

Agilent 6475 LC-Triple Quad User Manual

Table of Contents

I.	Introduction.....	3
II.	Safety.....	3
III.	Data Management	3
IV.	Software	3
V.	Sample Preparation.....	4
VI.	Quick Start Checklist	5
VII.	Mobile Phase Volume Check	6
VIII.	Data Acquisition	7
a.	Launch Program on Desktop	7
b.	Loading the Method, Column Installation and Purging Pumps	11
c.	Worklist Creation.....	14
i.	Worklist Creation – Setting up the Root Data Folder	14
ii.	Worklist Creation – Creating your Worklist using the IMSERC .csv Template.....	16
iii.	Option 1 - Uploading a .csv/.xlsx Template file	16
iv.	Option 2 – Starting from Scratch.....	18
d.	Running the Worklist and Post-Analysis Steps	18
IX.	Opening Files in Qualitative Analysis (to check raw data)	19
X.	Quantitative Analysis – Quantification Using Calibration Curves	21
XI.	Appendix 1 – Obtaining the Template File from the IMSERC Public Drive and Uploading in the Scratch Drive	31
XII.	Appendix 2 – Multisampler Map	31
XIII.	Version History	32

LC-TRIPLE QUAD USER MANUAL

I. Introduction

The use of this instrument is allowed only by qualified users after receiving training from IMSERC personnel. Do not run this instrument without approval from IMSERC staff. Failure to do so may cause damage to the instrument, produce invalid data, and result in additional fees and/or removal of all IMSERC privileges.

This short set of instructions is meant to serve as a guide for 'routine' data collection on the instrument. Please read this standard operating procedure and acquaint yourself with the instrument. If something happens that you do not understand while using the system, please **stop** and **get help**. In any event, be completely prepared to justify your actions. The cost of even minor repairs is considerable.

The **Agilent 6475 LC/TQ**, or **triple quad** for short, is coupled to an Agilent 1290 Infinity II UHPLC system and is mainly used as a highly sensitive quantitative M.S. solution using multiple reaction monitoring (MRM) or selected ion monitoring (SIM) modes. It is also capable of conducting full scan (MS1/MS-only) experiments. The UHPLC coupled to this M.S. has two locations where a User can install their own Column (based on their set Method) in the multicolumn thermostat and has the capability to use multiple mobile phase solvents connected to the external solvent selection valves and a binary pump. The autosampler (multisampler) can, in theory, have 432 vials in its 8-tray compartments. In practice, the top two trays, plates 7 and 8, are set up to accept 96-well plates, while plates 1 – 6 are set up to accept autosampler vials. The multisampler also has multi-wash capability, which utilizes three different solvent lines to wash to conduct seat back flush and needle wash before every sample injection.

The creation of MRM/SIM/Full Scan methods requires IMSERC personnel to use reference standards of the analytes-of-interest to validate the retention time(s) on the corresponding Column being used and determine the most selective product-ion to be used for detection/quantification. IMSERC personnel must create these methods to evaluate for the best chromatographic separation, the highest sensitivity, and the most accurate method for the experimental need before a User can effectively use it for their research.

II. Safety

All users of IMSERC must review the general safety policies at <http://imserc.northwestern.edu/about-policies.html>.

Familiarize yourself with the location of standard safety stations, such as eye wash and shower stations, just outside of BG70. When using the computer, gloves should be removed.

III. Data Management

Your personal data folders are created during training and are located under your supervisor's group folder (Project Folder). See a staff member if you do not have a personal folder on this instrument yet. Your personal files are located in either the Methods, Worklist, and Data subfolders and must contain your Lastname_Firstname as the main folder name. Inside these Data and Worklist subfolders, you will create a new folder that references the date the data was collected.

IV. Software

Data Acquisition is performed on the Agilent MassHunter Data Acquisition Program, which can be accessed through the Control Panel. The Control Panel icon can be accessed on the Desktop. Depending on the data output need and experimental design, the Agilent MassHunter Qualitative Analysis or the Agilent MassHunter Quantitative Analysis Programs are used. Agilent files can also be analyzed by third-party or open-source software packages. The Agilent program suite is available for use on the data analysis computers at the ground level of IMSERC. The use of IMSERCterm also makes it possible to access these programs, but one needs permission to access them. Request access through this [form](#) or the IMSERC FAQs on the IMSERC website.

LC-TRIPLE QUAD USER MANUAL

V. Sample Preparation

Before training on this instrument, sample preparation and your overall experimental design should have been discussed in great detail with IMSERC personnel. At a bare minimum, topics such as the use of internal standards, dilution factors during sample preparation, and concentration range for calibration curves should have been discussed.

All samples should have gone through some extraction procedure, centrifugation, and/or filtration processes. If you are not certain that your sample preparation matches IMSERC-MS expectations, contact IMSERC personnel immediately to discuss.

When conducting quantitative M.S. on this instrument, the use of internal standards greatly improves the accuracy of the quantitative M.S. assay as they can normalize any variations from sample preparation and ion suppression during data collection. When creating calibration curves for a particular analyte, there will be a specific linear range of concentration that can be generated for the curve to accurately determine concentrations of unknown samples. Due to the introduction of an internal standard and other processes in sample preparation, there will be a dilution factor that will be used across calibrator and Q.C. samples, as well as the actual samples themselves.

Any questions and concerns should be directed to IMSERC personnel immediately.

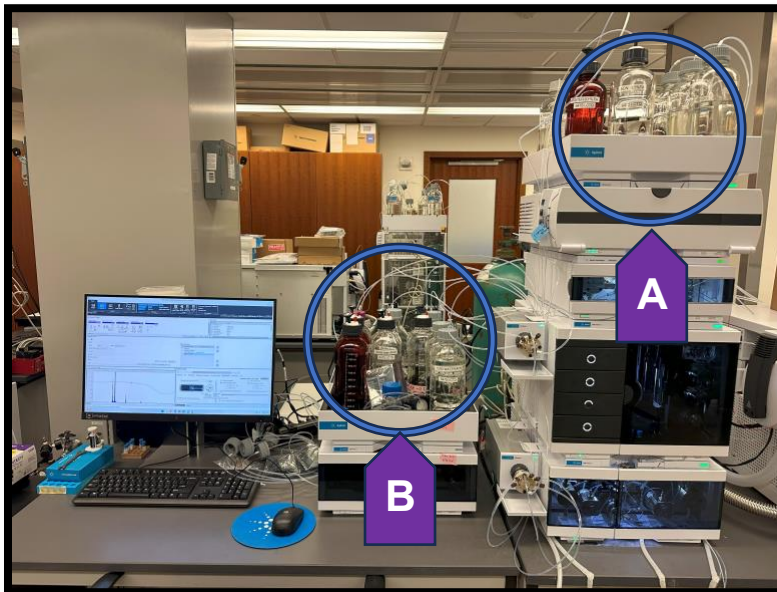
Detail	Recommendation	Consideration
Vials	Agilent Vial 5182-0714, Agilent cap 5185-5865	The Autosampler picks up the vial. With the wrong vial dimensions, the injection needle could miss or hit the top of the glass vial.
Solvent	HPLC-grade water, methanol, Acetonitrile, isopropanol, dichloromethane	Listed solvents include the mobile phase solvents located on instruments. Your Sample must be soluble in your chosen solvent and compatible with a combination of the mobile phases listed. Avoid DMF and high concentrations of DMSO.
Concentration / Purity	Varies by Method	Sample(s) may be diluted with modifiers to increase signal/ionization efficiency, such as 0.1% Formic or Acetic acid, ≤ 10 mM ammonium formate or acetate Sample(s) should have gone through some extraction procedure, centrifugation, and/or filtration processes.
Volume	500 µL	If the volume available is too low, use Agilent vial spring loaded insert 5182-8872
Labeling	Name and solvent	Sample identification & safety / proper disposal

VI. Quick Start Checklist

- a. Start Nucore reservation
- b. Check mobile phase volumes
- c. On the Desktop, Open the Agilent Control Panel and select Project (your P.I.'s last name). Skip to step f.
- d. If MassHunter Acquisition and Control Panel are already open, close all windows and **click NO** on all pop-up windows. On the Agilent Control Panel, click Close Connection.
- e. On the Agilent Control Panel, select Project (your P.I.'s last name).
- f. Launch. The MassHunter Acquisition Software will open.
- g. Select the correct layout (WALKUP layout)
- h. Doublecheck the actual volume of mobile phase bottles and update the Software's actual solvent volumes
- i. Install the needle wash solvents that complements your Method
- j. Load Method for your desired analysis
- k. Install your Column in the correct position in the MCT module based on the Method being used
- l. Turn On ALL Modules
- m. Add pump backpressure trace in the chromatogram plot
- n. Purge pump and Lines for 5 minutes
- o. While purging, start preparing your Worklist
- p. Set up Worklist default Method and Data Root Folders and activate Post-Worklist script
- q. Upload your Template file for the Worklist Table.
- r. Add the complementary WASH method for your analysis at the end of the Worklist
- s. Ensure that a post-worklist Script to turn off the pumps is Enabled in the Worklist Run Parameters Window.
- t. Click Play to start analysis.
- u. Adjust your NUcore reservation, if necessary.

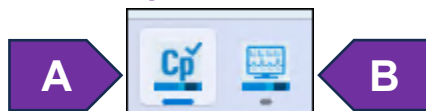
VII. Mobile Phase Volume Check

1. Ensure there are enough mobile phase solvents (A and B) for your analysis and wash solvents. You should know which channel is being used for the particular method you are using. If not enough, consult IMSERC-MS personnel to replace the bottle(s) immediately.



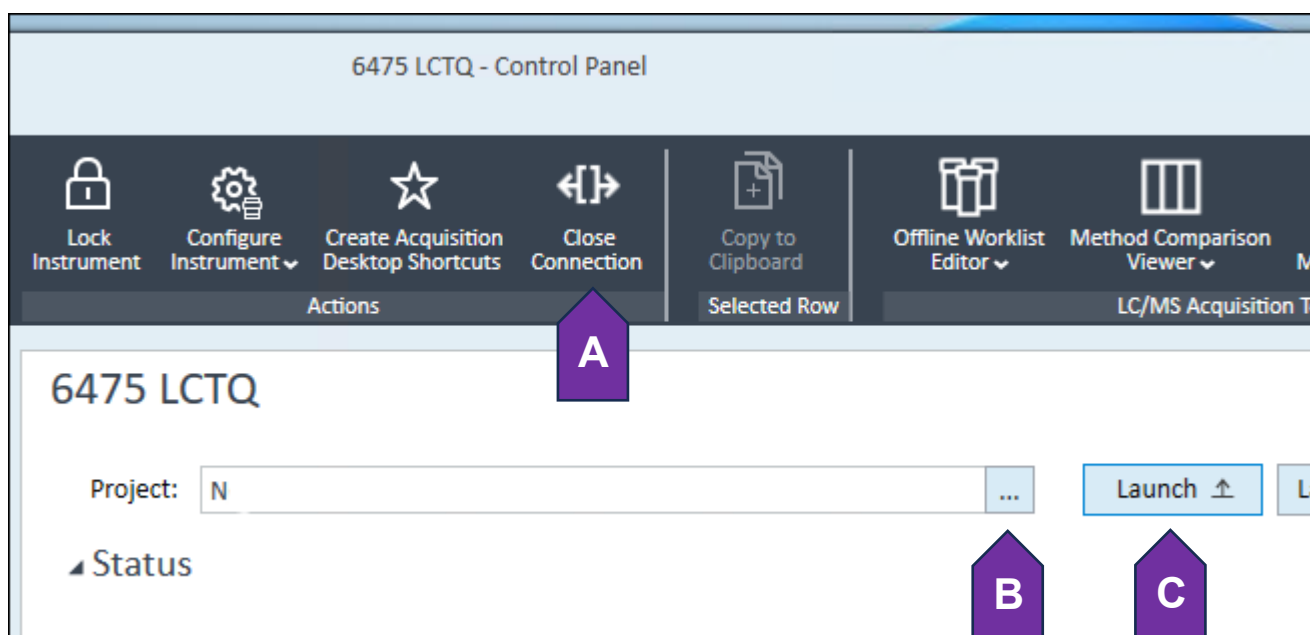
VIII. Data Acquisition

a. Launch Program on Desktop



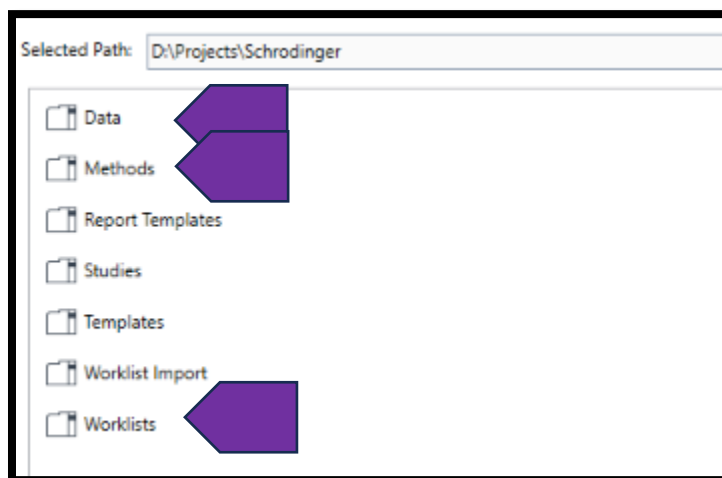
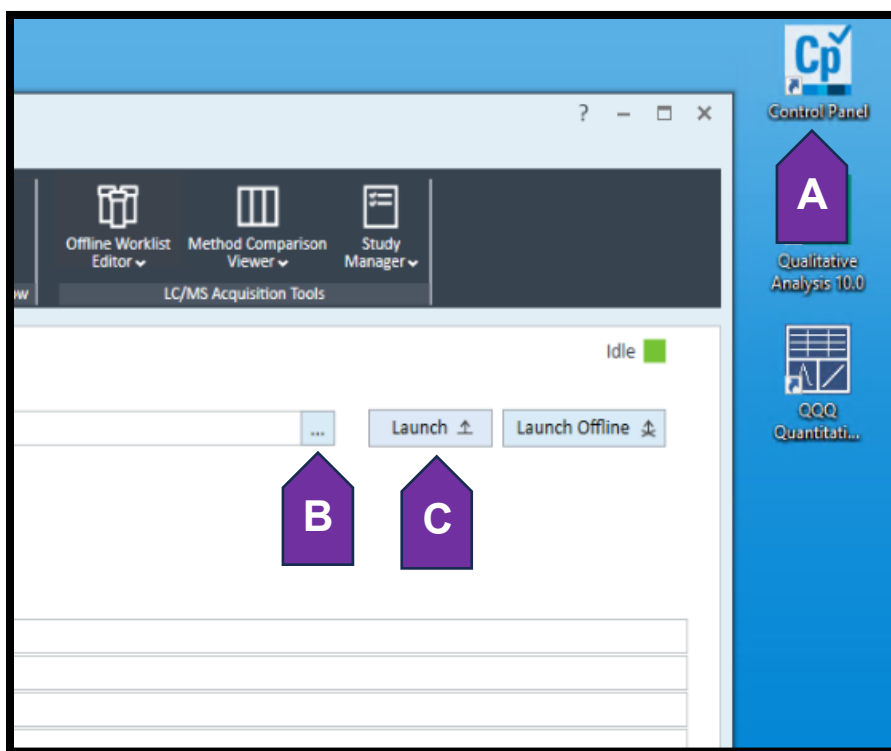
Program Icons of the Control Panel (A) and Acquisition Software (B) on the Taskbar

1. If the Acquisition Software is open and loaded with a Method but not collecting data, continue to step 2. If not, skip to step 4 to open the Control Panel Program.
2. You can close the Window. When asked to put the instrument on standby and save layout changes, click **"NO"** on all pop-up windows.
3. In the Control Panel program, which should be open as a Window, click **Close Connection** to close the Project. **(A)** After the Connection has been closed, you can now choose your P.I.'s name under P.I.'s name under Project **(B)**. Click **Launch (C)**. The Agilent MassHunter Data Acquisition window will open. Skip to step #4a.



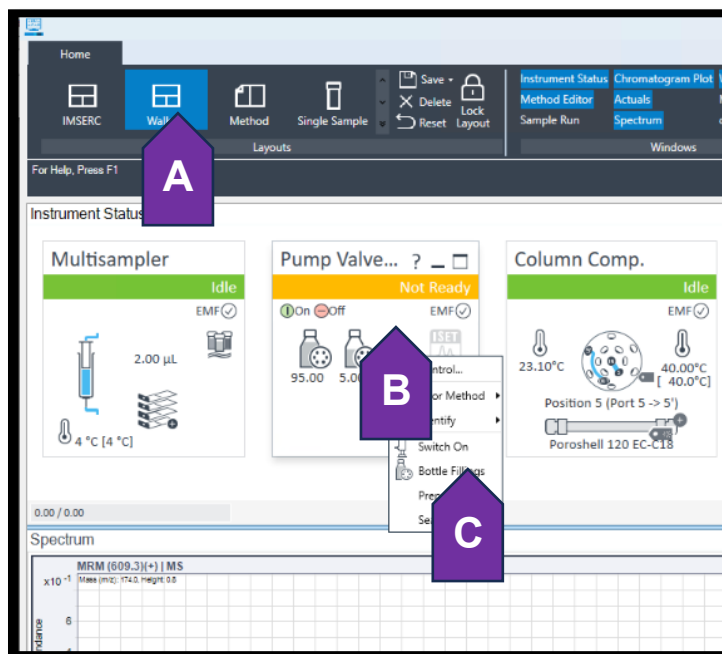
LC-TRIPLE QUAD USER MANUAL

4. Open the Control Panel program **(A)** on the Desktop and choose your P.I.'s name under Project **(B)**. Click Launch **(C)**. The Agilent MassHunter Data Acquisition window will open.
 - a. **IMPORTANT:** The Files created for each session will be saved in one of the three locations within your P.I.'s folder. Each subfolder contains a corresponding for every user (named LastName_FirstName). Save all corresponding files (data, worklists, methods) in those appropriate subfolders.

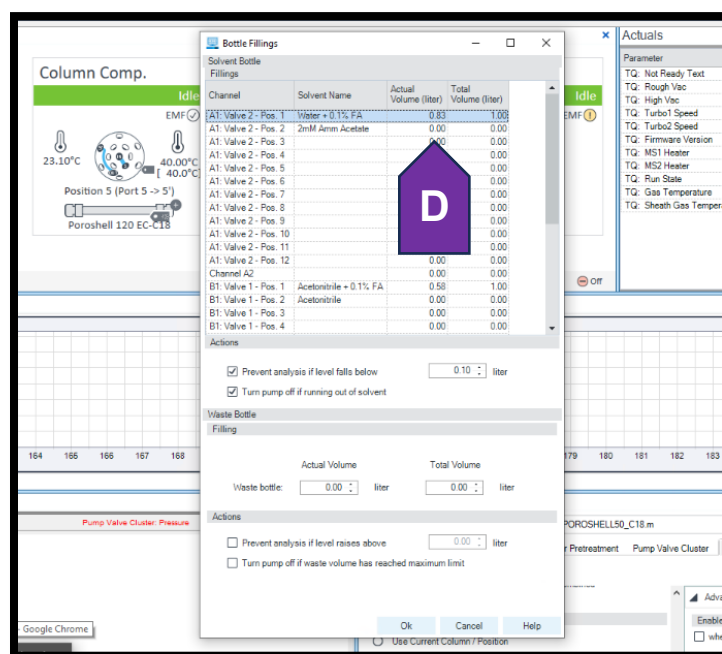


LC-TRIPLE QUAD USER MANUAL

- In the Acquisition software, at the top-left corner, click the **Walkup** icon to ensure you're in the correct user interface layout. **(A) When asked to save the layout, click "NO."**
- Double-check that the actual volume of your mobile phase matches the actual volume in the Software. Right-click on the Pump Valve Cluster (specifically the center blank area) **(B)**, and choose **Bottle Fillings** **(C)**. If not, continue to Step #5.

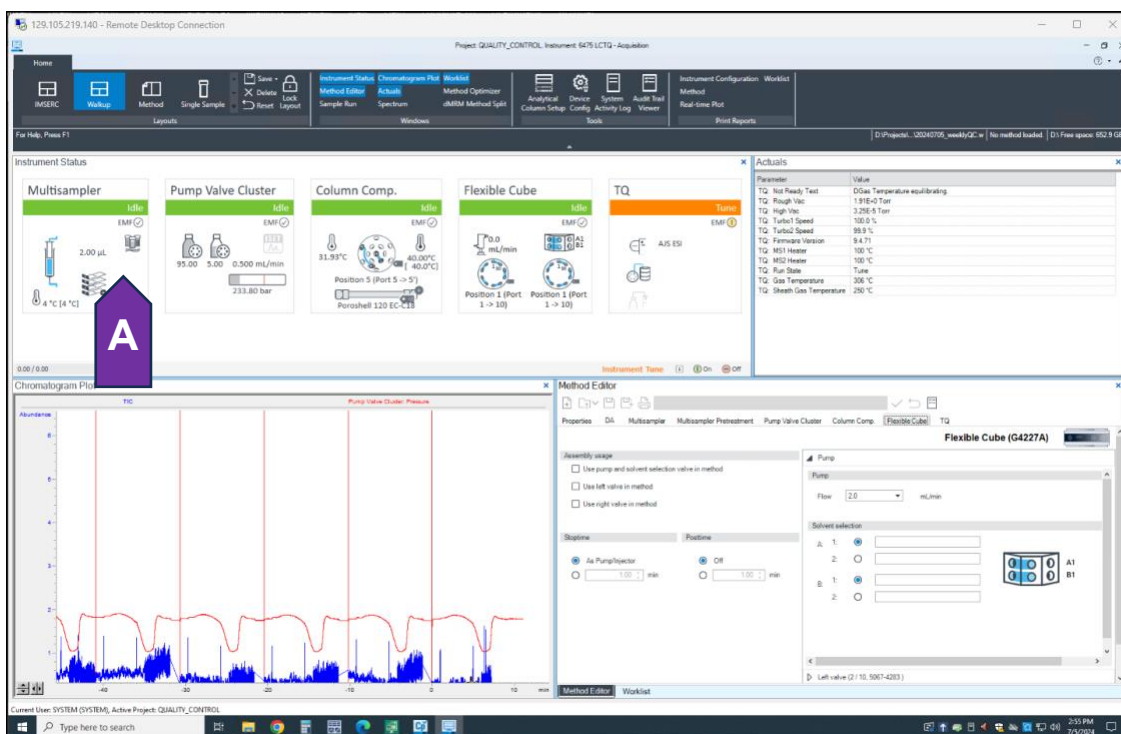


- In the Actual Volume (liter) column, enter the actual volume(s) of the mobile phase bottle(s) being used **(D)**. Click **OK** to continue.

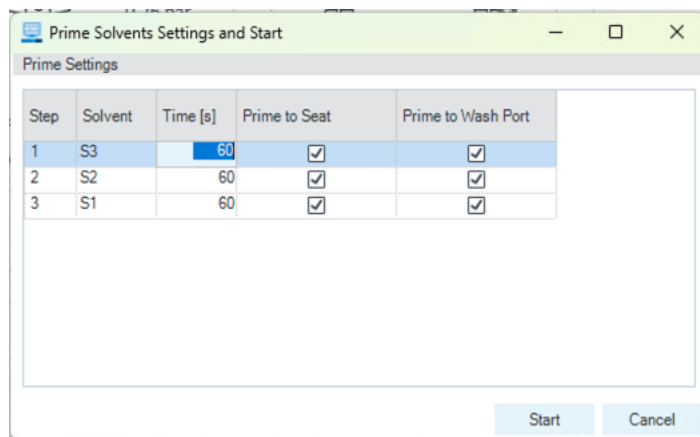


LC-TRIPLE QUAD USER MANUAL

8. **Ensure that the appropriate Needle Wash/ Needle Seat solvents are installed for your method.**
These are the solvent bottles attached to the lines on the right-side of the UHPLC stack. The wash solvent bottles installed will most likely change between usages. The correct wash solvents has been mentioned during your training or subsequent discussions with IMSERC-MS personnel. The installation of the appropriate wash solvents ensures that there is minimal sample carryover in between injections.
9. After the bottles have been installed, right-click on the Multisampler (specifically the center blank area), and choose Prime Solvents... **(A)**



10. Check all the boxes for each solvent line and set the Time(s) to 60 seconds as shown below. Click **OK** to continue to initiate the Priming steps. Continue to the next section.



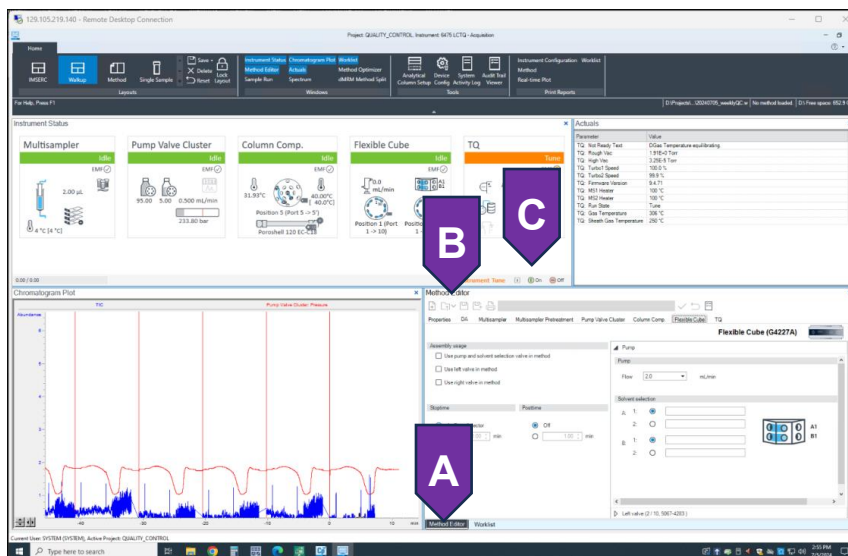
LC-TRIPLE QUAD USER MANUAL

b. Loading the Method, Column Installation, and Purging Pumps

1. At the bottom of the screen, click on the Method Editor Tab **(A)** and click the Folder icon to open your desired Method from Training. **(B)**
2. Install your Column following the instructions from your training. Depending on the column position assigned, your Column will be on **Position 3 or Position 6**. Each position uses a Quickconnect Assembly to easily connect and disconnect your Column. To install your Column, make sure the Quick Connect latch is in the open position, and screw your Column into the connection while following the flow diagram of the Column. Fingertight the blue screw, then close the latch. Screw the outlet of the Column to the brown plastic fitting. Keep the end plugs of your Column in a safe place to prevent from getting lost.

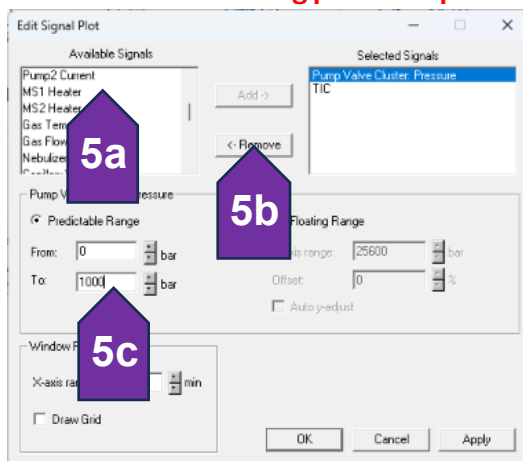


3. In the Acquisition Software, click the green "On" icon to turn on all LC-MS modules **(C)**. The Multisampler, the Pump Valve Cluster (binary pump and external solvent selection valves), the Column Compartment, and the Triple Quad (T.Q.) will now turn on and initialize to their starting conditions according to the **Method** selected.

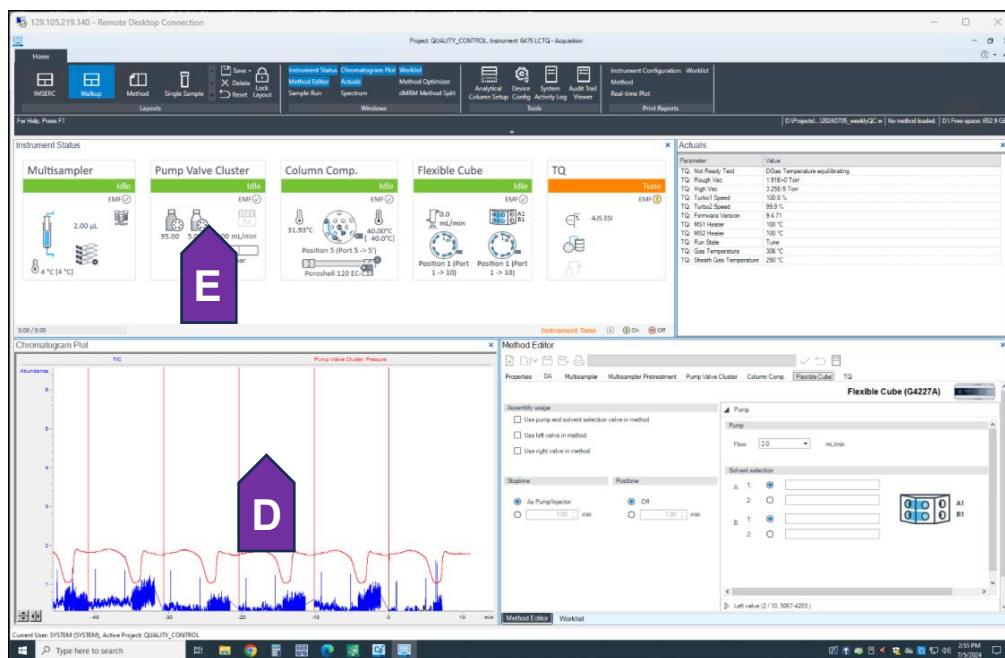


LC-TRIPLE QUAD USER MANUAL

- By default, MassHunter Acquisition displays only the TIC signal over time in the Chromatogram Plot Panel. Within the Chromatogram Plot panel, right-click on the white space and click on Change... (D)
- In the pop-up window, scroll down in the Available Signals box (5a) and select **Pump Valve Cluster Pressure** to add to the Selected Signals box (5b). Select **Predictable Range** in the middle section of the Window and change the range from 0 to **1000** bar. (5c) **You can now visualize the Pump Pressure over your usage, which will be important when assessing potential pressure increases due to clogs.**



- To purge the purge of new mobile phase solvent, right-click on the Pump Valve Cluster (specifically the center blank area) (B), and choose Prepare Pump... (E).



LC-TRIPLE QUAD USER MANUAL

7. In the pop-up window, choose **Purge**. Set the Duration, Flow, and Composition B, as shown below. Click **Start**. Note: the pump will now run at full flow for 5 minutes, purging the pumps of old mobile phase solvents and pushing through the new mobile phase in the loaded method to the waste lines. This will ensure that the mobile phase being used is filled up to the sample loop.

Prepare Pump

Purge

Use for changing mobile phases, drawing solvent or for removing air bubbles.

Duration: 5.00 min Composition A: 50.00 %
 Flow: 5.000 mL/min Composition B: 50.00 %

Conditioning

Minimize the pressure ripple by dissolving air bubbles in the pump heads.

Note: Solvents will flow through the LC system and column. Method parameters are applied for flow rate, composition and max. pressure.

Duration: 15.00 min

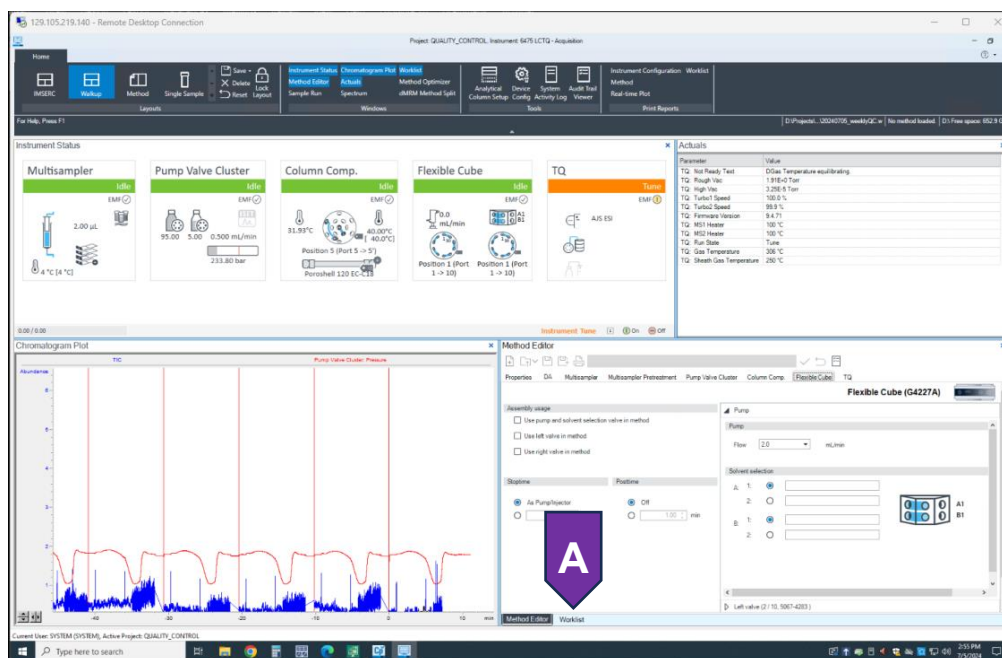
Prime

Draws solvent into (both) pump heads for removing air bubbles from the pump head and particles from valves. Flow goes to waste.

Do not use the Prime function for filling the solvent lines or changing the solvent type.

Start Cancel Help

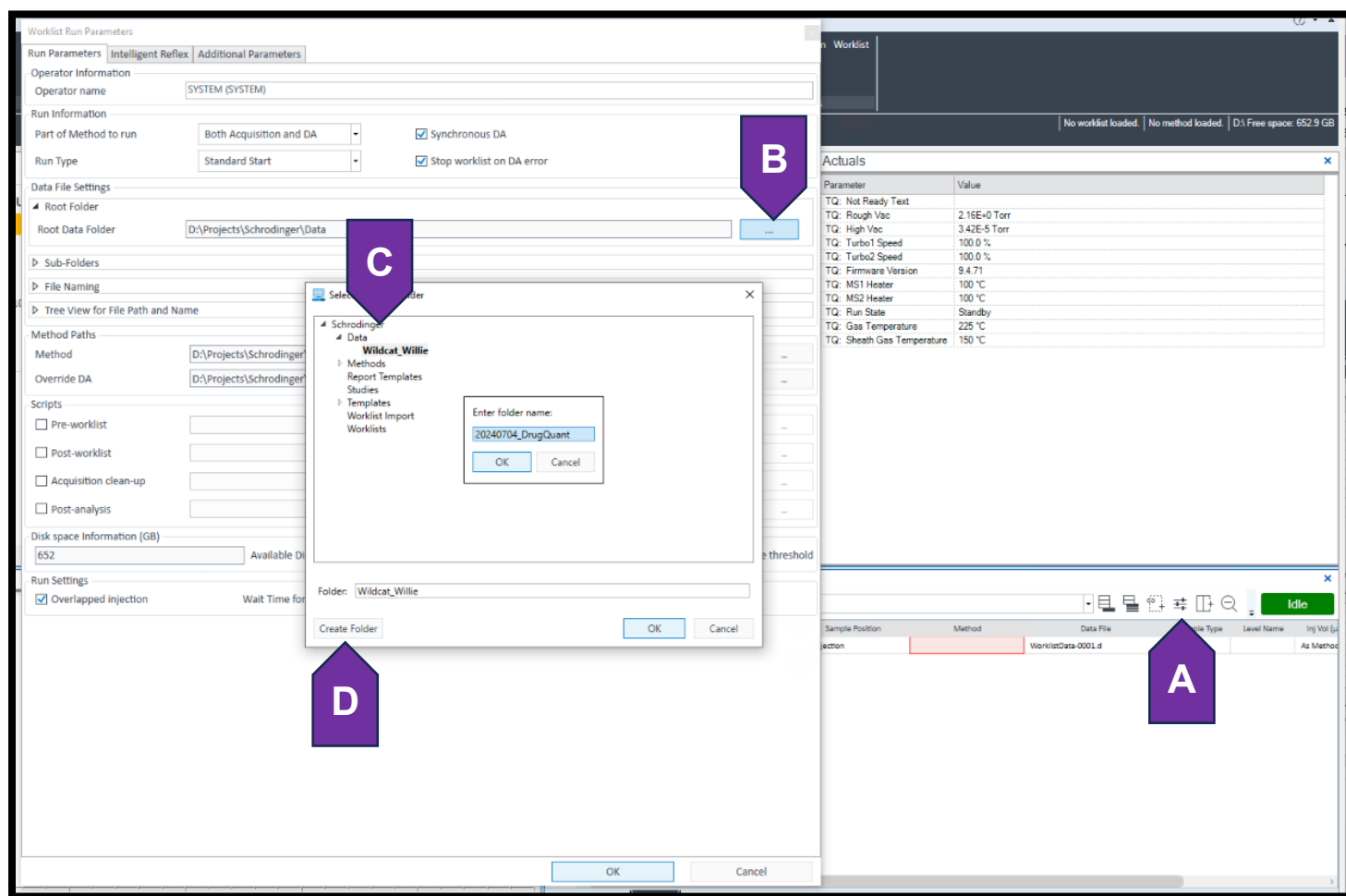
8. While the pumps are purging, click the Worklist tab to fill out your sample table. **(A)**



c. Worklist Creation

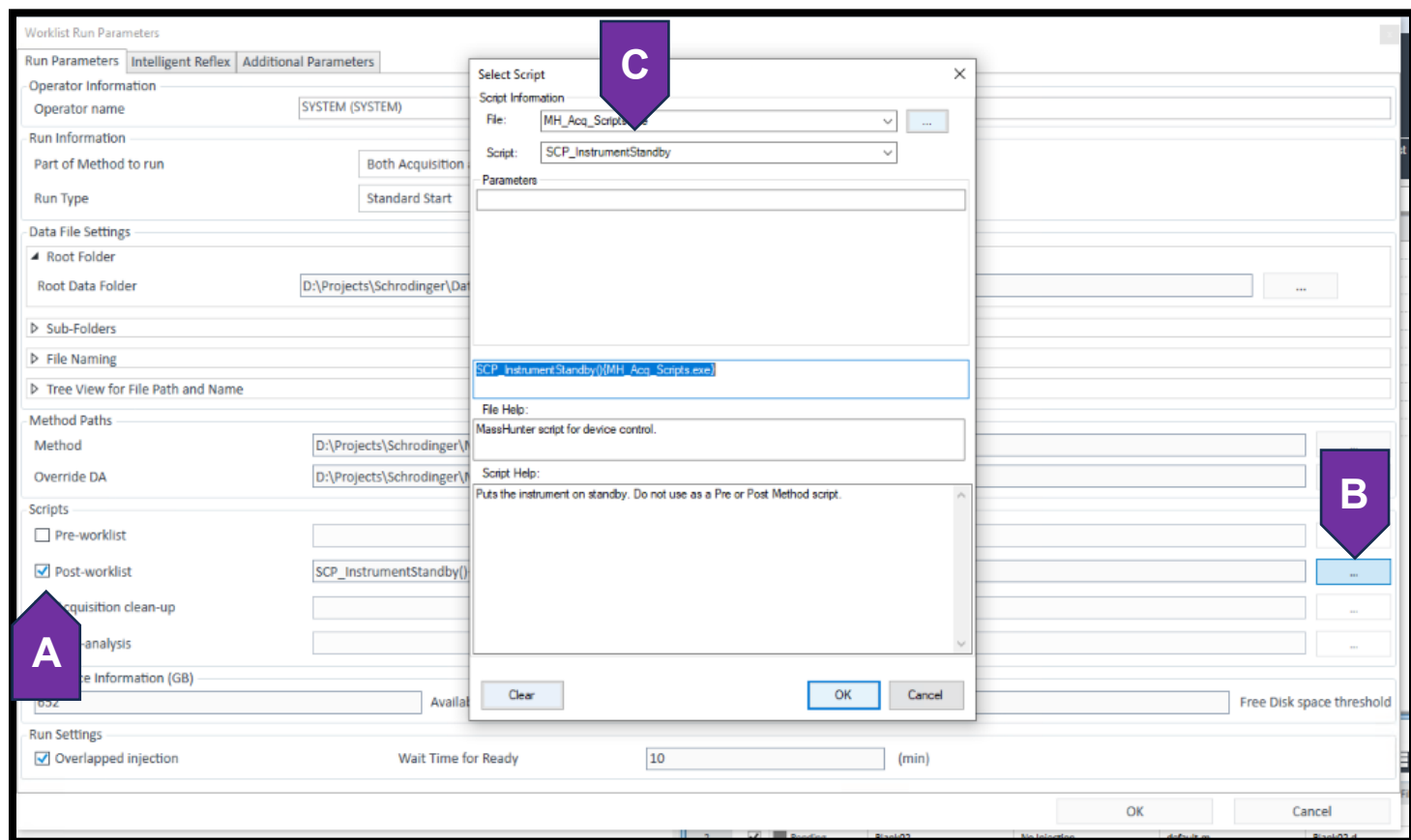
i. Worklist Creation – Setting up the Root Data Folder

1. In the Worklist Panel, there are several ways to fill out the sample queue: (1) load an existing Worklist, which contains the saved entries from when that worklist was created. If necessary, the user can update with new entries to match the sample analysis for the session. Post-analysis scripts will be rolled over from the original saved Worklist.
2. It is possible to start from scratch (see **section III** below). All Worklist files should be saved in the Worklist Folder under your name, with the format **yearmonthday_AnalysisName**.
3. For all sessions, **update the Root Data Folder** with the correct date with the format **yearmonthday_AnalysisName** for each session.
4. If updating the Root Data Folder or starting from scratch, click on the Worklist Run Parameter icon **(A)**. In the pop-up window, change the root data folder by clicking on the ".." icon. **(B)**
5. In the pop-up window, navigate to your data folder and **highlight your data Folder** and click "Create Folder." To create a folder, click on your folder first **(C)**, then click "Create Folder." **(D)** In the example below, Wildcat_Willie is the user folder. The user then clicks "Create Folder" and enters the name using the format **yearmonthday_AnalysisName**. Click **OK** to continue.



LC-TRIPLE QUAD USER MANUAL

6. In the Method Paths section, ensure that the Method folder is directed to your Folder (PILastName\LastName_FirstName). Do not assign a single method in this box.
7. In the Main Worklist Run Parameters window, check the Post-Worklist Script to activate **(A)** and click on the "... " icon. **(B)** In the pop-up Window, make sure **SCP_InstrumentStandby** is chosen. **(C)** Click **OK** to continue.



8. Click **OK** to continue on the Main Worklist Run Parameters window to go back to Acquisition.

ii. Worklist Creation – Creating your Worklist using the IMSERC .csv Template

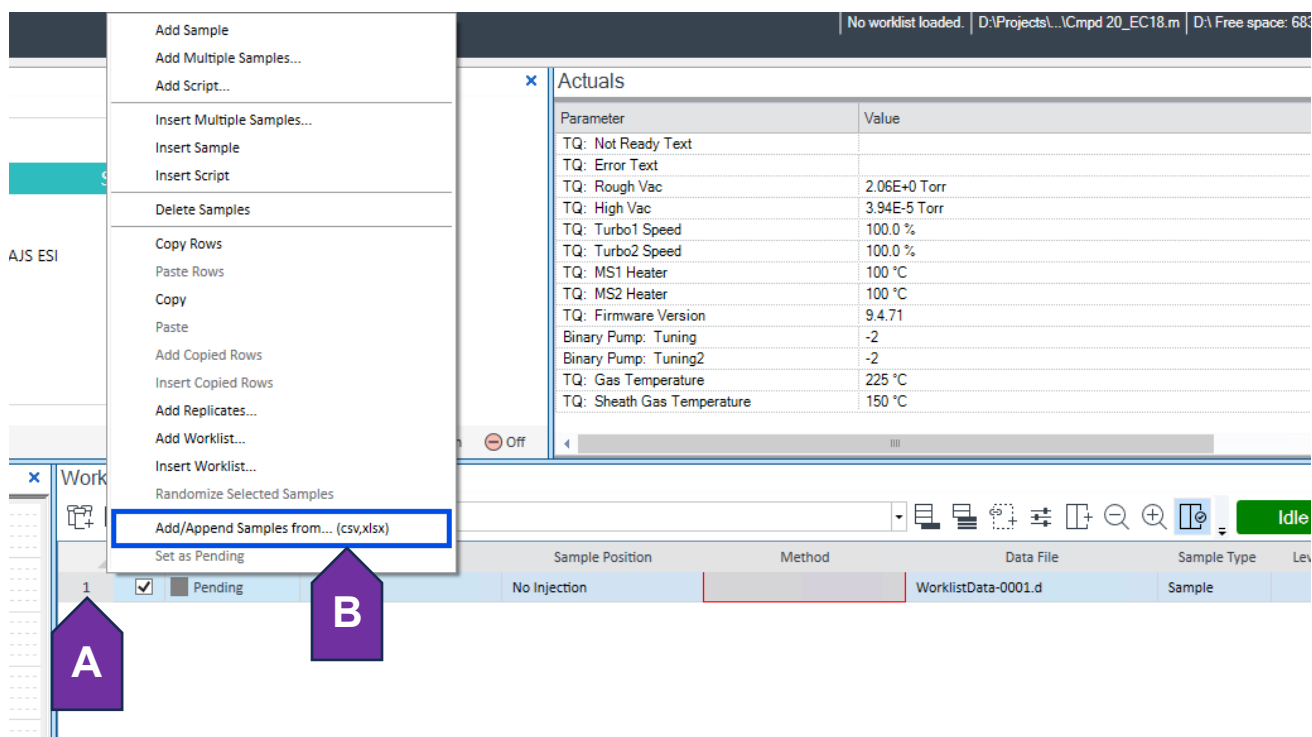
The IMSERC Worklist Template provides specific column headers that the Data Acquisition software will append to the correct Worklist columns.

1. Download the latest .csv Template via the LC-TripleQuad folder in the IMSERC Public Drive. To mount the Public Drive on your personal computer, follow the instructions in **Appendix I. Ensure that the column labels remain intact.**
2. Once you have obtained a copy of the Template, fill out the Template as shown below:
 - a. “Sample Name” should be a unique identification label for each sample you are injecting – CAL, QC, sample1, sample2, Blank, Blank-IS, etc. Each technical injection replicate will have an increment (‘_01’, ‘_02’, etc).
 - b. “Sample Position” is left blank until you are in front of the instrument. As a reference, **Appendix II** shows the map of the Autosampler and each vial tray.
 - c. “Method” is left blank until you are in front of the instrument
 - d. “Data File” will be unique for each acquired sample and is typically denoted as the sample name with an increment if there are multiple replicates. **The Sample Name can be copied and pasted to the Data File column.**
 - e. “Sample Type” options include the following: Blank, DoubleBlank, Cal, QC, Sample, ResponseCheck. Fill out each sample line appropriately (use the exact spacing and capitalization). True blanks are DoubleBlanks, Cal are calibrators, QC are QCs, Sample are study samples, Blank-IS are Blanks, and Equilibration/System suitability samples are ResponseChecks.
 - f. “Level name” applies to CAL or QC samples only. Identify each level of calibrator or QC appropriately (1,2,3,4, LQC, MQC, HQC, etc.)
 - g. “Inj Vol (μL)” should be denoted as “As Method”. Fill this entry all the way down for all your samples.
 - h. Each Sample vial (calibrator, QC, and study samples) should be added at least twice to the Worklist.
3. In addition to your samples (CAL, QC, Sample, etc.), add MS1-Only injections of your Blank sample, which uses the corresponding MS1-Only method for your experiment. The first MS1-only injection will be added after your initial Blanks and before your first calibrator injection. The second is added after you have injected all your samples and before the WASH injection.
4. The last injection will use the WASH method, which is created to wash and flow the appropriate mobile phase through your column. This will enable your column to be stored in the appropriate solvent upon removal.
5. Ensure that you save your Template file in your Group Folder in the Scratch Drive as a “.csv” file. To mount the Scratch Drive on your personal computer, follow the instructions in **Appendix I.**

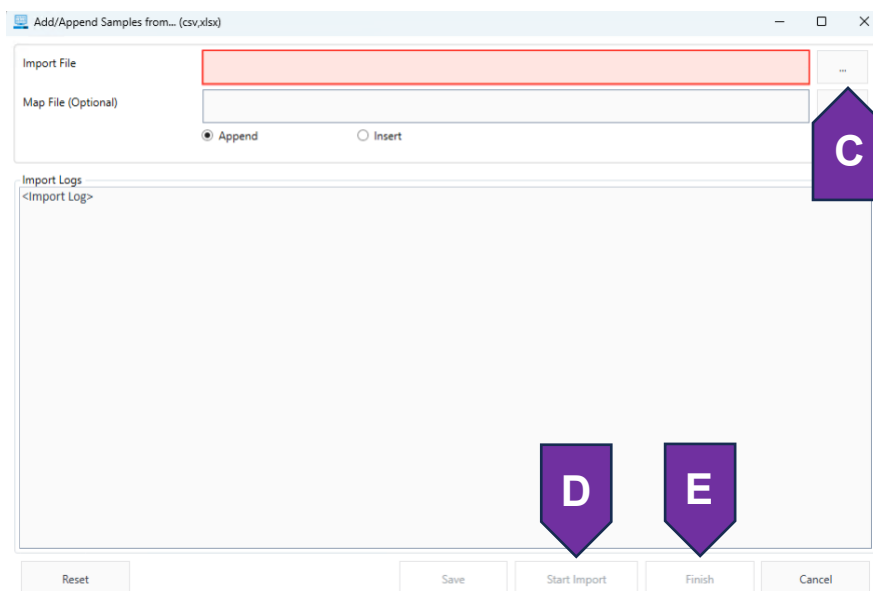
iii. Option 1 - Uploading a .csv/.xlsx Template file

1. Sample Worklists can be built using Microsoft Excel or OpenOffice Calc from a pre-built Template File supplied by IMSERC-MS. Your Worklist can be created on your computer prior to starting your reservation.
2. In order to upload to the instrument computer:
 - a. Mount IMSERC Scratch drive on your personal computer and
3. In the Worklist Panel, right-click on the left-most column where the row numbers are located **(A)** and select “Add/Append Samples from.. (csv,xlsx)” **(B)**.

LC-TRIPLE QUAD USER MANUAL



4. In the pop-up window, add the .csv/.xlsx file in the Import File section (C). Click Start Import (D).



5. Once Import is complete, click "Finish" (E) and validate that your samples are imported into the worklist queue with the appropriate parameters.
6. Once the Worklist has been uploaded, the Method column needs to be filled in. Using the same method, a simple copy-paste can be done for injections.

iv. Option 2 – Starting from Scratch

1. If needed, you can create the Worklist from the Data Acquisition. Follow the guidance in **Section iii** for the appropriate injection order for analysis.
2. **IMPORTANT:** Each Sample Name should be unique in the Worklist, as the entire column will be copied and pasted into the Data File column.
3. The Multisampler Map is available in **Appendix II** to assist in vial position assignments.
4. A general template/order for your Worklist should look something like this:
 - a. Add 2-3 "Solvent" blanks at the beginning of the Worklist.
 - b. Add 1 Injection of the "Solvent" Blank using the MS1-Only Method
 - c. Add 1 "Matrix + internal standard" blank sample.
 - d. Add a "System Suitability" injection(s). This can be a separate sample or the exact vial that is your lowest calibrator sample. This sample is to assess the system detection limit and be compared to the last usage. Retention time and peak intensity should be compared from the last analysis to ensure consistent performance.
 - e. Add your calibration curve (**Cal**) samples, starting with the lowest concentration and moving to the highest.
 - f. Add your quality control (**Q.C.**) samples, starting with the highest concentration and then the lowest.
 - g. Add 2 "No injection" blanks
 - h. For duplicate or triplicate injections, repeat steps b to e.
 - i. Add your samples after the **Cal** and **Q.C.** samples, preferably in a randomized order to minimize bias.
 - j. For **Cal** and **Q.C.** samples, input a label in the **Level Name** column, starting with the lowest concentration and moving to the highest. This will facilitate easy integration with the Quantitative Analysis Software.
 - k. For **Q.C.** sample injections, input "HQC" for the highest Q.C., "MQC" for the middle Q.C., and "LQC" for the lowest Q.C. sample in the Level Name column.
 - l. Add the final "Solvent" Blank using the MS1-Only Method before the Wash Metho
 - m. **IMPORTANT:** Add the WASH method at the end of the Worklist so the column can be stored with the appropriate solvent, ensuring a longer column life.

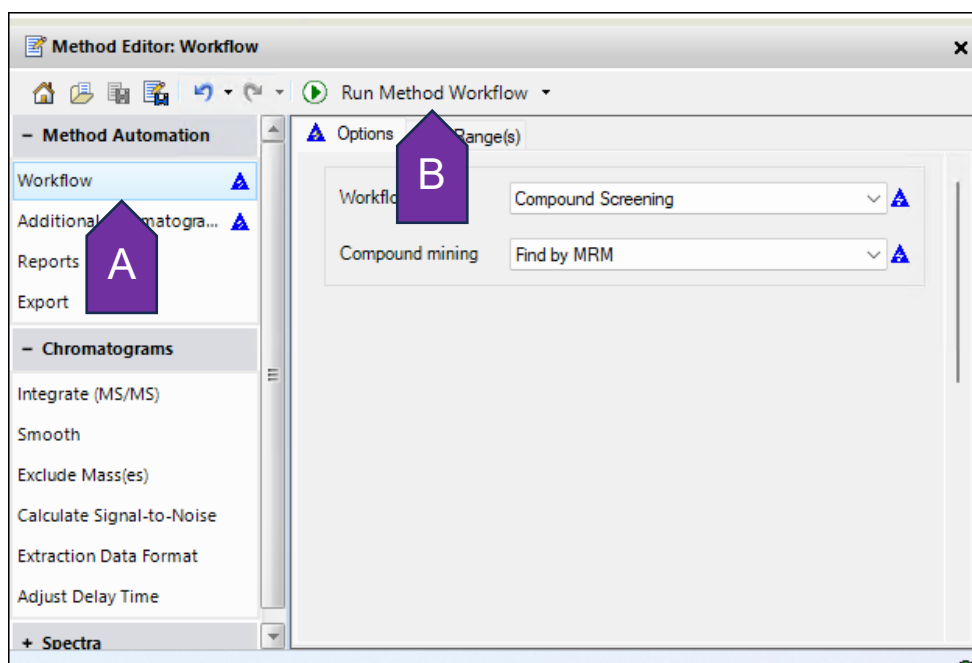
d. Running the Worklist and Post-Analysis Steps

1. Click **Play** to Run your Worklist. If your Worklist has not been saved, click Yes to Save. If a file needs to be saved, enter the name using the format **yearmonthday_AnalysisName** to be saved in your Worklist Folder, located in the (P.I./Worklists/LastName_FirstName) folder.
2. After the analysis is completed, carefully remove your Column from the MCT and close the MCT door carefully, making sure the door "clicks" upon closing.
3. Close the Acquisition Software and choose **NO** on all pop-up windows.
4. In the Control Panel Window, click "Close Connection" and choose YES in the pop-up Window.
5. End your reservation.
6. All files will be available for access in your Group Folder.
7. Data analysis should be done on the data analysis computers on the ground floor of IMSERC or by using the IMSERC terminal. IMSERC terminal access can be requested through the [FAQs section](#) of the IMSERC website.

IX. Opening Files in Qualitative Analysis (to check raw data)

The Qualitative Analysis software enables to look at individual injection files from your Worklist. You can use this check each file(s) for system suitability and method checks.

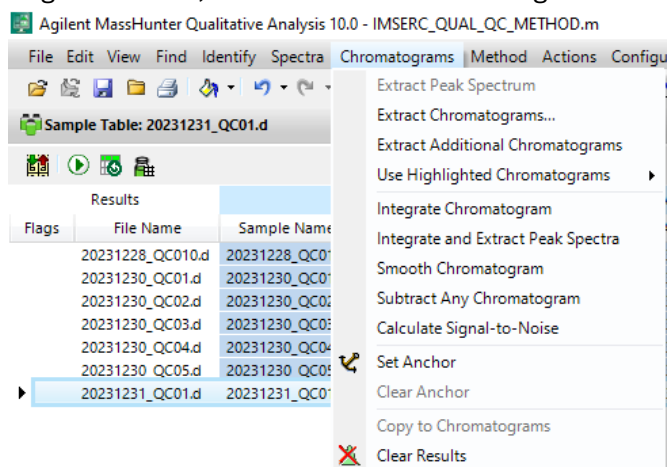
1. From the Desktop, open Qualitative Analysis. A pop-up window will open to sample files.
2. Select the file(s) you wish to open.
3. In The Method Editor Panel, go to Method Automation then Workflow **(A)**, and choose the following settings under the Options Tab:



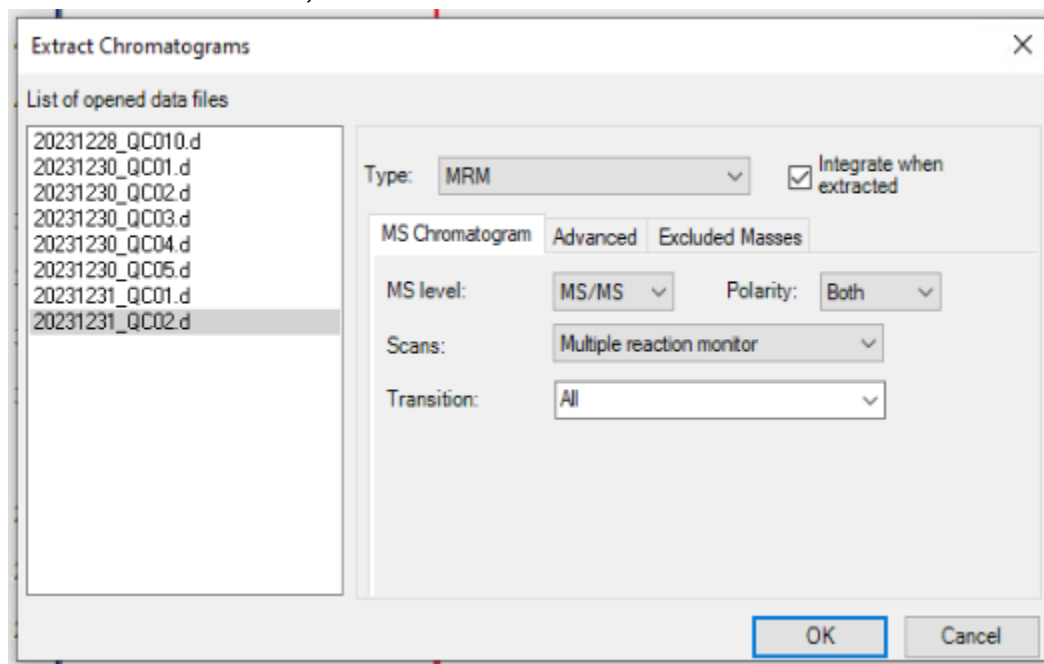
4. Click Run Method Workflow **(B)**. In the pop-up Window, choose which files to "Run Method Workflow."

LC-TRIPLE QUAD USER MANUAL

5. Alternatively, in the Chromatograms menu, choose Extract Chromatograms...



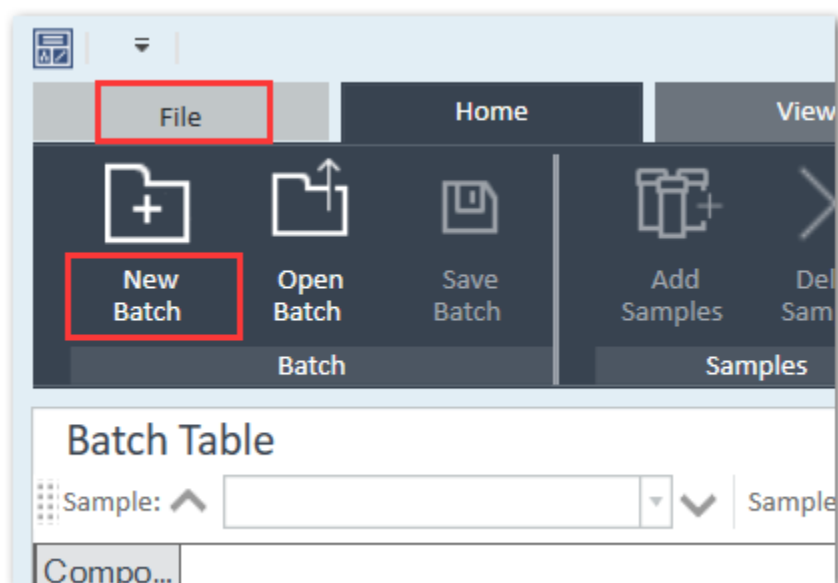
6. In the Extract Chromatograms window, choose which files to extract ion chromatograms (MRM) from and set the parameters as shown below, then click **OK** to continue.



X. Quantitative Analysis – Quantification Using Calibration Curves

The following steps in Quantitative Analysis detail how to set up the Software to quantify samples using a set of calibrator samples to generate the calibration curve. The calibrators and samples should have been analyzed in the same acquisition session to ensure the highest quantification accuracy.

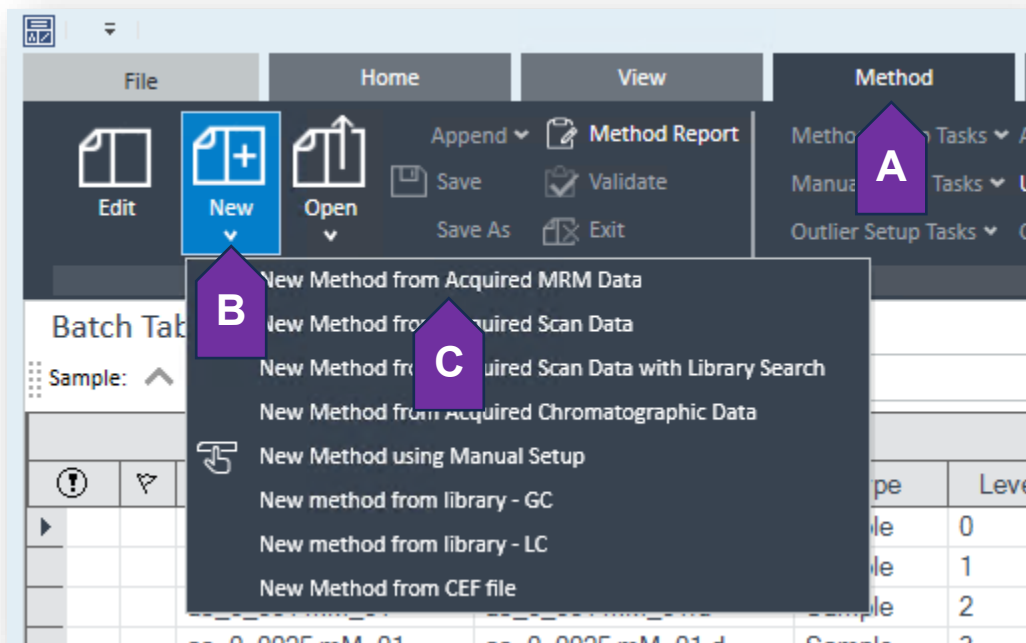
1. Click File or click New Batch, then click Browse to navigate to the folder where you want to create and store the batch file for the results of your analysis. Creating a batch in the same file folder with the original data files avoids having to copy the data files to the new batch folder. This also avoids duplicating data files and saves disk space. Enter a file name for the new batch (Batch files are identified by the batch.bin suffix) and click Create.



2. Quantitative Analysis Software will display a list of all the data files in the batch directory. Click Select All to select all the samples in the list, and click **OK** to continue.

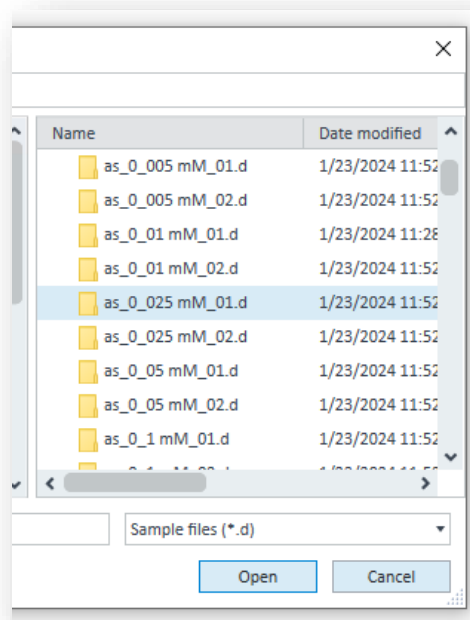
LC-TRIPLE QUAD USER MANUAL

- On the Method Tab **(A)**, Click "New" **(B)** and choose "New Method from Acquired MRM Data" **(C)**.



LC-TRIPLE QUAD USER MANUAL

- Choose a representative .d file from one of the injections for the present analysis. Below, one of the calibrators in the middle range was chosen, which has an abundant amount of the compound(s) and internal standard(s). Click Open.



- Upon clicking Open, the Method Task Window will open.
- MRM Compound Setup.** In this panel, confirm that the internal standard compound(s) have the type "ISTD" or "Surrogate" **(A)**. Choose the appropriate product ion to be the quantifier ion for each compound(s) **(B)**.

Method Tasks

- New / Open Method
- Workflow
- Method Setup Tasks
 - MRM Compound Setup**
 - Retention Time Setup
 - ISTD Setup
 - Concentration Setup
 - Qualifier Setup
 - Calibration Curve Setup
- Globals Setup
- Save / Exit
 - Validate
 - Save
 - Save As...
 - Exit
- Manual Setup Tasks
- Outlier Setup Tasks
- Advanced Tasks

Method Table

Time Segment: < All >
Compound: Melatonin
Reset Table View

Sample		Quantifier									
Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time						
Name	TS	Transition	Scan	Type	Precursor Ion	Product Ion	RT	Ion Polarity	Collision Energy	Collision Energy Delta	Accuracy Max. % Dev.
I-CC-1-4.d	I-CC-1-4.d										
Melatonin	1	233.1 -> 174.0	MRM	Target	233.1	174.0	7.087	Positive	12.0	0.1	20.0
Melatonin-d4	1	237.1 -> 178.0	MRM	ISTD	237.1	174.0	7.044	Positive	12.0	0.1	20.0

A

B

LC-TRIPLE QUAD USER MANUAL

7. **Retention Time Setup.** Confirm that the compound(s), including the internal standard(s), have the correct retention time (R.T.) **(A)**. If needed, this is the Window to narrow down the retention time window for each compound **(B)**.

Method Tasks

- ▶ New / Open Method
- ▶ Workflow
- ▶ Method Setup Tasks
 - MRM Compound Setup
 - Retention Time Setup**
 - ISTD Setup
 - Concentration Setup
 - Qualifier Setup
 - Calibration Curve Setup
- Globals Setup
- Save / Exit
 - Validate
 - Save
 - Save As...
 - Exit
- ▶ Manual Setup Tasks
- ▶ Outlier Setup Tasks
- ▶ Advanced Tasks

Method Table

Time Segment: <All> Compound: Melatonin-1 Reset Table View

Sample								
Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time			
I-CC-1-4.d	I-CC-1-4.d							
Quantifier								
Name	TS	Transition	Scan	Type	RT	Left RT Delta	Right RT Delta	RT Delta Units
Melatonin	1	233.1 -> 174.0	MRM	Target	7.087	1.000	1.000	Minutes
▶ Melatonin-d4	1	237.1 -> 178.0	MRM	ISTD	7.044	1.000	1.000	Minutes

A **B**

LC-TRIPLE QUAD USER MANUAL

8. **ISTD Setup.** For the target Compound(s) being quantified, choose the appropriate ISTD Compound Name in the drop-down list **(A)** for each compound. The internal standard compound should have <None>. Confirm that the internal standard(s) has the ISTD Flag checked **(B)** and that the concentration(s) values have the correct concentration typed into the ISTD Conc. box **(C)**.

Method Tasks

New / Open Method

Workflow

Method Setup Tasks

MRM Compound Setup

Retention Time Setup

ISTD Setup

Concentration Setup

Qualifier Setup

Calibration Curve Setup

Globals Setup

Save / Exit

Validate

Save

Save As...

Exit

Manual Setup Tasks

Outlier Setup Tasks

Advanced Tasks

Method Table

Time Segment: < All > Compound: Melatonin Reset Table View

Sample									
Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time				
I-CC-1-4.d	I-CC-1-4.d								
Quantifier									
Name	TS	Transition	Scan	Type	ISTD Compound Name	ISTD Flag	ISTD Conc.	Time Reference Flag	
Melatonin	1	233.1 -> 174.0	MRM	Target	<None>	☐		☐	
Melatonin-d4	1	237.1 -> 178.0	MRM	ISTD	<None>	☒	250.0000	☐	

A

B

C

LC-TRIPLE QUAD USER MANUAL

9. **Concentration Setup.** Under the Units column, choose the appropriate units for concentration **(A)**. The default settings should be appropriate for most studies. Go to the Calibration Curve drop-down menu and choose "Create Levels from Calibration Samples **(B)**". This will create a line for each "CAL" sample imported in Steps 1 – 3. For each Level, input the appropriate concentration (pre-dilution or post-dilution) for each compound being quantified in every calibrator sample **(C)**. The Software will also show the internal standards concentration for each Sample. There is no need to change this since the concentration should be the same.

The screenshot shows the software interface with the 'Method Setup Tasks' menu open. The 'Calibration Curve' option is highlighted. The 'Method Table' is visible, showing a sample named 'as_0_025 mM_0...' and a quantifier table. The quantifier table has columns for Name, TS, Transition, Scan, Type, Units, and Accuracy Max. % Dev. The 'Units' column for '3-Hydroxybutyric acid' is highlighted with a purple arrow labeled 'A'.

Name	TS	Transition	Scan	Type	Units	Accuracy Max. % Dev.
3-Hydroxybutyric acid	1	103.0 -> 59.0	MRM	Target	ng/ml	20.0
3-Hydroxybutyric acid-d5	1	107.1 -> 58.9	MRM	ISTD	ng/ml	20.0

Quantifier							
Name	TS	Transition	Scan	Type	CF	CF Origin	CF Weight
Melatonin	1	233.1 -> 174.0	MRM	Target	Linear	Ignore	None
Calibration							
Level	Δ	Conc.	Response				
1		1.0000					
1		1.0000					
2		2.0000					
2		2.0000					
3		3.0000					
3		3.0000					
4		4.0000					
4		4.0000					
5		5.0000					
5		5.0000					
6		6.0000					
6		6.0000					
7		7.0000					
7		7.0000					
H		8.0000					
H		8.0000					
L		10.0000					
L		10.0000					
M		9.0000					
M		9.0000					

LC-TRIPLE QUAD USER MANUAL

10. **Qualifier Setup.** If applicable, each compound will have a corresponding qualifier ion. Confirm that your target compounds have the correct ion, which should differ from the quantifier ion (**A**). The default settings should be appropriate for most studies. If false-positive I.D.s occur, it is recommended to tighten up Uncertainty values.

Method Tasks

New / Open Method

Workflow

Method Setup Tasks

MRM Compound Setup

Retention Time Setup

ISTD Setup

Concentration Setup

Qualifier Setup

Calibration Curve Setup

Globals Setup

Save / Exit

Validate

Save

Save As...

Exit

Manual Setup Tasks

Outlier Setup Tasks

Advanced Tasks

Method Table

Time Segment: < All >

Compound: < >

Reset Table View

Sample								
Name	Data File	Type	Level	Acq. Method File	Acq. Date-Time			
I-CC-2-4.d	I-CC-2-4.d							
Quantifier								
Name	TS	Transition	Scan	Type	Precursor Ion	Product Ion	Uncertainty	
Melatonin	1	233.1 -> 174.0	MRM	Target	233.1	174.0	Relative	
Qualifier								
Precursor Ion	Product Ion	Transition	Rel. Resp.	Uncertainty	Δ	Area Sum		
233.1	77.0	233.1 -> 77.0	22.9	20.0				
Quantifier								
Name	TS	Tr	Scan	Type	Precursor Ion	Product Ion	Uncertainty	
MelatoninD4	1	237.1	MRM	ISTD	237.1	178.0	Relative	

A

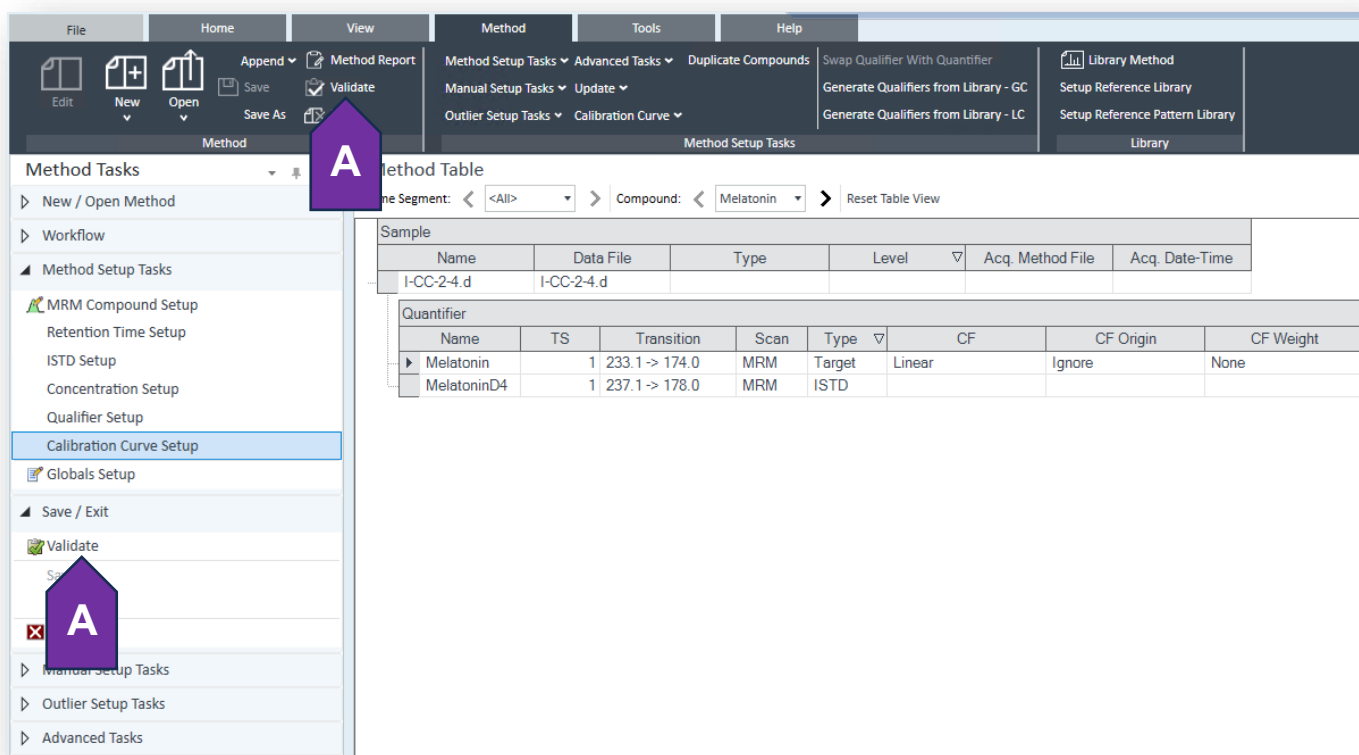
LC-TRIPLE QUAD USER MANUAL

11. **Calibration Curve Setup.** Choose Linear (for now) as the C.F., and 1/x as the C.F. Weight for each compound. You may have to go back to this menu afterward to change the C.F. or C.F. Weight if the R² value of the regression line is not close to linearity.

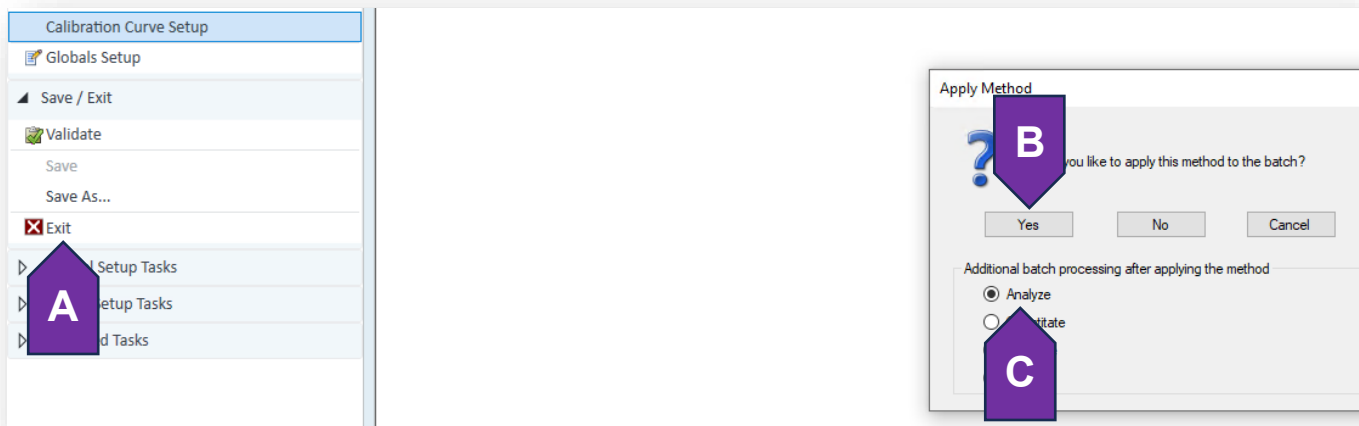
Quantifier							
Name	TS	Transition	Scan	Type ▾	CF	CF Origin	CF Weight
Melatonin	1	233.1 -> 174.0	MRM	Target	Linear	Ignore	None
Calibration							
Level	Δ	Conc.	Response				
▶ 1		1.0000					
1		1.0000					
2		2.0000					
2		2.0000					
3		3.0000					
3		3.0000					
4		4.0000					
4		4.0000					
5		5.0000					
5		5.0000					
6		6.0000					
6		6.0000					
7		7.0000					
7		7.0000					
H		8.0000					
H		8.0000					
L		10.0000					
L		10.0000					
M		9.0000					
M		9.0000					

12. Click Validate to confirm the Method settings **(A)** – no errors should be found. If there are errors, each issue will be highlighted at the bottom of the Window.

LC-TRIPLE QUAD USER MANUAL



13. Click Exit (A) and click Yes (B) and Analyze (C) in the pop-up Window.



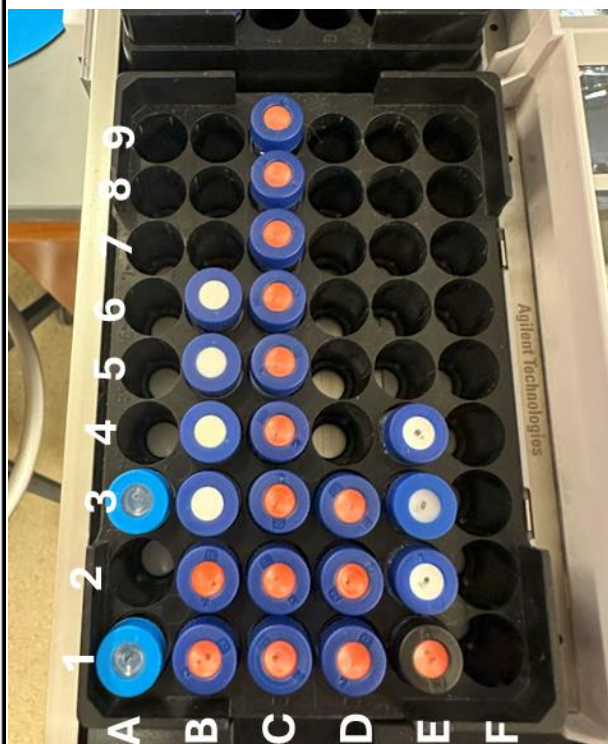
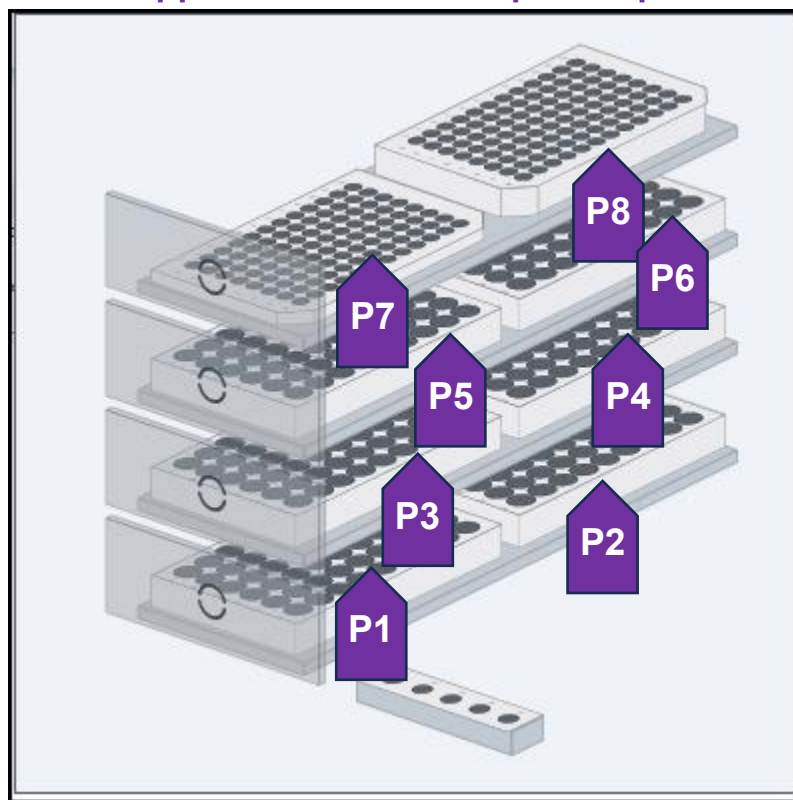
14. The Batch Table will now be populated with results. Below are some of a few parameters to check.
- Assess the accuracy of the concentration of your calibrator and Q.C. samples to determine the usefulness of the current assay. Acceptable values for accuracy should $\pm 10\%$.
 - If the lower calibrators or Q.C. do not show a value, it may be below the detection limit and may have to be excluded from the analysis.

- c. Assess the Curve Fit and C.F. weight of the calibration curve by comparing the R^2 value of each C.F. type.

XI. Appendix 1 – Obtaining the Template File from the IMSERC Public Drive and Uploading in the Scratch Drive

1. On a **Windows PC**, map a Network drive via the “This PC” window. Upon clicking, map '\\imsercdata.northwestern.edu\public' (without apostrophes). This is a read-only Drive meant for accessing software and Template files provided by IMSERC.
2. On a **Mac**, and from the Mac OS X Finder, hit Command+K to bring up the 'Connect to Server' window. Connect to the 'imsercdata.northwestern.edu' server, i.e., 'smb://imsercdata.northwestern.edu/public' (without apostrophes). This is a read-only Drive meant for accessing software and Template files provided by IMSERC.
3. Once the drive has been mounted, go to the folder LC_TripleQuad. The latest template is located in this folder.
4. Fill out the Template as instructed and upload it to your Group's corresponding folder in the Scratch drive. If your Group does not have a folder in the Scratch drive, create one with the name 'PILastName-working'.
5. To mount the Scratch drive in your personal computer, map/connect using the same location as you did to access the Public Drive, but replace 'public' with 'scratch'.

XII. Appendix 2 – Multisampler Map



Note: P1 through P6 can hold autosampler vials only, while P7 can hold short 96-well plates and P8 can hold tall 96-well plates. Autosampler vials and 96-well plates must be IMSERC-approved before being used.

XIII. Version History

1.0: Initial version 1/24/2024

2.0: Vastly improved Data Acquisition Steps to incorporate the UHPLC + external SSV upgrades. 7/5/2024

2.1: Edited Worklist creation after User feedback and "Find By MRM" Workflow for Qualitative Analysis. 8/29/2024

2.2: Expanded Worklist Creation section and added instructions to access the Worklist Template File 12/02/2024