MassHunter Metabolomics Dynamic MRM Database and Method

Method Setup Guide

For Research Use Only. Not for use in diagnostic procedures.

This guide describes an ion-paired reverse-phase method to separate anionic and hydrophobic metabolites. This method enables simultaneous analysis of hundreds of molecules, including amino acids, citric acid cycle intermediates and other carboxylic acids, nucleotides, nucleosides, nucleobases, phosphosugars, and fatty acids.

In conjunction with the MassHunter Metabolomics dMRM Database and Method, this separation enables rapid, robust, and reproducible quantitation of a large array of compounds in primary metabolism across a wide dynamic range. The MassHunter Metabolomics Dynamic MRM Database and Method product includes the data acquisition method file **Metabolomics Base dMRM.m**.

Use this guide and the method to set up your MRM methods. The method is installed by default when you install the complete MassHunter Metabolomics Dynamic MRM Database and Method product.

For more detailed instructions, see the *Quick Start Guide* for this database, the *Familiarization Guide*, the *Analysis Guide*, and the MassHunter Data Acquisition for 6400 Series Triple Quadrupole LC/MS *Familiarization Guide* and *online Help*.

Workflow Overview

Figure 1 summarizes the dMRM workflow used to set up a Metabolomics dMRM method with your analytes of interest.

CAUTION

Do not use the base method for positive mode analysis, including polarity switching. The method is not set up for positive mode analysis.

The general steps of the workflow are:

- Load the base MassHunter Data Acquisition method, which contains predefined parameters to ensure accurate reproduction of retention times.
- Import compounds of interest into the method from the Metabolomics dMRM Database.
- Save the method. The method is now ready for use with LC/MS data acquisition.

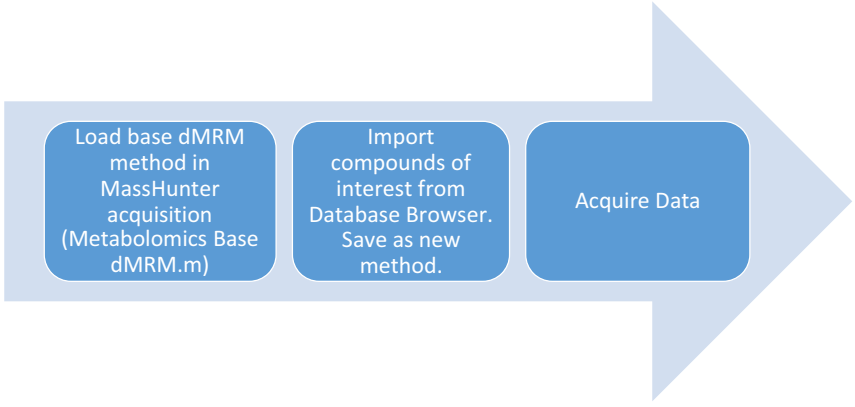


Figure 1. dMRM Method Development Workflow

System Preparation

This chapter describes the steps to set up your system for analysis with the MassHunter Metabolomics Dynamic MRM Database and Method.

For optimal results, make sure that you keep your system clean of contaminants. See the *Analysis Guide* for more information.

Before you begin, make sure you have:

Reagents

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

LC System

Module	Agilent p/n	Notes
1290 Infinity II LC Flexible Pump (Quaternary Pump)	G7104A	• Seal wash is used. • Disable JetWeaver/Solvent mixer if equipped
1290 Infinity II Multisampler	G7167B	• Standard needle wash is used (multiwash and dual needle not used) • Infinity II Sample Cooler required (Option #100, p/n G7167-60005)
1290 Infinity II Multicolumn Thermostat	G7116B	• Valve drive required (Option #058)

LC parts

Part	Agilent p/n	Notes
ZORBAX RRHD Extend-C18, 2.1 × 150 mm, 1.8 um	759700-902	Analytical column
ZORBAX Extend Fast Guards for UHPLC, Extend-C18, 2.1 mm, 1.8 um, 3/pk	821725-907	Guard column
2 pos/ 6 port ultra high pressure (1200 bar) valve head	5067-4117	6 port TCC valve for column backflushing
SS Capillary, 0.12 mm × 150 mm SL/SX	5067-4650	TCC Valve to Heat Exchanger
A-Line Quick Connect Assembly, ST 0.12 × 220 mm	5067-5959	Column tail to TCC valve
Finger-tight 1/16 in PEEK Fitting Kit	5065-4426	Fittings for waste line from port 5 of TCC valve, and PEEK tubing connections to port 1 TCC valve and MS inlet
PEEK tubing, 0.13 mm, cut to 700 mm	0890-1915	TCC valve to MS Divert Valve
Tubing/Sleeving-Flex	5062-2462	Waste line from TCC valve to waste
Blank lock nut, long	5067-6127	Blank nut to close unused port 6 of TCC valve
Heat exchanger, 1.6 uL*	G7116-60015	
A-Line Quick Turn LC Fitting*	5067-5966	Fitting for heat exchanger entry
A-Line Quick Turn LC Fitting*	5067-5966	Fitting for heat exchanger exit
A-Line Quick Connect Assembly ST 0.12 × 105 mm*	5067-5957	Heat exchanger to Fast Guard/Column Head
SS Capillary, 0.17 × 400 mm (green)†	5500-1245	Pump to autosampler
ST Capillary 0.12 × 500 mm (red)‡	5500-1157	Multisampler to TCC valve

* Included with Multicolumn Thermostat

† Included with Flexible pump

‡ Included with Multisampler

LC/MS instrument

- 6460/6470 Triple Quadrupole LC/MS (non-iFunnel instruments only) with Agilent Jet Stream Technology source

CAUTION

Do not use this method for positive mode analysis, including polarity switching. The method is not set up for positive mode analysis.

Step 1. Prepare stock Buffer Additive Mixture

The buffer additive needs to be prepared as a batch of concentrated mixture as described in this topic. With this approach, the measured volumes are easy to handle, and approximately 60 liters of mobile phase can be created from a single batch of additive. For optimum consistency within large experiments, use a single batch of additive. Additive preparation can be scaled to suit lab throughput.

CAUTION

Handle all solutions with glass serological pipettes or other clean glassware. Plastic lab ware, particularly disposable pipettes, is frequently contaminated with plasticizers, slip agents, and other leachable compounds.

CAUTION

The chemicals used in the following steps are hazardous. Exercise appropriate caution and wear gloves, protective eye wear and a lab coat. Prepare the buffers in a chemical fume hood.

You will need:

- 900 mL of LCMS-grade methanol
- 71.5 mL of GC-grade (>= 99%) tributylamine
- 25.75 mL of HPLC-grade glacial acetic acid
- 50-mL glass graduated cylinder
- 100-mL glass graduated cylinder

1. Rinse a clean borosilicate bottle with **LCMS-grade methanol**, then drain.
2. Add the **900 mL of LCMS-grade methanol**.
3. Use a **50-mL glass graduated cylinder** to add **25.75 mL of HPLC-grade glacial acetic acid** to the solution.
4. Swirl the solution to mix. Make sure no phase separation is visible.
5. Use a **100-mL glass graduated cylinder** to add **71.5 mL of GC-grade (>= 99%) tributylamine** into the **LCMS-grade methanol**.
6. Swirl the solution to mix. Make sure the mixture appears homogeneous with no precipitation or separation of phases.

If the buffer appears turbid or “cloudy”, most likely the pH of the buffer is above the threshold where tributylamine goes into solution. To avoid this problem:

- Ensure that glacial acetic acid is used for the preparation.
- Confirm the exact measurement of the reagents.
- Follow the order of buffer preparation steps exactly.

At this point, the stock buffer additive mixture is stable and can be stored for months in amber glass at 4°C away from light.

Step 2. Prepare Buffer A and Buffer B

You will need:

- Buffer Additive Mixture (see **"Step 1. Prepare stock Buffer Additive Mixture"** on page 6)
- HPLC-grade water
- LCMS-grade methanol
- 50-mL glass graduated cylinder (or suitable glass pipette)

1 Prepare **Buffer A**:

   a Use a **50-mL glass graduated cylinder (or suitable glass pipette)** to add **34.25 mL Buffer Additive Mixture** to 1 liter **HPLC-grade water**.

   Alternatively, to make exactly 1 liter of Buffer A, add **33.25 mL Buffer Additive Mixture** to 970 mL **HPLC-grade water**.

   b Swirl the solution to mix. Make sure no precipitate or miscible material is visible.

   c Measure the pH of the final aqueous solution. If the pH is not close to 5 ± 0.2 units, prepare fresh **Buffer Additive Mixture**.

   Make sure that the pH meter is properly calibrated at pH 4 and pH 7 before use.

   Final concentration of Buffer A is 97% **HPLC-grade water** and 3% **LCMS-grade methanol**, 10mM **GC-grade (>= 99%) tributylamine**, 15mM **HPLC-grade glacial acetic acid**

2 Prepare **Buffer B**:

   a Use a **50-mL glass graduated cylinder (or suitable glass pipette)** to add **34.25 mL Buffer Additive Mixture** to 1 liter **LCMS-grade methanol**.

   Alternatively, to make exactly 1 liter of Buffer B, add **33.25 mL Buffer Additive Mixture** to 970 mL **LCMS-grade methanol**.

   b Swirl the solution to mix. Make sure no precipitate or immiscible material is visible.

   Final concentration of Buffer B is 10mM **GC-grade (>= 99%) tributylamine**, 15mM HPLC-grade glacial acetic acid, prepared in **LCMS-grade methanol**.

If the buffer appears turbid or “cloudy”, most likely the pH of the buffer is above the threshold where tributylamine goes into solution. Check that the Buffer Additive Mixture described in **“Step 1. Prepare stock Buffer Additive Mixture”** on page 6 was correctly prepared and remake if necessary.

Buffers are stable on instrument for at least 72 hours after preparation. Discard unused buffer and rinse solvent bottles before re-filling. *Do not top-up bottles with additional solvent.*

Step 3. Configure the divert valve

- Configure your system as shown in **Figure 1** on page 10.

  The system is configured with a 6-port/2-position valve mounted in the thermostatted column compartment plumbed to enable on-line flow reversal. This configuration removes contaminants at the column head for each and every analytical run.

  Failure to regenerate the column in this way will result in progressive retention time drift and premature column failure.

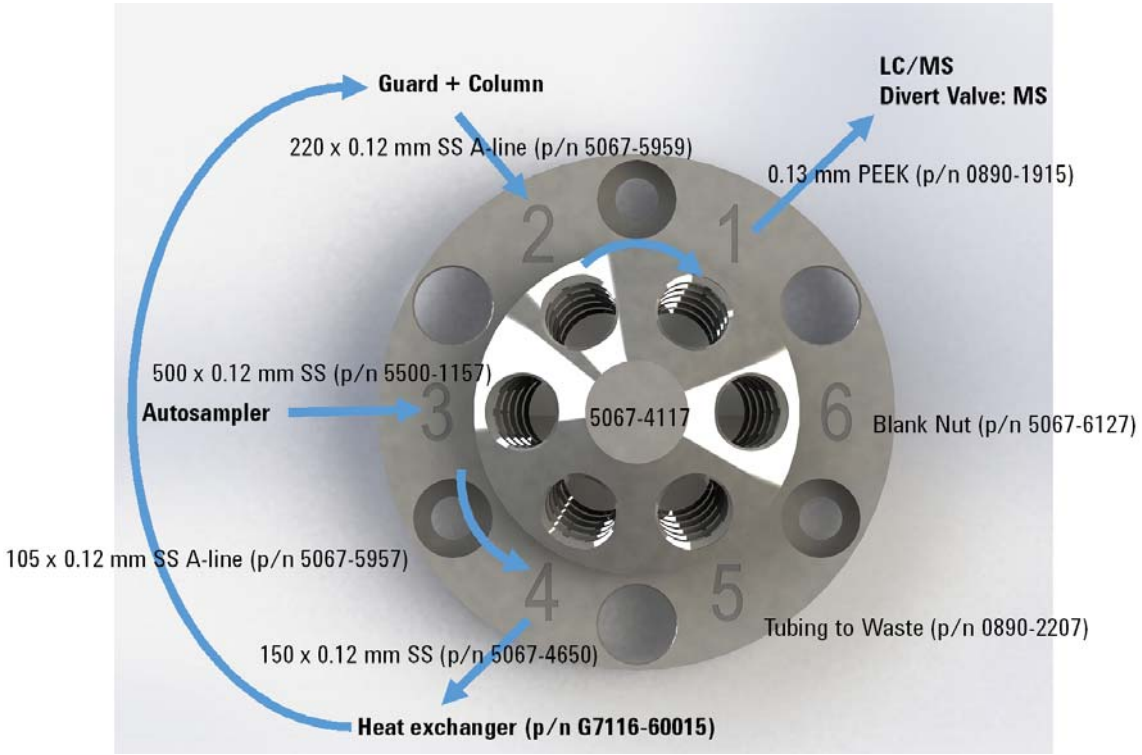


Figure 1. Valve configuration for analytical method

During the analytical gradient (valve position 1-2, forward), sample flows

- from Multisampler (port 3) to heat exchanger (port 4) and to guard column head in forward flow direction
- from column tail back into valve (port 2) and from valve to mass spectrometer (port 1)

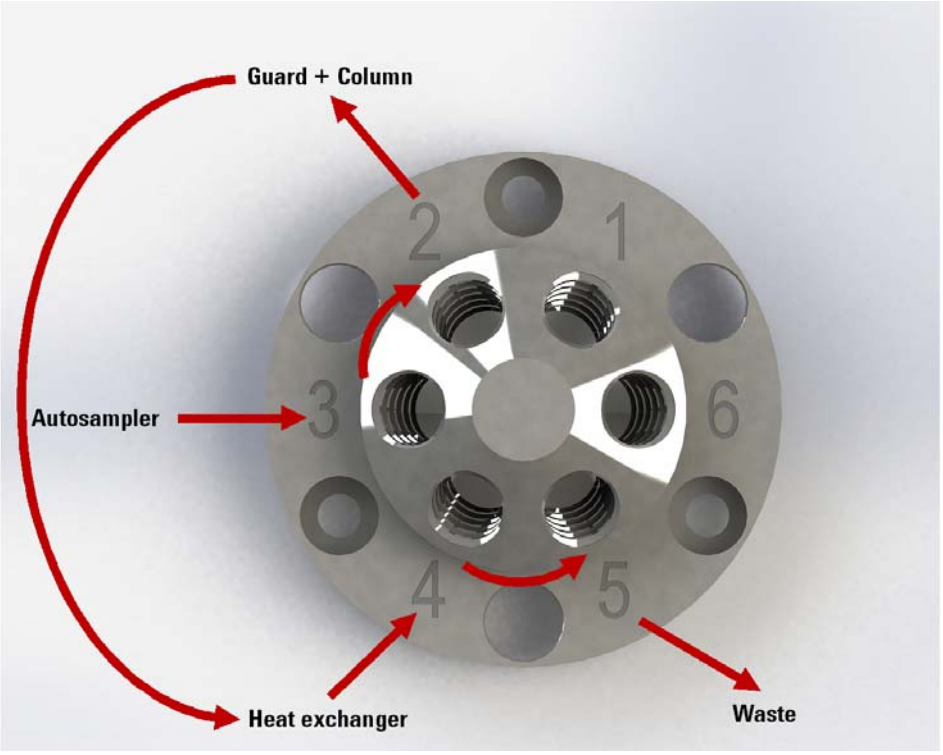


Figure 2. Valve configuration for regeneration method

During regeneration (valve position 1-6, reverse), solvent flows

- from Multisampler outlet (port 3) into the tail of column (port 2)
- through column in reverse flow direction
- from column head back to heat exchanger/valve (port 4)
- from port 5 to waste through waste line

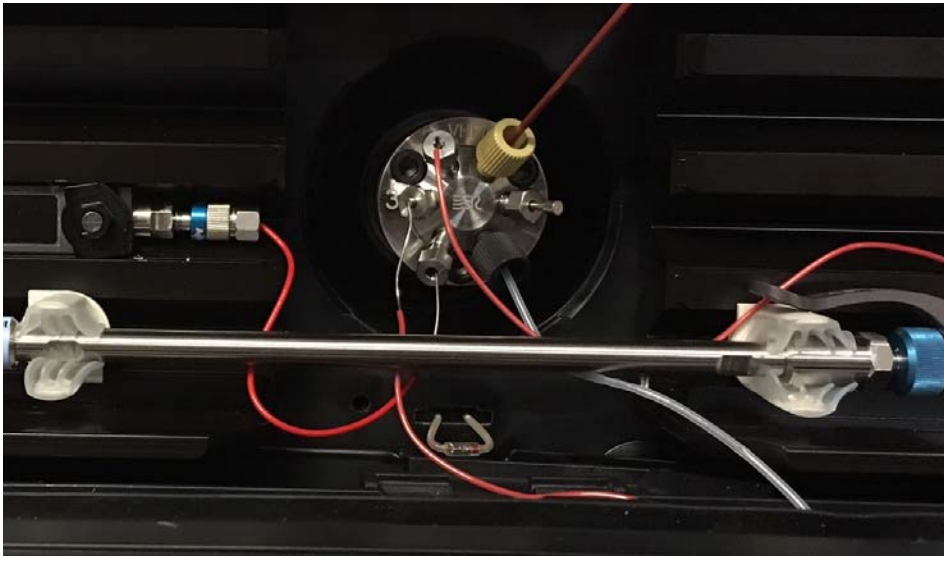


Figure 3. Divert valve properly configured.

Method Setup

Step 1. Set up the LC part of the method

1 Prepare the samples. Refer to the *Analysis Guide* for sample preparation.

2 Set up the mobile phases. See **"Step 2. Prepare Buffer A and Buffer B"** on page 8.

- Channel A: Buffer A (**HPLC-grade water** with TBA additive, or **HPLC-grade water** that contains 3% **LCMS-grade methanol**, 10mM TBA, and 15mM **HPLC-grade glacial acetic acid**)
- Channel B: (**LCMS-grade isopropanol**, to prevent algae growth when not in use)
- Channel C: Buffer B (**LCMS-grade methanol** with TBA additive, or **LCMS-grade methanol** that contains 10mM TBA and 15mM **HPLC-grade glacial acetic acid**)
- Channel D: 100% **LCMS-grade acetonitrile**

3 Open the method Metabolomics Base dMRM.m.

4 Verify that the parameters in the method for these modules exactly match those that are shown in the figures and table indicated:

- Multisampler (**Figure 1** on page 14)
- Flex pump (**Figure 2** on page 15)
- Column Comp (**Figure 3** on page 16)
- TCC valve (**Table 1** on page 16)

CAUTION

The method parameters must exactly match those shown in the next figures. Any difference in these settings can result in retention time discrepancies with the database retention time values.

The autosampler temperature should be set to 4ºC.

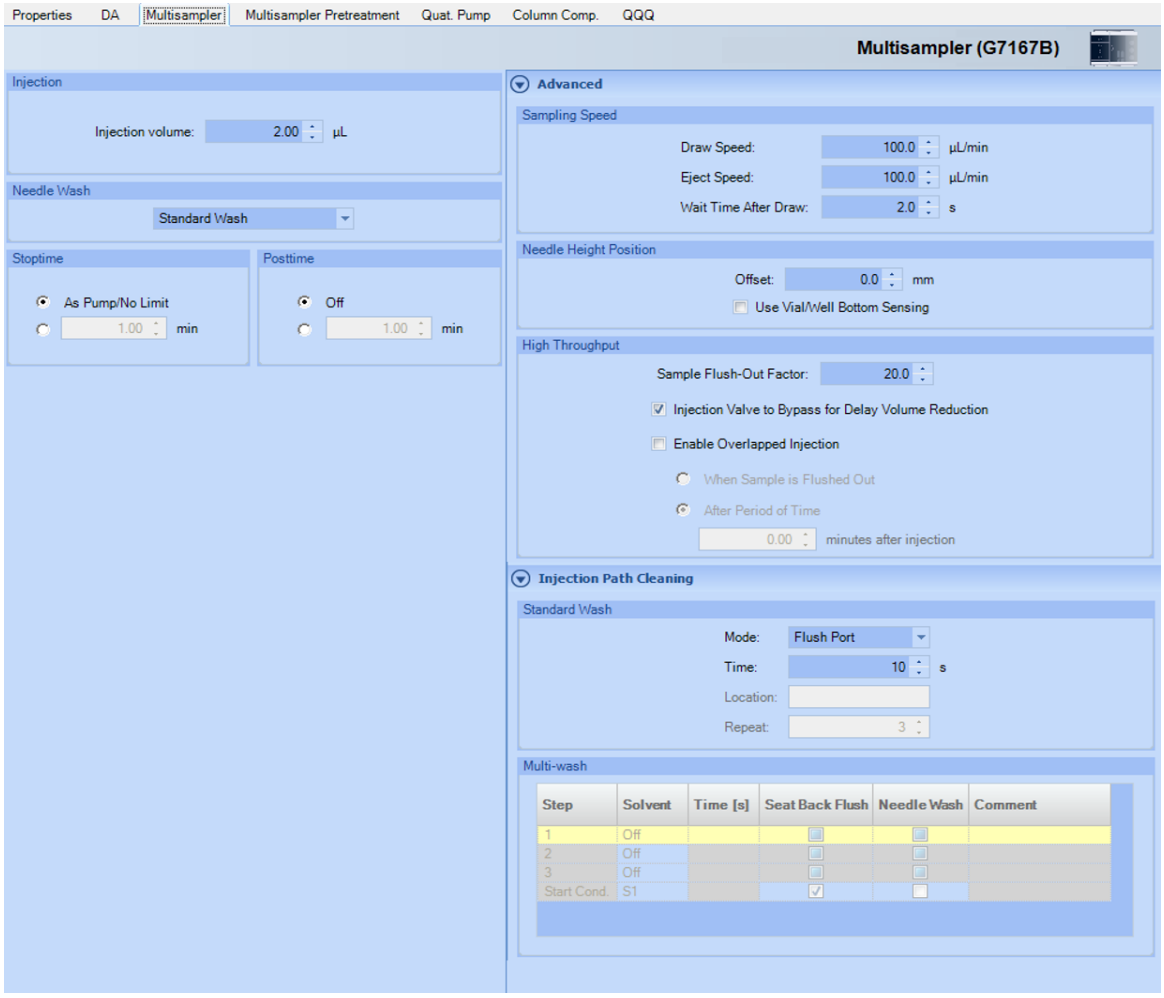
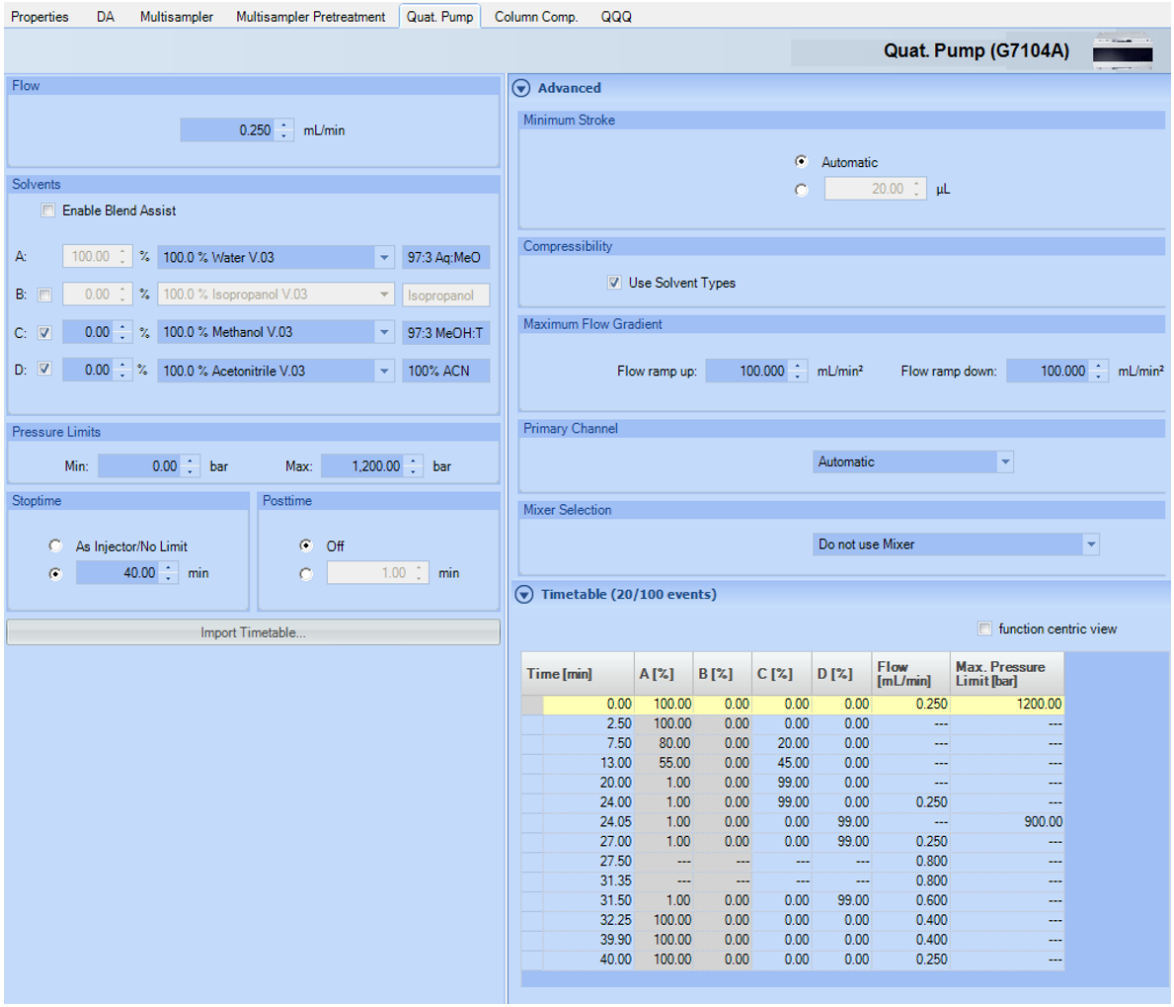


Figure 1. Multisampler tab (multiwash is disabled if equipped)



Time [min]	A [%]	B [%]	C [%]	D [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	100.00	0.00	0.00	0.00	0.250	1200.00
2.50	100.00	0.00	0.00	0.00	---	---
7.50	80.00	0.00	20.00	0.00	---	---
13.00	55.00	0.00	45.00	0.00	---	---
20.00	1.00	0.00	99.00	0.00	---	---
24.00	1.00	0.00	99.00	0.00	0.250	---
24.05	1.00	0.00	0.00	99.00	---	900.00
27.00	1.00	0.00	0.00	99.00	0.250	---
27.50	---	---	---	---	0.800	---
31.35	---	---	---	---	0.800	---
31.50	1.00	0.00	0.00	99.00	0.600	---
32.25	100.00	0.00	0.00	0.00	0.400	---
39.90	100.00	0.00	0.00	0.00	0.400	---
40.00	100.00	0.00	0.00	0.00	0.250	---

Figure 2. Quat. Pump (Flex Pump) tab

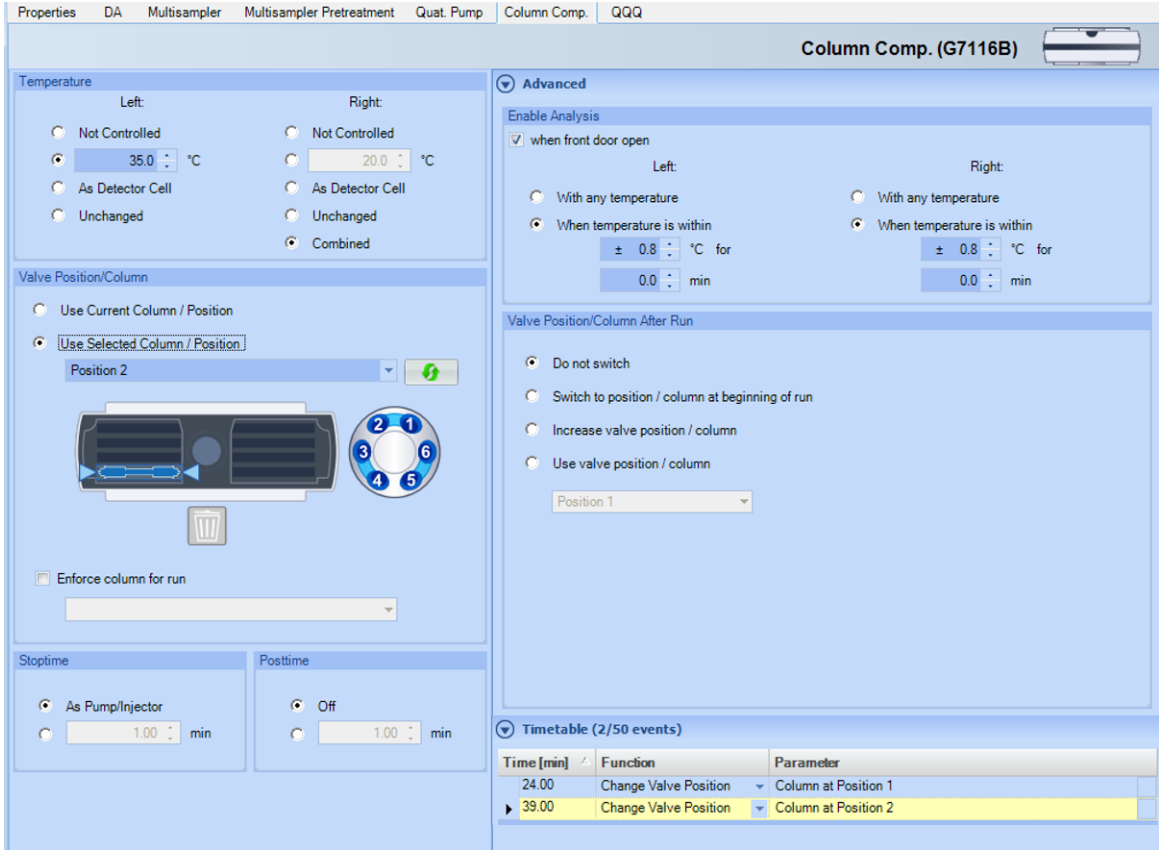


Figure 3. Column Comp. (Multicolumn Thermostat, MCT) tab

The flow rate is 0.25 mL/min throughout the analytical gradient, and is then increased during regeneration to reduce overall run time. During regeneration, the direction of flow through the guard and analytical columns is reversed.

Table 1 TCC valve settings

Stage	Valve position
Analysis	1 => 2 (forward)
Column regeneration	1 => 6 (reverse)
Equilibration	1 => 2 (forward)

5 Set up needle and seal wash solvents:

- For needle wash solvent, use 50:50 LCMS-grade methanol:HPLC-grade water with 15mM HPLC-grade glacial acetic acid.
- For seal wash solvent, use 50:50 LCMS-grade isopropanol: HPLC-grade water.
- Right-click Flex Pump, and then in the Control tab, set up Seal Wash parameters as shown in **Figure 4**.

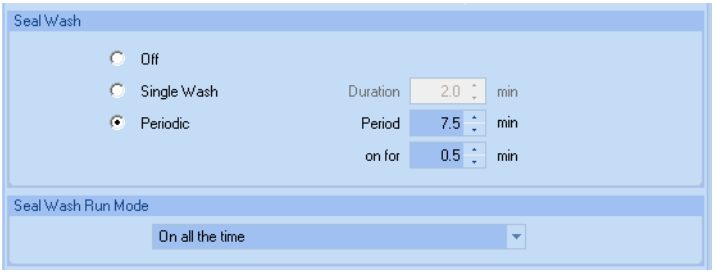


Figure 4. Seal wash settings

NOTE

Seal wash must be used to extend pump seal life span.

Step 2. Check the LC/MS parameters

CAUTION

Do not use this method for positive mode analysis, including polarity switching. The method is not set up for positive mode analysis.

- Verify that the time segments and ion source parameters loaded in the method tab match the parameters shown in **Figure 5**.
- Ensure the LC eluent is plumbed to MS divert valve and not plumbed directly to the ion source.
- System source parameters provided are optimized to provide the best overall response for the metabolites covered by this method. For methods focused on a subset of all analytes, optimization of ion source parameters for a specific set of compounds may provide sensitivity gains.
- Use a Delta EMV setting of 200 V as a starting point. Exact setting will depend on the application and instrument condition. Increasing Delta EMV increases signal, but can result in unacceptable decreases in signal to noise ratio. For more details, refer to the online Help.

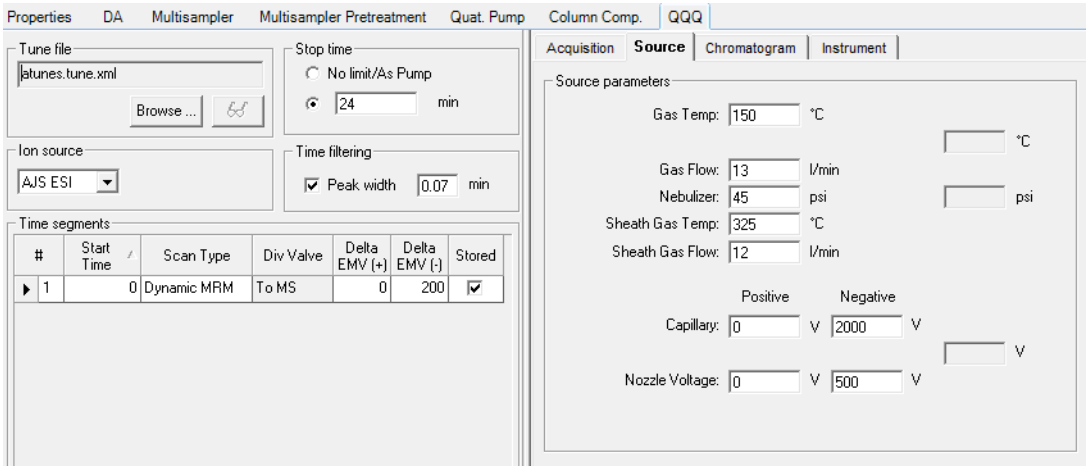


Figure 5. LC/MS parameters

Step 3. Import compounds from the Metabolomics Dynamic MRM Database

The base method does not specify any transitions for acquisition. All transitions can be imported for a single dMRM method. You can also acquire only a subset of compounds or transitions to optimize some applications. For an example with detailed steps, see the *MassHunter Metabolomics Dynamic MRM Database and Method Familiarization Guide*.

Sample Analysis

Step 1. Determine system background and carryover

Check solvents, additives, and consumables for contaminants on a lot-wise basis.

- Run blanks (solvent only, no injections) to evaluate solvent suitability.
- Perform mock extractions without media or biological material to evaluate filter membranes, when used, and all plastics and extraction solvents.
- Include biological control samples in sample runs to assess day-to-day reproducibility, air (or no-injection blanks) and mock-extraction solvent blanks.
- To assess system carryover, run biological sample followed by one or more solvent-only blanks. If system carryover is found, increase the duration of the needle wash, or add an extra wash vial to more completely solubilize contaminants.

Step 2. Run new acquisition method

- Run the acquisition method that you just saved, with your compounds of interest.

This sample analysis method determines the relative quantity of selected metabolites in different samples. The quantity of each metabolite is measured by integrating the area under the peak in the chromatogram, to determine the total signal of the compound. Any background associated with a compound needs to be subtracted by determining the signal from a blank injection (solvent only, no injections).

Step 3. Analyze the data

- Run data analysis. Refer to the *Analysis Guide* for data analysis recommendations and software workflows.

Reference

Method Overview

The MassHunter Metabolomics Dynamic MRM Database and Method provides MRM transitions for over 200 compounds.

Use of dynamic MRM (dMRM) acquisition enables efficient targeted analysis because the mass spectrometer is acquiring signals for a given compound within a time window flanking compound elution. Where possible, two unique transitions are monitored for individual metabolites in order to provide spectral matching in addition to retention time.

Compound-Specific Notes and Tips

A number of these compounds exist in isomeric compound pairs. Some of these compounds have the exact same MRM transitions but can be separated in retention time. **Table 1** lists these isomer pairs and their respective elution order.

Table 1 Closely eluting isomers and their elution order

Compound names and elution order	CAS	Comments
1. L-Isoleucine 2. L-Leucine	73-32-5 61-90-5	Closely eluting isomers.
1. 2-2-Dimethyl Succinic acid 2. Adipic acid	597-43-3 124-04-9	Closely eluting isomers.
1. cis-Aconitic acid 2. trans-Aconitic acid	585-84-2 4023-65-8	Closely eluting isomers. Note: Appears as a doublet
1. Citramalic acid 2. L-Hydroxyglutaric acid	2306-22-1 13095-48-2	Closely eluting isomers.
1. D-Gluconic acid 2. Galactonic acid	526-95-4 576-36-3	Closely eluting isomers.
1. Itaconic acid 2. 4-Methyl-2-oxovaleric acid	97-65-4 816-66-0	Closely eluting isomers.

Table 1 Closely eluting isomers and their elution order (continued)

Compound names and elution order	CAS	Comments
1. D-Ribose 5-phosphate 2. Arabinose-5-phosphate 3. D-Xylulose-5-phosphate	4300-28-1 13137-52-5 60802-29-1	Closely eluting isomers. Note: Several closely eluting peaks of pentose phosphate isomers are apparent in extract. Spike-in of standards is recommended for verification.
1. D-Glucose 6-phosphate 2. D-Fructose 6-phosphate 3. D(+)Mannose 1-phosphate 4. alpha-D-Glucose-1-phosphate	54010-71-8 643-13-0 27251-84-9 59-56-3	Closely eluting isomers. Note: Several isomers of hexose phosphate are present in cells. Spike-in of standards is recommended for verification.
1. 3-Methylglutaric acid 2. alpha-Ketoglutaric acid	626-51-7 328-50-7	Closely eluting isomers. Note: 3-methylglutaric acid elutes first, but signal for alpha-ketoglutaric acid is typically much higher in biological samples.

Some of the isomer pairs cannot be separated in retention time and cannot be distinguished from each other when using this chromatographic method. **Table 2** lists these isomer pairs.

Table 2 Co-eluting isomers that are not distinguished in this chromatography

Compound names	CAS	Comments
L-Arabitol Xylitol	7643-75-6 87-99-0	Co-eluting isomers.
N-Acetyl-D-glucosamine 6-phosphate N-Acetyl-D-glucosamine 1-phosphate	1746-32-3 28446-21-1	Co-eluting isomers. Note: In extract two isomeric peaks are seen, the second peak is n-acetyl-glucosamine-phosphate.
Deoxyguanosine 5-triphosphate Adenosine 5-triphosphate	2564-35-4 56-65-5	Co-eluting isomers. Note: ATP levels are typically higher in most cells.
Citric acid DL-Isocitric acid	77-92-9 320-77-4	Co-eluting isomers.
2-Deoxyguanosine 5-diphosphate Adenosine 5-diphosphate	3493-09-2 58-64-0	Co-eluting isomers. Note: ADP levels are typically higher than dGDP levels in most cells.
2-Deoxyguanosine 5-monophosphate Adenosine 5-monophosphate	902-04-5 61-19-8	Co-eluting isomers. Note: AMP levels are typically higher than dGMP levels in most cells.
D-Mannose L-Sorbose	31103-86-3 87-79-6	Co-eluting isomers.
Uridine 5′-diphosphogalactose Uridine 5′-diphosphoglucose	2956-16-3 133-89-1	Co-eluting isomers.

Table 3 lists useful chromatographic and MRM transition notes for some compounds.

Table 3 Chromatographic and MRM transition tips

Compound Names	CAS	Comments
L-Carnitine	541-15-1	Appears as a doublet in some extracts, the L-Carnitine authentic standard elutes first.
N-acetyl D-galactosamine	1811-31-0	Appears as a doublet in some extracts, the N-acetyl D-galactosamine authentic standard elutes first.
Guanosine 3,5-cyclic monophosphate	7665-99-8	Two isomeric compounds elute in many cell extracts, the guanosine 3, 5 cyclic monophosphate authentic standard elutes first.
Cytidine 5-diphosphate Uridine 5-diphosphate	63-38-7 58-98-0	Isotopic interference. Note: CDP is one mass unit less than UDP and will experience some cross-talk from the M+1 isotope of UDP.
Cytidine-5-monophosphate Uridine 5-monophosphate	63-37-6 58-97-9	Isotopic interference. Note: CMP is one mass unit less than UMP and will experience some cross-talk from the M+1 isotope of UMP.
Cytidine 5-triphosphate Uridine 5-triphosphate	65-47-4 63-39-8	Isotopic interference. Note: CTP is one mass unit less than UTP and will experience some cross-talk from the M+1 isotope of UTP.
2-Deoxycytidine 5-diphosphate	800-73-7	Isotopic interference. Note: dCDP is one mass unit less than dUDP and will experience some cross-talk from the M+1 isotope of dUDP. dUDP is not contained in the dMRM DB and will be at very low levels in most cells.
2-Deoxycytidine 5-monophosphate	1032-65-1	Isotopic interference. Note: dCMP is one mass unit less than dUMP and will experience some cross-talk from the M+1 isotope of dUMP. dUMP is not contained in the dMRM DB and will be at very low levels in most cells.
Deoxycytidine 5-triphosphate 2-Deoxyuridine 5-triphosphate	2056-98-6 1173-82-6	Isotopic interference. Note: dCTP is one mass unit less than dUTP and will experience some cross-talk from the M+1 isotope of dUTP. dUTP will be at very low levels in most cells.

While most compounds have shown excellent retention time reproducibility and agreement with database retention times over thousands of injections, a small subset of compounds can exhibit drifting and/or disparate retention times compared with the database retention times. These molecules mainly encompass the nucleosides (guanosine, thymidine, others) that retain to the stationary phase independent of ion-pairing mechanisms.

In extreme cases these compounds may fall outside of the dynamic MRM retention time (RT) windows specified in the database. If these compounds are of high interest, Agilent recommends that you:

1. Analyze authentic chemical standards to obtain the observed RT on your system.
2. Update the database with the observed RTs.
3. Widen the dMRM RT windows.

Due to retention time drift, these measurements may need to be repeated over time. These recommendations may be extended to any compound of high interest where confirmation of retention times is desired.

Please consult the troubleshooting section of the *Analysis Guide* for further tips on assessing the system performance and investigating possible causes for retention time changes.

In this Book

The *Method Setup Guide* describes how to create MRM methods for your specific LC/MS set up. The MRM methods are used το create Dynamic MRM (dMRM) methods for use with the MassHunter Metabolomics Dynamic MRM Database and Method product.

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