1. Agilent QQQ or AB Sciex QQQ

1. On the RapidFire computer, switch to *Sequences* mode on the main *RF UI* page.
2. In *Miscellaneous Settings,*
   - select the *Plate Configuration* "96_Standard",
   - uncheck *Use Plate Handler*,
   - uncheck *Use Barcode Scanner*,
   - uncheck *Mass Spec Standby After Run*,
   - input 1 for *Plates Between Flushes*, and
   - type in a *Missed Sip Tolerance* of 10.

   Click on *Apply.*
3. Load *Concentration Test.rfbat*
   - from *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QQQ\ RF* or
   - from *C:\ Program Files\ Agilent\ RapidFire\ QC\ AB Sciex QQQ\ RF*
4. Press the *Play* green arrow to start the run.
5. After data acquisition has completed, launch the *RapidFire Integrator* software (RFI).
6. In RFI, under *File\ Load RF300 Data Set*, navigate to the MS computer data folder to perform data processing:
   - *D:\ MassHunter\ Data\ RapidFire\ year\ month\ day\ #* or
   - *D:\ Analyst Data\ RapidFire\ year\ month\ day\ #*
7. Open the *Concentration test calculation X.xlsx* file (X= 6460, 6490, 4000, or 5500)
   - from *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QQQ\ Data Processing* or
   - from *C:\ Program Files\ Agilent\ RapidFire\ QC\ AB Sciex QQQ\ Data Processing*
8. Determine the background intensities of both MRM transitions (Leu-Enk: 556 → 120, and Met-Enk: 574 → 120), and fill in cells C2 and C6 of *Concentration test calculation X.xlsx* with their respective values (see *Figure 2 a*nd *Figure 4*).
9. Determine the peak intensities of both MRM transitions (Leu-Enk and Met-Enk) at all four concentrations (20 ng/mL, 100 ng/mL, 500 ng/mL and 2500 ng/mL), and fill in cells B2-B9 accordingly (see *Figure 3 a*nd *Figure 4*).
10. Refer to *Table 2*, *Table 3* and column F in *Concentration test calculation X.xlsx* to select the optimal concentration for performing the QC test.

    Note: If several sample concentrations meet the criteria defined in *Table 2* and yield data whose ratios of [peak height/ background height] fall within the [minimum; maximum] range described in *Table 2*, then refer to *Table 3* to select the optimal concentration for the RapidFire QC test.

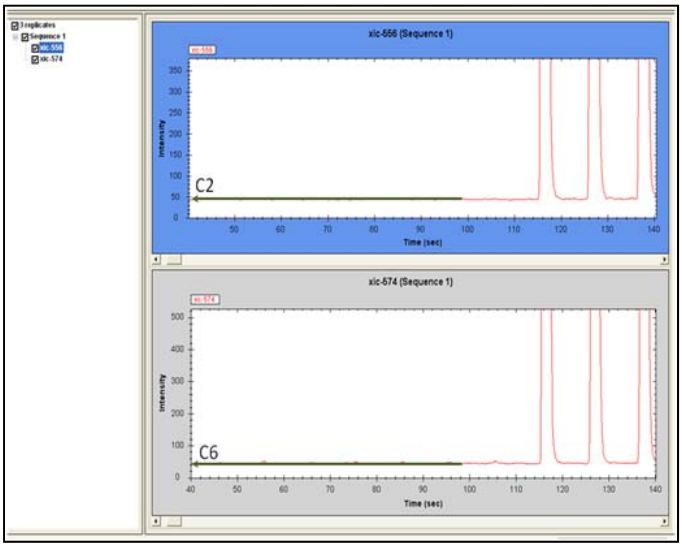


Figure 2

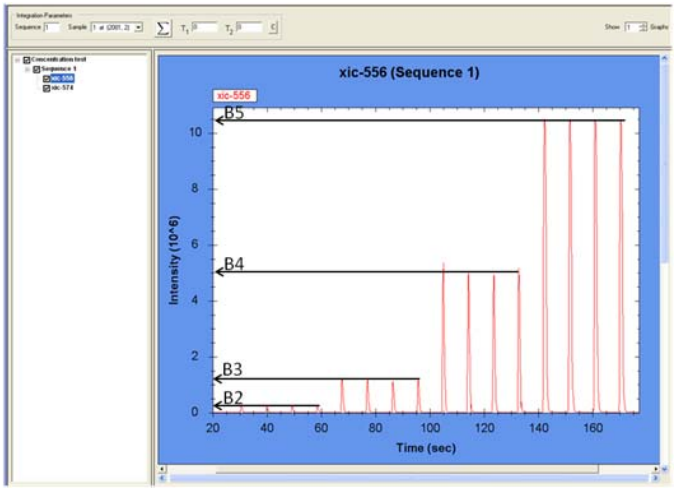
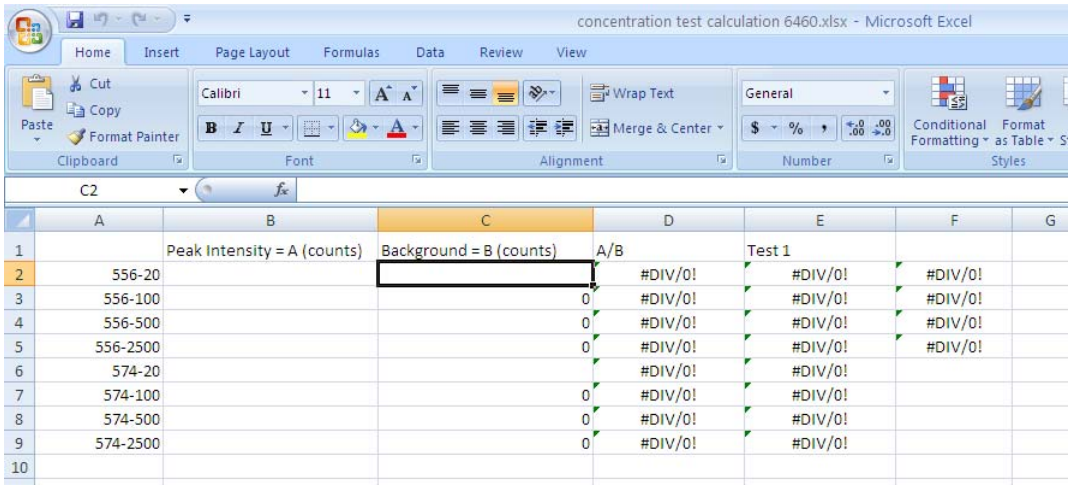


Figure 3



	A	B	C	D	E	F	G
1		Peak Intensity = A (counts)	Background = B (counts)	A/B	Test 1		
2	556-20			#DIV/0!	#DIV/0!	#DIV/0!	
3	556-100		0	#DIV/0!	#DIV/0!	#DIV/0!	
4	556-500		0	#DIV/0!	#DIV/0!	#DIV/0!	
5	556-2500		0	#DIV/0!	#DIV/0!	#DIV/0!	
6	574-20			#DIV/0!	#DIV/0!		
7	574-100		0	#DIV/0!	#DIV/0!		
8	574-500		0	#DIV/0!	#DIV/0!		
9	574-2500		0	#DIV/0!	#DIV/0!		
10							

Figure 4

Table 2: The ratio of [peak height/ background height] must fall within a definite range for a sample concentration to be suitable for the RapidFire QC test.

MS model	Minimum ratio	Maximum ratio
Agilent 6460 QQQ	50	12,500
Agilent 6490 QQQ	50	50,000
Agilent 6530 QTOF	25	25,000
AB Sciex 4000 QQQ/QTRAP	400	100,000
AB Sciex 5500 QQQ/QTRAP	400	200,000

*Table 3: The optimal concentration at which to perform the RapidFire QC test depends on the "column F readout" pattern and is shown in **blue**.*

Concentration	Column F readout					
[1] 20 ng/mL	Y	N	Y	N	N	Y
[2] 100 ng/mL	Y	Y	**Y**	N	Y	**Y**
[3] 500 ng/mL	**Y**	**Y**	Y	Y	**Y**	N
[4] 2500 ng/mL	Y	Y	N	**Y**	N	N

D. 2. Agilent TOF or QTOF

1. On the RapidFire computer, switch to *Sequences* mode on the main *RF UI* page.
2. In *Miscellaneous Settings,*
   - select the *Plate Configuration* "96_Standard",
   - uncheck *Use Plate Handler*,
   - uncheck *Use Barcode Scanner*,
   - uncheck *Mass Spec Standby After Run*,
   - input 1 for *Plates Between Flushes*, and
   - type in a *Missed Sip Tolerance* of 10.

   Click on *Apply.*
3. Load *Concentration Test.rfbat* from *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QTOF\ RF*.
4. Press the *Play* green arrow to start the run.

5. After data acquisition has completed, through the MassHunter Qualitative Analysis software (MHQual) on the MS computer, open *sequence1.d* from *D:\ MassHunter\ Data\ RapidFire\ year\ month\ day\ #*.
6. Generate extracted ion chromatograms (EIC) for Leu-Enkephalin (556.2771) and Met-Enkephalin (574.2335).
7. Determine the background intensities of both EICs, and fill in cells C2 and C6 of *Concentration test calculation 6530.xlsx* with their values (see *Figure 2 a*nd *Figure 4*).
8. Determine the peak intensities of both EICs at all four concentrations, and fill in cells B2-B9 accordingly (see *Figure 3 a*nd *Figure 4*).
9. Refer to *Table 2, Table 3* and column F in *Concentration test calculation 6530.xlsx* to select the optimal concentration for performing the QC test.
   Note: If several sample concentrations meet the criteria defined in *Table 2* and yield data whose ratios of [peak height/ background height] fall within the [minimum; maximum] range described in *Table 2Table*, then refer to *Table 3* to select the optimal concentration for the RapidFire QC test.

E. Perform the RapidFire QC test at optimal concentration

1. Load *QC Concentration X.rfbat* (X= 1,2, 3, or 4; refer to *Table 3*) from the relevant location on RapidFire computer:
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QQQ\ RF* or
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ AB Sciex QQQ\ RF* or
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent TOF\ RF*
2. Press the *Play* green arrow to start the run.

F. Process the QC plate data

1. After data acquisition has completed, launch the *RapidFire Integrator* software (RFI) on the MS computer.
2. Review the latest data folder and its contents:
   - on Agilent QQQ MS computers, *D:\ MassHunter\ Data\ RapidFire\ year\ month\ day\ #* contains *sequence1.d* and *batch.rftime*
   - on AB Sciex QQQ MS computers, *D:\ Analyst Data\ RapidFire\ year\ month\ day\ #* contains *sequence1-xic-556---120.rfxic*, *sequence1-xic-574---120.rfxic*, and *batch.rftime*
   - on Agilent QTOF MS computers, *D:\ MassHunter\ Data\ RapidFire\ year\ month\ day\ #* contains *sequence1.d*, *batch.rftime* and *platemap.tofmap.txt*
     Open *platemap.tofmap.txt* in Excel and add two columns to each row of data, listing the exact masses for Leu-Enkephalin: 555.2766 a.m.u. and Met-Enkephalin: 573.2257 a.m.u.

3. Process the data in RFI and export the integrated results by selecting *File*\ *Export peak data plate-wise* and save the file "using current plate names". The RFI-generated data file is thus called *QC plate.csv.*

Refresher on QTOF-generated data analysis by RapidFire Integrator

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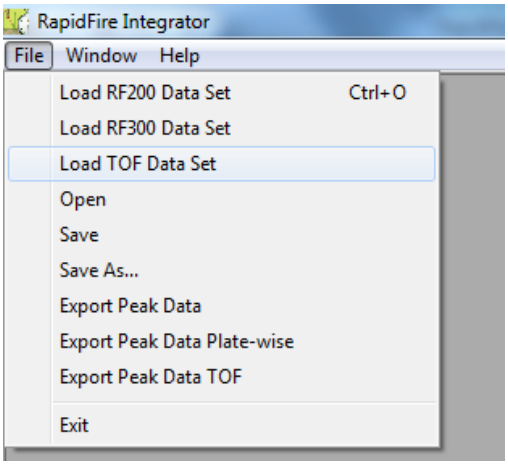
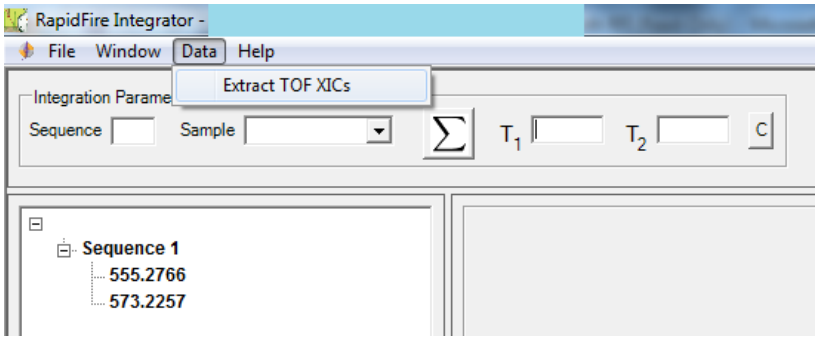
3.a. Open *platemap.tofmap.txt* in Excel and add two columns to each row of data, listing the exact masses for Leu-Enkephalin: 555.2766 a.m.u. and Met-Enkephalin: 573.2257 a.m.u.

	A	B	C	D	E
1	seq	row	col	mass	n
2	----------				
3	1	5	1	555.2766	573.2257
4	1	5	2	555.2766	573.2257
5	1	5	3	555.2766	573.2257
6	1	5	4	555.2766	573.2257
7	1	5	5	555.2766	573.2257
8	1	5	6	555.2766	573.2257
9	1	5	7	555.2766	573.2257
10	1	5	8	555.2766	573.2257
11	1	5	9	555.2766	573.2257
12	1	5	10	555.2766	573.2257
13	1	5	11	555.2766	573.2257
14	1	5	12	555.2766	573.2257



3.b. Launch *RapidFire Integrator* (RFI) on the MS computer, and under *File*\ *Load TOF Data Set*, navigate to *D:*\ *MassHunter*\ *Data*\ *RapidFire*\ *year*\ *month*\ *day*\ *#*\ (see F.2.).

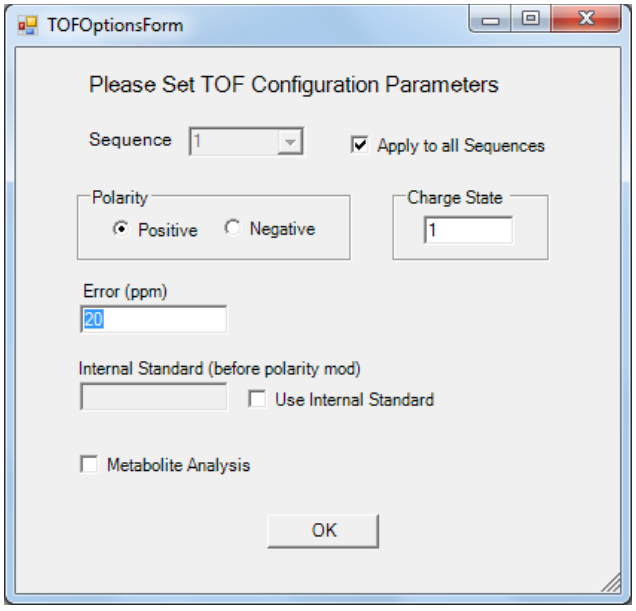
3.c. Under *Data*, select *Extract TOF XICs*.



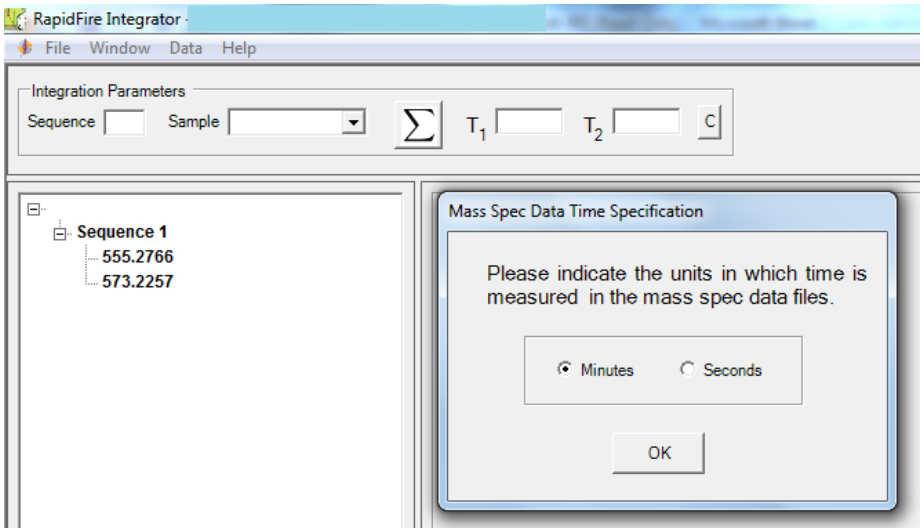
3.d. In the *TOF Options* window, enter

- *Polarity: Positive*
- *Charged State:* 1
- *Error:* typically 10 – 50 ppm
- no *Internal Standard*
- no *Metabolite Analysis*

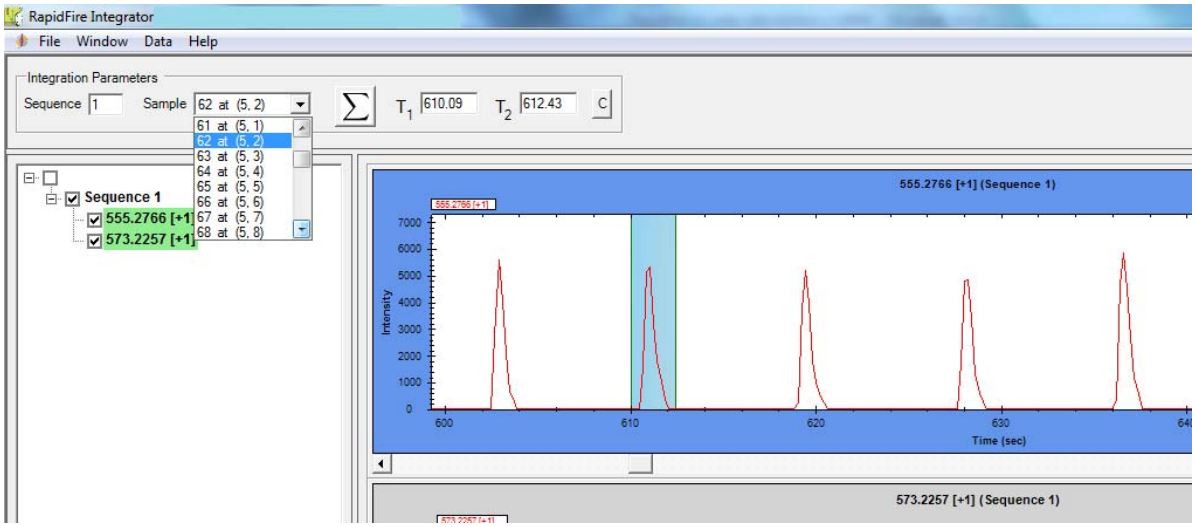
and click *OK.*



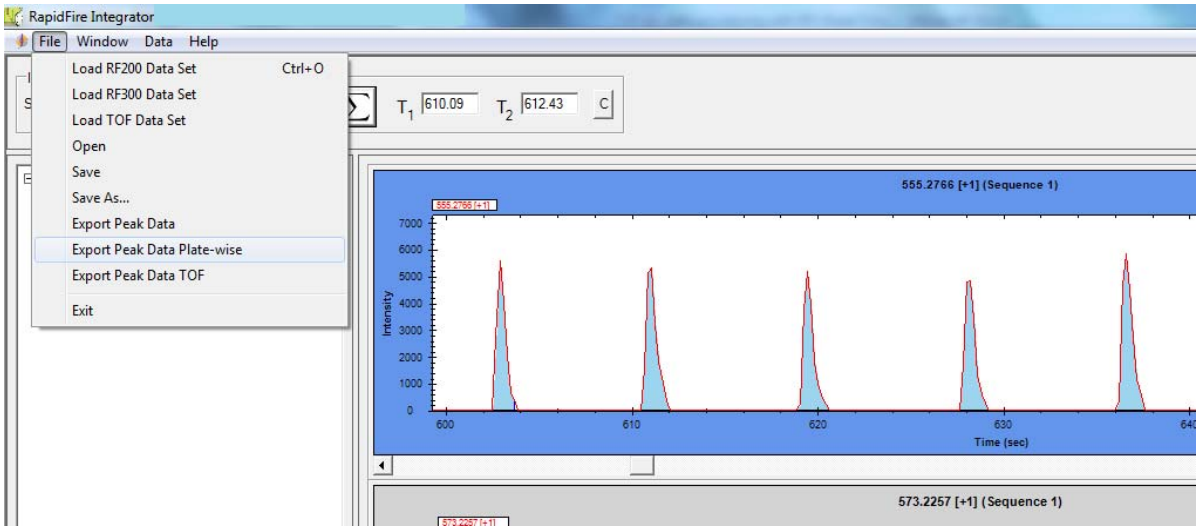
3.e. Confirm an acquisition time unit of *Minutes* and click *OK.*



3.f. Left-click on the mouse to zoom onto a peak of known identity, define its width via a slim rectangle dragged across its base, ascertain its identity in the drop-down *Sample* list, and press the Σ summation button to process the data and calculate areas under the curves.



3.g. Under *File*\ *Export peak data plate-wise,* save the file "using current plate names". The RFI-generated data file is thus called *QC plate.csv.*



4. Open *QC plate.csv* from step 3.
5. Copy *QC data processing.xlsx* to the same location as *QC plate.csv* from
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QQQ\ Data Processing* or
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ AB Sciex QQQ\ Data Processing* or
   - *C:\ Program Files\ Agilent\ RapidFire\ QC\ Agilent QTOF\ Data Processing*
6. Highlight and copy cells A1-M21 from *QC plate.csv* file (*Figure 5*).
7. Paste the cells copied in step 6 in cells A1-M21 of *QC data processing.xlsx* (*Figure 6*).
8. Save the file from step 7 as *QC plate.xlsx*.
9. Check column Q of *QC plate*.xlsx. If the CV value of the tested concentration is smaller than 5 (%), the RapidFire system passes the post-installation QC test (*Figure 6*).

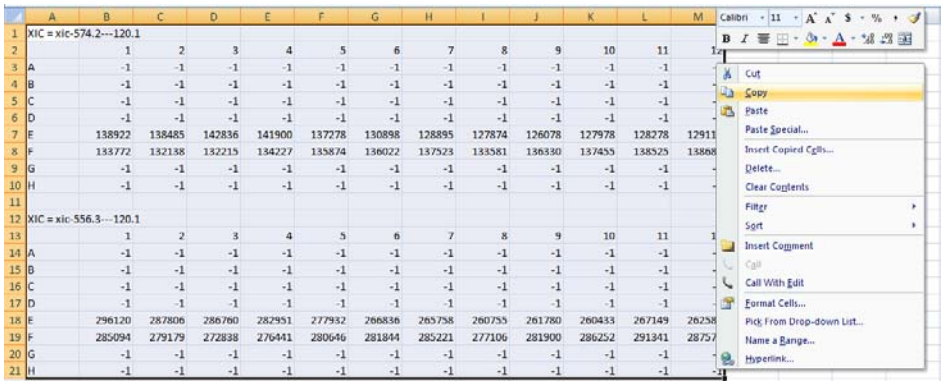


Figure 5

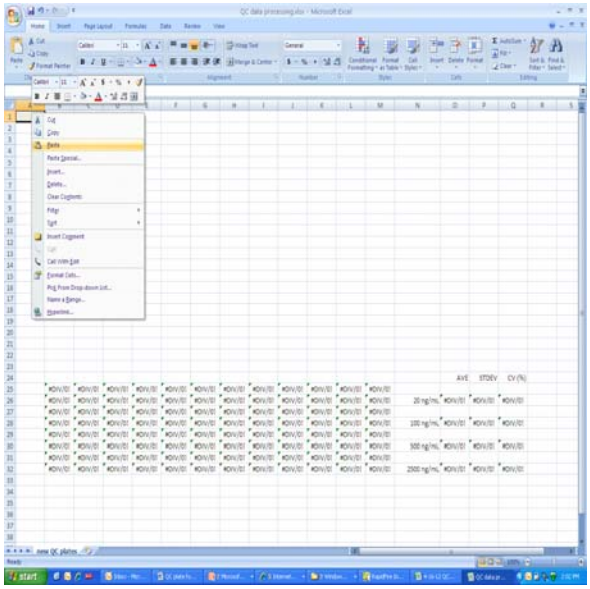


Figure 6