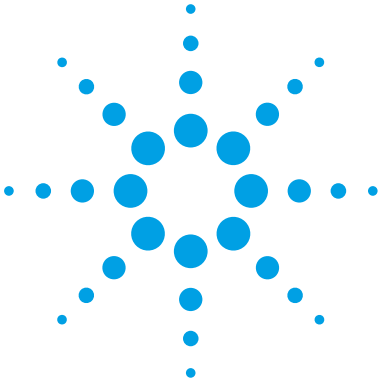


MassHunter Metabolomics dMRM Database and Method

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This guide provides instructions to prepare samples; prepare, maintain and clean instruments; and perform an example data analysis workflow using the Metabolomics Dynamic MRM Database and Method. Downstream data analysis can be done with MassHunter Quantitative Analysis, MassHunter Qualitative Analysis, and the Mass Profiler Professional software suite, including the Pathway Architect module.

Targeted analysis of metabolites using ion-pairing reverse phase chromatography

Metabolomics is a growing analytical field that characterizes the biochemical contents and reactions of the cell. The determination of metabolic state requires separation and measurement of a diverse set of small molecules.

The analytes covered by the provided method are common to a wide range of biological systems that extend from industrial and pathogenic bacteria and yeast to plant and animal cells. Details of metabolite sample preparation are necessarily experiment-specific. This guide serves as a starting point for tissue cell culture and microbial cell suspensions.

Before You Begin

Make sure you have these tools and consumables.

Sample Preparation Tools and Reagents

Equipment

- centrifuge
- vacuum concentrator
- nitrogen gas

Parts

- 15-mL polypropylene conical centrifuge tubes
- 5-mL microcentrifuge tubes
- 1.5-mL microcentrifuge tubes
- Agilent autosampler vials
- nylon 0.2 µm, 0.45-µm, and 1-µm supported filters

Reagents

- LCMS-grade methanol
- GC-grade (>=99%) tributylamine
- HPLC-grade (or higher) glacial acetic acid
- HPLC-grade water
- LCMS-grade acetonitrile

Sample Preparation

This chapter describes the steps to prepare your samples prior to data acquisition and analysis to be used with the MassHunter Metabolomics dMRM Database and Method.

This protocol describes two alternative ways to extract metabolites: one from cell suspensions and one from adherent cells.

To ensure complete recovery of metabolites, *use speed and efficiency* when you extract cells, remove cells from the growth medium, and quench and lyse the cells.

Step 1a. Extract polar metabolites from cell suspensions

This topic describes how to harvest cells grown in suspension by filtration, followed by a rapid extraction with cold extraction solvent.

The cold extraction solvent simultaneously lyses the cells and quenches metabolism.

CAUTION Do not wash cells. Even a rapid PBS dispense and aspiration step can result in significant changes in metabolic profile.

1. Chill aliquots of extraction solvent (80:20 LCMS-grade acetonitrile:HPLC-grade water or 40:40:20 LCMS-grade acetonitrile:LCMS-grade methanol:HPLC-grade water) in polypropylene tubes at -20°C for at least two hours or overnight.
2. Prepare a filter holder/manifold for 25-mm filter disks (such as a Hoefer FH225V) with one nylon filter membrane for each sample to be collected.
   - For microbial samples, use a 0.45-µm filter.
   - For yeast and mammalian cultures, use 1-µm filter.
3. At intervals appropriate for your experiment, remove culture from incubator and transfer an aliquot of sample into the filtration apparatus.

   Amount of culture and number of cells needed will vary based on many parameters, and should be optimized.

NOTE For actively growing microbial and eukaryotic cell cultures, 10 mL is generally sufficient for multiple injections.

4 Apply vacuum to begin filtration.

5 After the media completely passes through the filter, discontinue filtration and immediately proceed to the next step.

CAUTION Cells starve rapidly, so in the next step, you must quickly transfer the cells to solvent for quenching and extraction.

6 Remove the filter with collected cells and immediately immerse in chilled extraction solvent. Use a vortex mixer to disperse cells into extraction solvent.

7 Freeze/thaw samples by cycling between -80°C and -20°C for a total of three freeze-thaw cycles.

Samples are stable at -80°C until further processing.

Step 1b. Extract polar metabolites from adherent cells

For adherent tissue culture cells, extraction solvent can be added directly to cells immediately after the aspiration of growth medium.

CAUTION Do not wash cells. Even a rapid PBS dispense and aspiration step can result in significant changes in metabolic profile.

1. Chill extraction solvent (80:20 LCMS-grade acetonitrile:HPLC-grade water) at -20°C for at least two hours or overnight. Place dry ice in a large container suitable for holding tissue culture plates during processing.
2. Remove culture vessel from incubator and do the next steps in an appropriate biosafety cabinet.

   For most cell lines, one well of a >50% confluent six-well cluster provides sufficient material for multiple analyses. You can scale up to 10-cm or 15-cm dishes as required to generate large amounts of material for repeated analysis.
3. Use vacuum aspiration to remove culture media from monolayer of cells. Quickly remove culture media, but do not let monolayer dry out.

   Work on a single extraction at a time. Quickly remove culture media and proceed to the next steps. Delays in processing can cause cells dry out or starve, which can adversely affect results.
4. Use a pipette to immediately transfer extraction solvent onto aspirated monolayer. Place plate on dry ice.

   A solid metal plate can be put on top of the dry ice to provide a flat surface and enable rapid heat transfer.
5. Repeatedly draw cell mixture into and out of the pipette to fully detach cells from plate. Transfer cells and solvent to a fresh polypropylene tube, store at -80°C.
6. Collect cells from a parallel sample for counting and normalization.

Step 2. Measure quantity of input cells

Do this step to enable quantitative comparisons between samples and improve reproducibility. Collect an equal amount of cells from a parallel sample for sample normalization.

- Use one of these processes to measure cellular input.
  - Direct Coulter sizing:

    Coulter measurement directly measures cell volume and number, and is the gold standard for determination of cell size.
  - Packed cell volume:

    Measurement of packed cell volume using calibrated centrifuge tubes can provide a population average and can be sufficient where sample input is not limiting.
  - Quantification of total or specific protein concentrations:

    Determination of total protein content in a sample, by BCA or Bradford assay, can provide measurements that are relatively well-correlated with total integrated cell volume, but are confounded by proteins present in serum. Sample-specific proteins can be quantitated by ELISA, Western blots or similar methods. Well-chosen antigens will vary in abundance as a function of cell volume and not other confounding factors.
  - Simple cell counting:

    Cell counts using a hemocytometer are inexpensive and rapid, but fail to account for changes in cell size.
  - Bulk sample weighing:

    Bulk samples such as tissue biopsies can be assessed by weight in addition to the other methods described in this topic.

Step 3. Process harvested cells for LC/MS analysis

For the ion-paired reverse-phase method described here, extracts are clarified to remove protein, dried, and resuspended in aqueous buffer for injection in water according to the quantity of cells in each sample.

1 Remove extracts from -80°C and thaw (if applicable) at -20°C.

2 Remove filters (if applicable) and spin in a bench-top centrifuge at maximum permissible speed to clarify metabolite extract and turn insoluble material into pellets.

   In general:

   - Spin 15-mL polypropylene conical centrifuge tubes in a swinging bucket rotor up to 4,000 × g for 15 minutes.
   - Spin 5-mL microcentrifuge tubes in a specialized centrifuge rotor up to 12,000 × g for 5 minutes.

3 Carefully transfer 80% of the supernatant (extract) to a clean fresh 1.5-mL tube.

   Do not disturb the insoluble pellet. The 20% of extract left in the original tube allows you to avoid cross-contamination with pellet material.

4 Evaporate the extract to dryness under pure nitrogen gas at room temperature.

   You can also use a vacuum concentrator, such as a Speedvac.

   Labile and volatile compounds can be lost during vacuum concentration. Use no heat during evaporation, and test for adequate compound recovery on limited samples before use.

5 After evaporation, resuspend extracts in HPLC-grade water that contains internal standards and spike-in controls, if any.

   Use sample normalization to guide volume for resuspension. See "Step 2. Measure quantity of input cells" on page 7.

   If the sample is from adipose tissue, you will need to add organic solvents, such as acetonitrile, to the resolubilization solution. Adipose tissue extract does not dissolve in water, and sediments can cause high back pressure in the column or clog the column. But too much organic solvent can cause metabolites not to bind to the column. Use care when optimizing the resolubilization solvent for adipose tissues.

6 Filter resuspended extract through a hydrophilic polypropylene membrane to remove any solids before LC/MS analysis. Use 0.22-µm or 0.45-µm filters to remove particulate matter.

- For small numbers of samples, you can use centrigual filters..
- For routine processing, use 96-well filter plates. 96-well plates can be rapidly processed using a vacuum manifold.

7 Immediately transfer filtrate to autosampler vials or plates.

Samples can be stored at -80°C until analysis.

Data Analysis

This chapter introduces data analysis software and describes a workflow with an example data set acquired with the MassHunter Metabolomics dMRM Database and Method.

For optimal results, make sure that you keep your system clean of contaminants. See "Cleaning and preparing the LC and LC/MS instruments" on page 17.

Step 1. Acquire LC/MS data

- Use the MassHunter Data Acquisition program to acquire data from prepared biological samples. Use the included method or one based on an included method. Refer to the *Method Setup Guide*.

Step 2. Run downstream analysis

- Data sets acquired with the Metabolomics dMRM Method can be analyzed with one or a combination of the following software programs:
  - MassHunter Qualitative Analysis
  - MassHunter Quantitative Analysis
  - MassHunter Mass Profiler Professional with Pathway Architect

MassHunter Qualitative Analysis is useful to quickly evaluate data integrity when running the **Find by MRM** feature.

MassHunter Quantitative Analysis allows the simultaneous analysis of many data files and is useful for analyzing the data from typical projects. MassHunter Quantitative Analysis includes tools for inspecting data, for editing peak integration, and provides the capability of automatically calculating the relative or absolute concentration of metabolites in a sample by comparing biological sample data with data containing known quantities of metabolites.

MassHunter Quantitative Analysis is also used for more in-depth analyses of multiple data files (batch analysis). In addition, Quantitative Analysis methods provide compound identifiers for immediate data analysis and pathway visualization in Mass Profiler Professional and Pathway Architect.

See “Example Workflow” on page 13 and the software user guides for these programs for further details.

To determine metabolite concentration

In general, comparison among similar biological samples allows identification of relative changes in metabolite concentration between samples.

This relative quantitation is sufficient for many metabolomic studies. In situations where absolute quantitation (determination of concentration of compounds in molar or mass terms) is required, additional experiments are required, as described here:

- Prepare a series of spike-in controls containing increasing amounts of target compound spiked into biological sample. Use these spike-in samples to determine instrument response as a function of concentration added, and then determine the amount of compound present in the original sample by measuring the intercept point where no added compound -- only endogenous levels -- are present. Care must be taken to avoid signal saturation or analysis beyond the instrument linear range.

  The efficiency of electrospray ionization, used by this method, is affected by the presence of co-eluting compounds. These compounds can compete for or enhance ionization and affect the resulting signal of targeted metabolites. Consequently, the same concentration of a compound can give a different signal when the compound is part of a biological sample than when the compound is present as a single, pure compound.

Example Workflow

The steps in this topic are used to perform a typical data analysis workflow designed for data sets generated from the Agilent Metabolomics dMRM Database and Method. The workflow enables multivariate differential analysis and pathway visualization based on relative compound abundances, without the use of chemical standards in the experimental design.

Step 1. Create a Quantitative Analysis Batch

1 Open the Quantitative Analysis program.
   - Click **Start > All Programs > Agilent > MassHunter Workstation > Quantitative Analysis (QQQ)**.
   - Click the QQQ Quantitative Analysis icon ().

2 Create a new batch.

3 Add samples for the dMRM data files of interest.

4 Click **Method > Open > Open method from existing file**.

5 Select **Metabolomics dMRM quant method.m**, the quantitative analysis method that was provided with the installation disk.

6 In the Method Tasks window click **MRM Compound Setup** in the Method Setup Tasks section.

7 In the Method Table right-click and click **Add/Remove Columns**.

8 Add the columns **CAS#** and **User Annotation** if not already visible. Click **OK**.

   Note that comments in the **User Annotation** column provide information regarding closely resolved isomers, compounds that are not distinguished, and transitions where crosstalk is observed. Further compound information may be found in the Reference section of the Method Setup Guide.

9 Retention times can be adjusted for compounds using qualifier co-elution and **User Annotation** notes, if available, as guidance.

10 Compounds with low or zero abundance can be deleted from the method or filtered out later with MPP software.

11 If changes are made to the method, save the method as a new method name. Click **Method > Save As**. Enter a new method name and click **Save**. If the method is accidentally overwritten, the original quant method can be recovered from the installation disk.

12 Click **Method > Exit**. Click **Yes** to apply the method to the batch.

13 Click **Analyze Batch**.

14 Review the batch and edit or manually integrate as appropriate. The Compounds-at-a-Glance window is helpful to quickly review large numbers of compounds and samples.

15 Save the batch.

Step 2. Create a Quantitative Analysis Report

A Quantitative Analysis Report is required to serve as input for MPP. Compound Names, CAS #s, and compound abundances (height/area) are critical information required for the report. The following steps produce a .pdf report and an .xml file, with the latter serving as the input file for MPP.

1 Click **Report > Generate**. The **Generate Report** dialog box opens.

2 Note the **Report folder** which is the location where the report is saved.

3 Under Report method, click **New**.

4 Click **Add Template**.

5 Go to the folder **MassHunter\Report Templates\Quant\PDF-Reporting**. Select **Gen_Complete.report.xml**. Then, click **Open**.

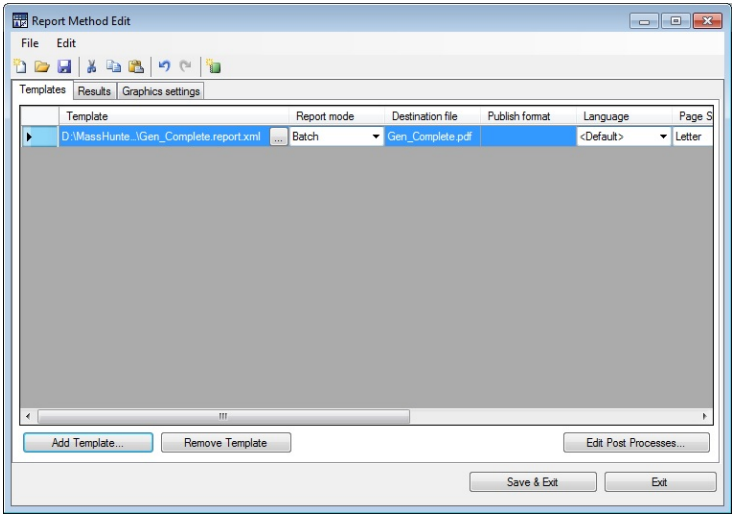


Figure 1 Template tab in the Report Method Edit dialog box

6 Click the **Results** tab. Verify that **QQQ** is selected as the **Instrument type**. Click **Yes - Always generate results file**.

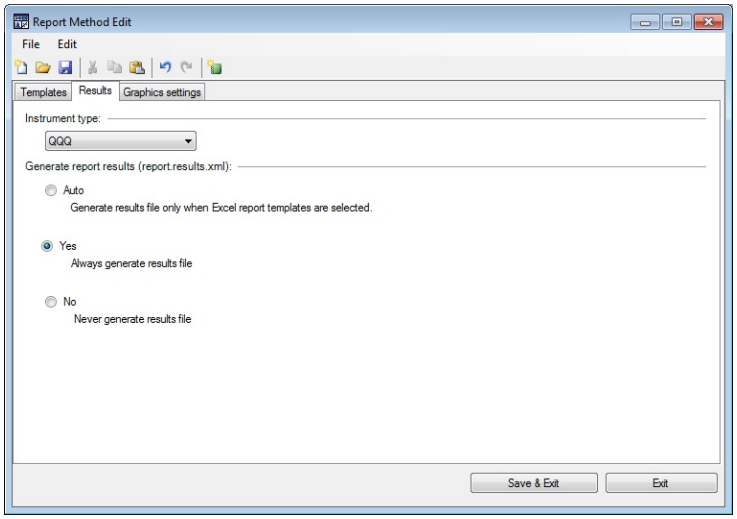


Figure 2 Results tab in the Report Method Edit dialog box

7 Click **Save & Exit**. Save the method to an appropriate location.

8 Click **Choose Samples** or **Choose Compounds** if a subset of samples or compounds is desired for the report.

9 Click **OK** to generate a report. It is recommended to generate the report with the **Queue report task** option (selected by default). Mark the **Start Queue Viewer** check box.

10 The Task Queue Viewer program opens in the bottom right on the taskbar of Windows. If necessary, click the program to open and view progress. Once the **Status** column is set to **Done**, you can open and review the .pdf quant report from the saved location.

Step 3. Import Data into Mass Profiler Professional (MPP)

This section describes only the critical steps to bring in the reviewed data from Quantitative Analysis into the Mass Profiler Professional (MPP) software program. Please refer to MPP User Guide and the *Integrated Biology with Mass Profiler Professional - Workflow Guide* for further details in setting up differential and pathway analyses.

1. Open Mass Profiler Professional.
2. Click **Create new project** and click **OK**.
3. Type the **Project name** and **Notes**, and click **OK**.
4. Click **Create new experiment**, and click **OK**.
5. Type an appropriate **Experiment name**.
6. For Analysis type, select **Mass Profiler Professional**.
7. For Experiment type, select **Identified**.
8. For Workflow type select either **Analysis: Significance Testing and Fold Change** or **Data Import Wizard**, and click **OK**.
9. On the **MS Experiment Creation Wizard (Step 1 of 12)** page select the data source. Click **MassHunter Quant**. Select the **Organism** of interest. Click **Next**.
10. On the **MS Experiment Creation Wizard (Step 2 of 12)** page click **Select Data Files**. Navigate to the location where you saved the Quantitative Analysis report, and choose the file **report.results.xml**. Click **Open**. Click **Next**.
11. On the **MS Experiment Creation Wizard (Step 4 of 12)** page click either **Height** or **Area**.
12. Create the MPP experiment as appropriate. Many of the settings and parameters will depend on the specifics of your experimental design. Note that CAS# contained in the imported **report.results.xml** file will serve as pathway identifiers when creating experiments with the Pathway Analysis workflow.

    Please refer to the MPP User Guide and the *Integrated Biology with Mass Profiler Professional - Workflow Guide* for further details in setting up differential and pathway analyses.

Cleaning and preparing the LC and LC/MS instruments

The system needs to be clean of impurities and contaminants to run metabolomics analysis. Make sure you follow the cleaning steps in this guide to keep your system optimized for analysis.

To passivate the LC system

Do these steps to improve chromatographic performance for phospho-containing metabolites.

CAUTION This passivation step involves the use of acidic solvents. Acidic conditions can significantly impact negative ionization. If negative ionization is needed after passivation, thoroughly flush the LC system after passivation to neutralize the pH.

Before you begin, make sure you have:

- Agilent LC/MS Cleaning Solution
- HPLC-grade water
- HPLC-grade isopropanol
- concentrated (65%) nitric acid
- pH paper for range 4.0-7.0
- clean 250-mL beaker
- frit for the outlet filter (p/n 5067-5716)
- 25% aqueous ammonia
- Restriction Capillary (p/n 5022-2159)

Prepare the LC system

- Disconnect the tubing to the LC/MS and place the PEEK tubing in a safe waste container.
- Replace the column with a zero-dead-volume union and Restriction Capillary.
- Put the autosampler in mainpass mode.
- Set the 2-position/6-port MCT valve to forward-flush mode.
- Clean the LC system if the system has never been used. Use the Agilent LC/MS Cleaning Solution. Refer to the 6400 Series LC/MS *Installation Guide* for more details.
- Connect all four solvent lines directly to the solvent selection valve to bypass the degasser in the 1290 pump.

CAUTION Failure to bypass the degasser during the passivation procedure can lead to damage.

Passivation Solution

1 Dilute the concentrated (65%) nitric acid in an equal volume of HPLC-grade water to create the **Passivation Solution** (32.5% nitric acid).

Ammonia Solution

1 Dilute 60 mL 25% aqueous ammonia in 40 mL of water to create **15% Ammonia Solution.**

Passivation for New Systems

CAUTION Failure to bypass the degasser during the passivation procedure can lead to damage.

1 Condition the LC instrument. Refer to the LC/MS system *Installation Guide*.

2 Put the solvent inline filters for all 4 channels into a clean 250-mL beaker that contains HPLC-grade isopropanol.

3 Set the pump to 25% for each channel and a flow rate of 0.5 mL/minute.

4 Purge the pump (all 4 channels) for 5 minutes at 5 mL/minute (1.25 mL/minute per channel).

5 Allow the pump to run HPLC-grade isopropanol for 10 minutes (25% of each channel).

6 Repeat step 4 and step 5 but with a clean 250-mL beaker of HPLC-grade water.

7 Repeat step 4 but with the Passivation Solution.

8 Change the flow rate to 0.25 mL/minute and pump 25% of each channel for at least 4 hours but *no more than 8 hours*.

9 Set the pump to a flow rate of 0.5 mL/minute.

10 Repeat step 4 and step 5 with a fresh beaker of HPLC-grade water.

11 While pumping, lift all solvent inline filters from the water to allow the volume in the filters to be pumped out and create an air gap. Then place the solvent inline filters into a clean 250-mL beaker of HPLC-grade water and repeat step 10.

12 Repeat step 4 and step 5 with a fresh beaker of Ammonia Solution.

13 Repeat step 4 and step 5 with a fresh beaker of HPLC-grade water.

14 While pumping, lift all solvent inline filters from the water to allow the volume in the filters to be pumped out and create an air gap. Then place solvent inline filters into a clean 250-mL beaker of HPLC-grade water and repeat step 13.

15 Repeat step 10 and step 11 with clean 250-mL beaker of HPLC-grade water until the pH of the LC effluent from the PEEK tubing outlet has a pH > 4.5.

16 Put the degasser back in-line. Connect the solvent bottle lines to the appropriate degasser channels. Connect the degasser channel lines to the solvent selection valve.

17 Replace the frit for the outlet filter.

Passivation for Existing Systems

CAUTION Failure to bypass the degasser during the passivation procedure can lead to damage.

1. Put the solvent inline filters for all 4 channels into a beaker that contains HPLC-grade water.
2. Set the pump to 25% for each channel and a flow rate of 0.5 mL/minute.
3. Purge the pump (all 4 channels) for 5 minutes at 5 mL/minute (1.25 mL/minute per channel).
4. Allow the pump to run HPLC-grade water for 10 minutes (25% of each channel).
5. Repeat step 3 and step 4 but with a clean 250-mL beaker of HPLC-grade isopropanol.
6. Repeat step 3 and step 4 but with a clean 250-mL beaker of HPLC-grade water.
7. Repeat step 3 but with the **Passivation Solution** that you prepared in "Passivation Solution" on page 19.
8. Change the flow rate to 0.25 mL/minute and pump 25% of each channel for at least 4 hours but *no more than 8 hours*.
9. Set the pump to a flow rate of 0.5 mL/minute.
10. Repeat step 3 and step 4 with a clean 250-mL beaker of HPLC-grade water.
11. While pumping, lift all solvent inline filters from the water to allow the volume in the filters to be pumped out and create an air gap. Then place the solvent inline filters into a clean 250-mL beaker of HPLC-grade water and repeat step 10.
12. Repeat step 3 and step 4 with a fresh beaker of **15% Ammonia Solution**.
13. Repeat step 3 and step 4 with a fresh beaker of HPLC-grade water.
14. While pumping, lift all solvent inline filters from the water to allow the volume in the filters to be pumped out creating an air gap. Then place solvent inline filters into a fresh beaker of water and repeat step 13.
15. Repeat step 10 and step 11 with clean 250-mL beaker of HPLC-grade water until the pH of the LC effluent from the PEEK tubing outlet has a pH > 4.5.

16 Put the degasser back in-line. Connect the solvent bottle lines to the appropriate degasser channels. Connect the degasser channel lines to the solvent selection valve.

17 Replace the frit for the outlet filter.

To prepare the LC column and column guard

NOTE This column "phosphation" procedure can provide added chromatographic peak shape improvement for metabolites including, but not limited to, phosphate-containing compounds.

Include the nebulizer in this procedure to improve peak shape, especially when a new nebulizer needle has been installed.

Do these steps when:

- A new guard cartridge or analytical column is installed on the LC system.
- Phosphorylated compounds show increased peak tailing or decreased sensitivity.

Before you begin, make sure you have:

- HPLC-grade water
- HPLC-grade isopropanol
- clean 250-mL beaker
- concentrated (~85%) phosphoric acid
- HPLC-grade acetonitrile
- pH paper for range 4.0-7.0

LC System Preparation

1. Clean and passivate the LC system. See "To passivate the LC system" on page 17.
2. Make sure that the guard cartridge and analytical column are in-line with the LC flow path.
3. Disconnect the LC system from the MS instrument. Connect the nebulizer to the end of the PEEK tubing connected to the multicolumn thermostat. Collect the nebulizer effluent in an appropriate waste container.

CAUTION Set the LC flow to waste for the entire protocol, off-line from the mass spectrometer. Spraying phosphoric acid into the mass spectrometer can damage the instrument.

Column Treatment Solution

1 Dilute the concentrated (~85%) phosphoric acid to 0.5% with 90:10 HPLC-grade acetonitrile:HPLC-grade water to create the **Column Treatment Solution**.

Column/Guard Treatment

1 Place the solvent line in a clean 250-mL beaker of HPLC-grade isopropanol.

   Use a single solvent channel. You can use Solvent line B because this channel is not used in the analytical method.

2 Purge the solvent channel with HPLC-grade isopropanol for at least 5 minutes at 5 mL/minute.

3 Run the pump with HPLC-grade isopropanol for 1 hour at 0.25 mL/minute.

4 Purge the channel with HPLC-grade water for 5 minutes at 5 mL/minute.

5 Allow the pump to run with HPLC-grade water for 1 hour at 0.5 ml/minute.

6 Purge the channel with Column Treatment Solution for 5 minutes at 5 mL/minute.

7 Allow the pump to run with Column Treatment Solution overnight at 0.1 mL/minute.

8 Purge the channel with HPLC-grade water for 5 minutes at 5 mL/minute. Repeat several times. Rinse and replace beaker with fresh HPLC-grade water each time.

9 Allow the pump to run with HPLC-grade water for 1 hour at 0.5 mL/minute.

10 Check that the pH of the LC effluent from the PEEK tubing is > 4.5. If not, repeat step 8 and step 9.

11 Purge with mobile phases A-D as detailed in the provided analytical method (see the *Method Setup Guide*).

12 Connect the nebulizer to the ion source. Connect the LC PEEK tubing to the MS divert valve.

The system is ready for analysis.

To clean the nebulizer needle

The frequency at which the needle needs to be cleaned depends on the type of sample, sample preparation, and the number of samples analyzed.

Parts needed

- 10-mL glass graduated cylinders
- 50-mL conical polypropylene tubes
- floating support (such as a foam tube rack with a cut-out to accommodate a tube).
- synthesis-grade dicholoromethane
- LCMS-grade isopropanol
- 0.1% Alconox in LCMS-grade water (p/n 5190-1401)
- LCMS-grade water
- molecular-biology grade 0.5M EDTA
- sonicator
- syringe with 10-32 Luer adapter
- PTFE tubing

Refer to the animated *6000 Series LC/MS Maintenance Guide* for complete instructions.

1 Remove the nebulizer. Follow all safety procedures described in the *6000 Series LC/MS Safety Guide*.

CAUTION Do the rest of the steps in this topic under a fume hood. The solvents used can be hazardous.

2 Do the wash steps in Table 1 in the order indicated. Specific details are found after Table 1.

3 Inspect the needle to make sure that the needle is centered and intact.

Table 1 Nebulizer needle wash steps

Do this step	With this solvent	In this container/port	For details, see
1 Sonicate for 5 minutes	1:1 synthesis-grade dicholoromethane: LCMS-grade isopropanol	10-mL glass graduated cylinder	"To sonicate the nebulizer assembly"
2 Sonicate for 5 minutes	0.1% Alconox in LCMS-grade water	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"
3 Flush the needle	1 mL molecular-biology grade 0.5M EDTA	nebulizer liquid port (10-32 fitting)	"To flush the needle"
4 Sonicate for 5 minutes	LCMS-grade water	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"
5 Sonicate for 5 minutes	LCMS-grade water	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"
6 Sonicate for 5 minutes	LCMS-grade water	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"
7 Sonicate for 5 minutes	LCMS-grade isopropanol	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"
8 Flush the needle	1 mL LCMS-grade water	nebulizer liquid port	"To flush the needle"
9 Flush the nitrogen inlet port	5 mL LCMS-grade water	nitrogen inlet port	
10 Sonicate for 5 minutes	LCMS-grade isopropanol	fresh 50-mL conical polypropylene tube	"To sonicate the nebulizer assembly"

To sonicate the nebulizer assembly

a Put the nebulizer needle into the indicated container.

b Pour the solvent to up to 2 cm from the top of the container.

c Insert the container into the floating support and put the whole unit into a sonicator.

d Sonicate in a sonicator for 5 minutes.

e Remove the nebulizer and discard solvent in appropriate waste container.

To flush the needle

a Fill a syringe with 10-32 Luer adapter with the indicated solvent.

b Attach the syringe to the LC effluent port.

c Apply gentle pressure to flush the needle.

d If the needle is clogged, resonicate. If the clog persists, replace the needle as directed in the *animated 6000 Series LC/MS Maintenance Guide*.

To clean the source

The frequency at which the source needs to be cleaned depends on the type of sample, sample preparation, and the number of samples analyzed.

Parts Needed

- 50-mL conical polypropylene tube
- 0.1% Alconox in LCMS-grade water
- 4000-grit abrasive mesh (p/n 8660-0827, supplied with instrument)
- LCMS-grade water
- LCMS-grade isopropanol
- clean lint-free cloth (p/n 05980-60051)

In addition to the daily source cleaning steps that are found in the *6000 Series LC/MS Maintenance Guide*, do these steps.

1. Sonicate the spray shield in a fresh 50-mL conical polypropylene tube with 0.1% Alconox in LCMS-grade water for 5 minutes.
2. Inspect the spray shield and rub with gloved fingers to remove any stubborn residue. Use an 4000-grit abrasive mesh with water to remove stubborn residue.
3. Sonicate the spray shield in a fresh 50-mL conical polypropylene tube with LCMS-grade water for 5 minutes.
4. Repeat step 3.
5. Open the spray chamber, and then:
   - Clean spray shield with LCMS-grade isopropanol from a wash bottle.
   - Flush the source with LCMS-grade water. Make sure to fully wet the spray chamber and cage standoffs. See Figure 3.



Figure 3 Spray chamber and cage standoff

CAUTION Do not squirt liquid into the ion transfer capillary when you spray cleaning solution close to the source body. The Stage 1 turbo pump can be damaged if too much liquid enters the capillary directly.

- Flush spray shield, capillary cap, end plate and surrounding areas with LCMS-grade water.
- Scrub source and end plate with clean lint-free cloth. Use additional LCMS-grade water to wash until surfaces no longer discolor cloth.
- Flush source and end plate with LCMS-grade isopropanol and scrub with a clean lint-free cloth.
- Replace capillary cover (if removed for cleaning) and spray shield.

6 Check the LC/MS waste levels and dispose if necessary.

7 Run a standard sample to confirm system performance.

To prepare for system downtime

Part Needed

- LCMS-grade isopropanol

Do this step if the system will not be in use for more than three or four days.

1. Equilibrate the column in 99% Buffer B for storage.
2. Put all solvent lines in LCMS-grade isopropanol and purge all channels of the system with LCMS-grade isopropanol to prevent algae growth.
3. When resuming use of the system, purge all channels into appropriate buffers.

To clean the system (monthly)

Parts Needed

- LCMS-grade isopropanol
- LCMS-grade acetonitrile
- LCMS-grade water
- LCMS-grade methanol

1. Purge channels per Table 2, in the order listed.

Table 2 Channel purge

Channel A	Channel C	Channel D
LCMS-grade isopropanol	LCMS-grade isopropanol	LCMS-grade isopropanol
Do cross-channel flushing of the aqueous and organic lines (run aqueous through the organic lines and vice versa. This procedure helps to remove contaminants from the degasser membranes):		
LCMS-grade acetonitrile (>50 mL)	LCMS-grade water (>50 mL)	LCMS-grade water (>50 mL)
Buffer A (LCMS-grade water with TBA Additive)	Buffer B (LCMS-grade methanol with TBA Additive)	LCMS-grade acetonitrile

2. Do a system pressure test on the 1290 Infinity II Flex pump.
3. Back-flush the multisampler injection seat. See "If contamination is suspected" on page 36 for details.

To remove tributylamine (TBA) from the MS

To avoid contamination, use a separate, dedicated LC system for TBA.

This procedure is designed to remove TBA from the ion source of the LC/MS instrument to enable positive-ion mode analysis. For this purpose, disconnect the dedicated TBA LC system, and connect a second LC system that will be used for non-ion-pairing separations. Make sure that solvent bottles are not shared between ion-pairing and non-ion-pairing systems to avoid contamination.

The nebulizer is a significant source of TBA contamination. Dedicate a nebulizer (p/n G1958-67098) for the Metabolomics dMRM Method, and use a second clean nebulizer for the cleaning procedure described in this topic and subsequent analysis in positive ion mode.

Cleaning of the mass spectrometer is performed by physical cleaning and by solvent flow.

Parts Needed

- clean lint-free cloth (p/n 05980-60051)
- LCMS-grade acetonitrile
- HPLC-grade water
- formic acid

1. Put the LC/MS instrument in standby mode and allow source components to cool.
2. Disconnect the dedicated TBA LC system from the LC/MS instrument up to and including the PEEK tubing that runs to the MS inlet.
3. Remove the two M3 screws that attach the metal cage electrode directly opposite the capillary inlet. See Figure 4 on page 32.

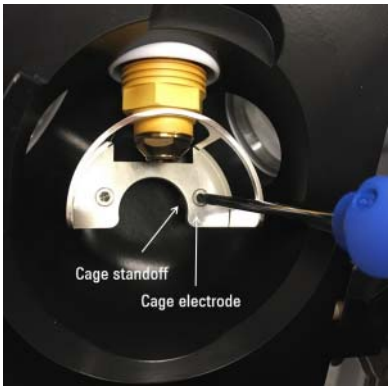


Figure 4 Cage electrode

4 Put the cage electrode into an appropriately-sized beaker and add 90:10 LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid. Sonicate for at least one hour with several changes of fresh solution.

5 While the cage electrode is sonicating:

   a Clean the source with a clean lint-free cloth soaked in 90:10 LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid.

   b Gently scrub the inner wall of the source and glass windows. Do not scratch the PTFE coating of the source.

   c Remove the spray shield and capillary cover from the LC/MS and sonicate in several changes of the 90:10 LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid solution.

   d Use a clean lint-free cloth and liberal amounts of LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid to clean all surfaces of the inlet assembly. Pay particular attention to the steel desolvation plate and PTFE mounting block. For more thorough cleaning, vent the system, remove the front source components and sonicate.

6 Reassemble the source and inlet, re-connect pneumatic and electrical fittings.

System flush and overnight cleaning

1 Connect the second LC system (without prior TBA exposure) to the divert valve of the LC/MS instrument. Remove column if present and replace with ZDV union.

2 Exchange the dedicated TBA nebulizer with a second nebulizer that has not been exposed to TBA.

3 Purge and prime all channels of the LC/MS system (90:10 LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid).

Purge for at least 5 minutes per channel at 5mL/min.

4 Create a new acquisition method with the following key parameters:

- 8 minute method delivering flow at 0.5 mL/min (90:10 LCMS-grade acetonitrile:HPLC-grade water with 0.1% formic acid)
- A positive-ion mode MRM method as shown below containing two time segments, which serve to actuate the MS Divert Valve. Note LC flow is entering mass spectrometer from 0.1 to 8.0 minutes.

Source conditions are shown in the figures that follow.

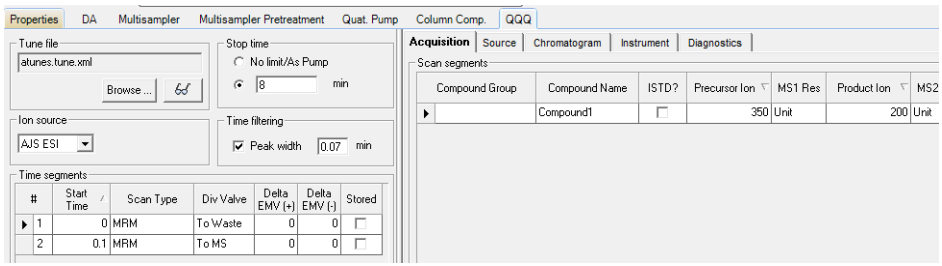


Figure 5 Time segments table and Acquisition tab in the Method Editor window

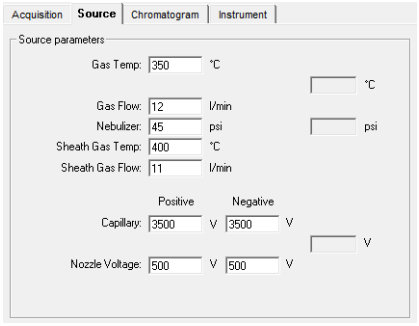


Figure 6 Source parameters in the Method Editor window on a 6470 QQQ

5 Create a worklist with the method for 120 sample replicates. Define the sample position as “No injection”. The worklist will require approximately 16 hours to complete.

Following completion of the worklist, TBA spectral counts in a positive-ion MS2 scan should be minimal when assessing with a non-TBA LC system, or when assessing calibrant bottle delivery.

Note that high levels of TBA ion (*m/z* 186) are commonly observed when the system has been left in standy mode and the mass spectrometer is turned on. The *m/z* 186 quickly reduces intensity and returns to minimal levels after several minutes.

Troubleshooting

This chapter describes the steps to troubleshoot your analysis.

To monitor system performance

Changes in the performance of the chromatography system and of the mass spectrometer can affect system performance over time.

- Include one or more quality control samples with every batch of sample run to assess system performance.
  - Use a representative biological sample prepared in large quantity, aliquoted, and frozen for inclusion in each set of samples run on the system.
  - Use a mixture of standard compounds to get useful confirmation of retention time performance. Comparison of mass spectral signal and chromatographic retention time in these controls over time can help to identify changes in system performance.

If retention time changes over time

- ✔ Check that the analytical gradient and system configurations are correct.
- ✔ Prepare and use fresh buffer.
- ✔ Check for leaks in the LC pump. Use Lab Advisor or volumetric measurement of post-column LC flow.

If contamination is suspected

Do these steps if:

- Sample carry-over is observed between successive injections (> 0.1%).
- Persistent contamination is observed between runs.

✔ Clean or replace the Multisampler needle.

The Multisampler needle or seat may have become contaminated by inadequate or missing wash, sample build-up on the needle surface, or wear on the needle or seat assembly.

✔ If seat contamination is suspected, back-flush the needle seat. Seat contamination is indicated by carry-over peaks that are present with air blank injections, but are absent with no-injection runs.

To prevent seat contamination, make sure that the needle wash solvent does not run dry during a run.

If system tune fails in positive mode after use of tributylamine

Contaminating tributylamine (TBA) at 186 *m/z* can cause system tunes to fail in positive mode. For positive-mode auto tunes, the level of 186 *m/z* ion (tributylamine [M+H]) must be below that of the 118 *m/z* ion (glycine-betaine [M+H]) present in the tune mix.

✔ Manually switch between "LC ➜ MS" and "LC ➜ Waste" 10 or more times to clear persistent contamination.

TBA contamination can persist on valve rotor and stator faces.

✔ For Agilent Jet Stream sources, rinse with HPLC-grade water and LCMS-grade acetonitrile. Dampen a clean lint-free cloth and gently wipe the source and spray chamber walls.

Auto tune immediately after cleaning. If the system is left idle, TBA will continue to be extracted from system components and will contribute to additional signal at 186 *m/z* in positive mode.

If you do not detect compounds that you expect

✔ Save the method as a new method and widen the RT window to encompass the chromatographic run. If you need to adjust your RTs, refer to the *MRM/dMRM/tMRM Database Familiarization Guide* for details on how to update an MRM method to a dMRM method.

A number of reasons can cause your retention times to change from those determined by Agilent, such as different instrument delay volume, dead volumes, or configuration. Make sure you follow exactly the instructions in the *Method Setup Guide*.

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In This Guide

This Analysis Guide provides instructions to prepare samples, configure instruments, and do an initial data analysis.

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