Comprehensive Test Mix for MassHunter Forensics and Toxicology Triggered MRM Database and Library

Method Setup Guide

NOTE The Comprehensive Forensics and Toxicology Test Mix is included with the G1734BA Forensics and Toxicology Triggered MRM Application Kit.

Agilent does not provide the actual acquisition methods to use with the Comprehensive Test Mix, due to the large number of instrument configurations that are possible.

Instead, Agilent provides this guide to explain how to create MRM methods that are used to create dMRM and tMRM methods for the test mix.

You must copy the values from the file **MRM_Methods_ForTox.xlsx**, found on the *Support Disc*, to set up your MRM methods.

Before you begin, make sure that your system meets the installation requirements that are described in the *MassHunter Forensics and Toxicology Triggered MRM Database and Library Quick Start Guide*.

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

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Test Mix Overview

The Comprehensive Forensics and Toxicology Test Mix is composed of 139 compounds, provided in 10 submixes. The methods described in this guide are suitable for the analysis of 135 of these compounds.

The remaining four compounds consist of two isobaric compound pairs, in which each pair has the same transitions. These compounds require dedicated LC/MS methods and are not covered in the general screening method included in this kit. These four compounds are found in Submix 6:

- Amitriptyline and Maprotiline
- Nortriptyline and Protriptyline

Of the remaining 135 compounds in the comprehensive test mix, several are isobaric pairs. All of these compounds have unique transitions that can be used in identification. Table 1 lists the elution order for these isobaric pairs on a C18 reverse phase column.

Table 1 Elution Order

Submix	Elution Order
Submix 2	**1** Methamphetamine **2** Phentermine
Submix 3	**1** Clobazam (Urbadan) **2** Temazepam
Submix 4	**1** Promethazine **2** Promazine
Submix 8	**1** Oxcarbazepine **2** Phenytoin
Submix 9	**1** Naltrexone (Submix 9c) **2** Acetylcodeine (Submix 9a)
Submix 9	**1** Codeine (Submix 9a) **2** Hydrocodone (Submix 9c)
Submix 9	**1** Morphine (Submix 9b) **2** Hydromorphone (Submix 9a)

Note that all the isobaric compound pairs in Submix 9 are provided in separate vials (Submix 9a through 9d) to aid in method development.

Workflow Overview

This Method Setup Guide contains LC and MS acquisition parameters to easily set up multiple standard mixes in the Comprehensive Forensics and Toxicology Test Mix.

Refer to the *Quick Start Guide* for more details. The *Quick Start Guide* uses example data from the Checkout Mix to illustrate the workflow and familiarization exercises. The general workflow (Figure 1) from the *Quick Start Guide* can easily be adapted to work with your unique analyses, including the analysis of the Comprehensive Test Mix included in this kit.

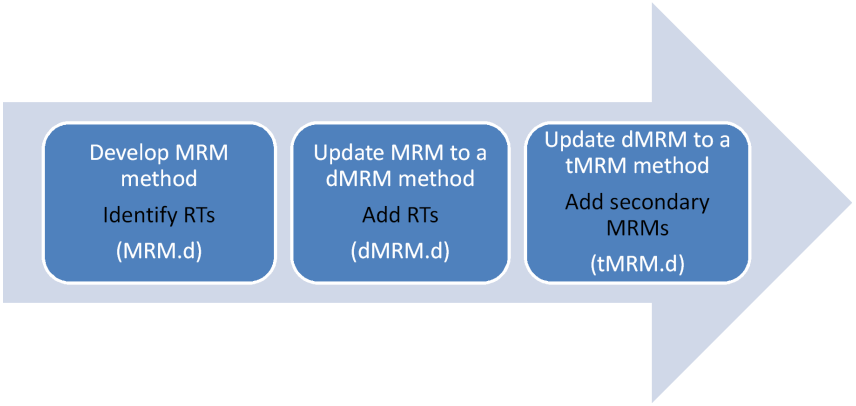


Figure 1 tMRM Method Development Workflow for single standard mix

Single Standard Mix Workflow

The workflow to analyze a single standard mix is:

1. Use the database to create the MRM method for the primary transitions.
2. Establish the Retention Times, and then create a new method using the **Update DMRM Method** command. Save as a DMRM method.
3. Check the dMRM editor for any overlaps and adjust accordingly the dwell time settings and or the Retention Time windows.
4. Acquire data to make sure that the dMRM method is valid.
5. Save the dMRM method as a tMRM method.
6. Add the secondary transitions.

After you have set up methods to analyze a single standard mix, you can adapt the same procedures for your unique multi-component analysis.

Multiple Standard Mix Workflow

To develop a method to analyze multiple standard mixes in one analytical run:

1. Optimize each tMRM method for each standard mix separately. Use the same LC chromatographic method.
2. Combine these tMRM methods.
3. Re-optimize the parameters for overlapping tMRM transitions for compounds that co-elute.

For ease of use, optimize no more than 50 compounds at a time in each **MRM -> dMRM -> tMRM** workflow.

Step 1. Set up the LC part of the method

1 Prepare the standards.

The concentration of the test mix stock solution is 100 µg/mL (100 ppm).

a Dilute 1 µL of the stock solution to 1.0 mL with acetonitrile.

For more accurate results, and if conservation of sample is not a concern, dilute 10 µL of the stock solution to 10.0 mL of solvent instead.

NOTE Submix 9 consists of 4 vials. Submix 10 consists of 3 vials. When you dilute either of these submixes, combine 1 µL from each vial, and then dilute to 1.0 mL with acetonitrile.

b Transfer 1 mL of the final sample solution to a standard 2-mL sample vial for analysis.

The final solution is a 100 ng/mL (100 ppb) working solution.

2 Set up the mobile phases.

This step is identical for all LC modules.

- Solvent A: 5 mM ammonium formate/0.01% formic acid in water
- Solvent B: 0.01% formic acid in methanol

3 Check that the method is set up to make a 1-µL injection.

4 Set up the gradient.

The gradient setup is dependent upon the LC configuration. The parameters that follow are examples.

5 Make sure that the Column Compartment temperature is set to 40°C.

1290 Infinity LC system

1290 Infinity LC system with Agilent Eclipse Plus C18, 2.1 mm × 100 mm, 1.8-µm ZORBAX LC column (p/n 959758-902), included in the G1734BA Forensics and Toxicology Triggered MRM Application Kit.

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	1200.00
0.50	95.00	5.00	---	---
1.50	70.00	30.00	---	---
6.50	40.00	60.00	---	---
9.00	5.00	95.00	---	---
10.00	5.00	95.00	---	---
10.10	95.00	5.00	---	---

Stop time is 12 minutes with a post time of 2 minutes.

1260 Infinity LC system

The 1260 Infinity LC system can have a lower backpressure limit (up to 600 bar) and a higher dead volume than the 1290 Infinity LC system.

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	95.00	5.00	0.400	600.00
1.50	95.00	5.00	---	---
2.00	70.00	30.00	---	---
8.50	40.00	60.00	---	---
11.00	5.00	95.00	---	---
12.00	5.00	95.00	---	---
12.10	95.00	5.00	---	---

Stop time is 14 minutes with a post time of 2 minutes.

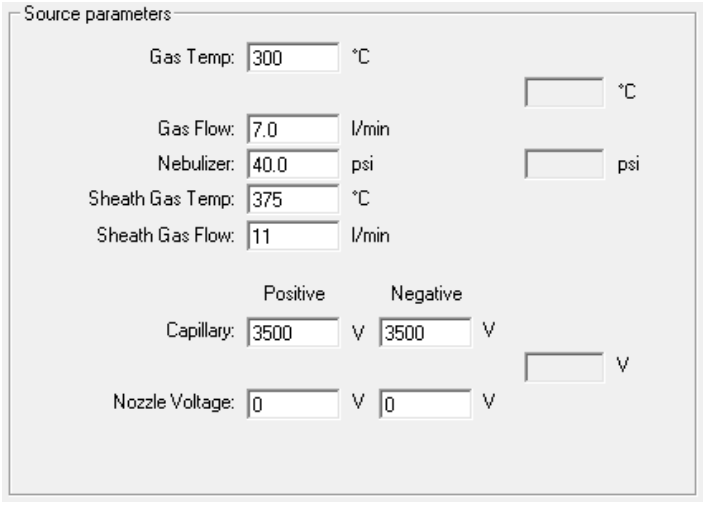
These settings are optimized over the whole Comprehensive Test Mix. For best sensitivity of Submix 5, use pure water and methanol in negative mode.

Step 2. Set up LC/MS ion source parameters

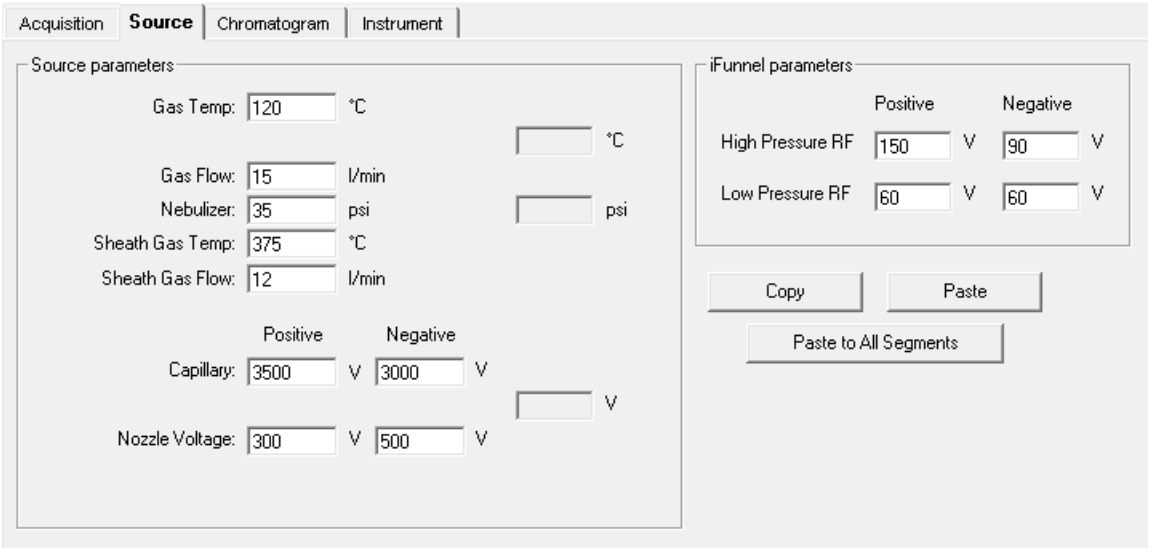
- Set up the ion source parameters in the MS part of the method tab.

  For a multicomponent method, the ion source parameters shown in the next figure are used to achieve the best overall sensitivity for all of the compounds in the Comprehensive Test Mix. You can adjust the method to optimize for individual compounds or submixes.

Ion source parameters for an 6400 Series LC/MS with an Agilent Jet Stream source



Ion source parameters for a 6400 Series LC/MS with Agilent iFunnel technology and an Agilent Jet Stream source



For instruments that are equipped with iFunnel technology, the **Source** tab includes iFunnel parameters. These iFunnel parameters ensure the best overall sensitivity for all of the compounds in the Comprehensive Test Mix. You can use the Source and iFunnel Optimizer program to optimize for individual compounds or submixes. Refer to the MassHunter Data Acquisition for 6400 Series Triple Quadrupole online Help.

Step 3. Set up the MRM method

1 From the *Support Disc*, open the file **MRM_Methods_ForTox.xlsx**.

This spreadsheet file contains several tabs, one for each of the standard mix in the Comprehensive Test Mix.

The Forensics and Toxicology Triggered MRM database contains both positive and negative polarity transitions for some compounds. The better polarity to use for a particular compound is often the one that gives the most selective transitions, not the most sensitive or most abundant transitions.

Selectivity depends on the sample matrix and to a lesser degree the mobile phase composition. The **MRM_Methods_ForTox.xlsx** spreadsheet contains the most generally selective polarity and transitions to use in your dMRM and tMRM methods.

NOTE

During method development, the inclusion of both polarities for one compound is often desirable. Do not include both negative and positive polarity transitions for qualifier and quantifier ions of one compound. The compounds that contain both polarities in the Triggered MRM database must be renamed in the method to "*compoundname*_pos" and "*compoundname*_neg"

When the best polarity and transitions are found for a compound, remove from the method all other transitions for the compound. Then remove "_pos" or "_neg" from the remaining compound name.

Make sure that you remove the secondary transitions that are not required for confirmation. Secondary transitions required for confirmation must be as unique to a particular analysis and/or have intense ion peaks.

NOTE

Some mixes contain two compounds which have one or more common MRM transitions. See Table 1 on page 2 for the elution order of isobaric compound pairs.

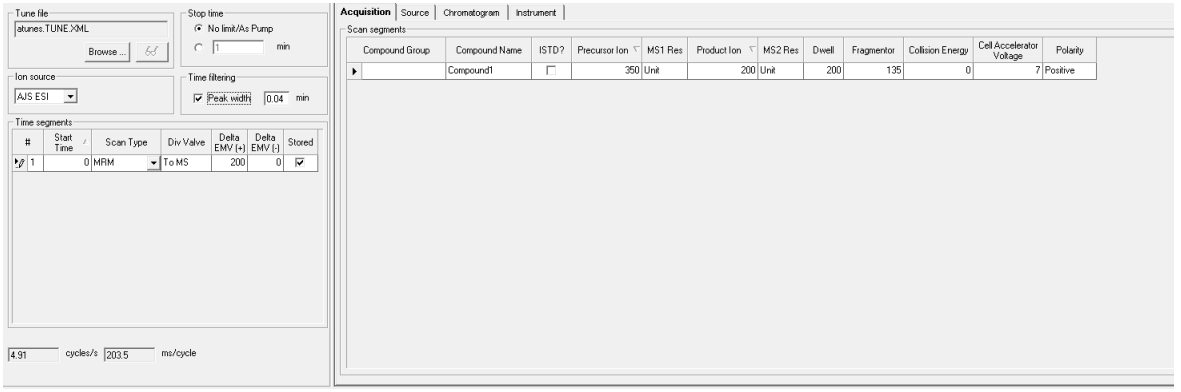
	A	B	C	D	E	F	G	H	I	J	K	L
1	MRM											
2	Compound Group	Compoun	ISTD?	Precursor	MS1 Res	Product Ic	MS2 Res	Dwell	Fragment	Collision E	Cell Accel	Polarity
3	SubMix 1	HU-210	FALSE	387.3	Unit	43.1	Unit	10	151	56	3	Positive
4	SubMix 1	HU-210	FALSE	385.3	Unit	367.2	Unit	10	285	24	3	Negative
5	SubMix 1	HU-210	FALSE	385.3	Unit	301.2	Unit	10	285	36	3	Negative
6	SubMix 1	JWH-200	FALSE	385.2	Unit	155	Unit	10	184	20	3	Positive
7	SubMix 1	JWH-200	FALSE	385.2	Unit	114.1	Unit	10	184	28	3	Positive
8	SubMix 1	JWH-018	FALSE	342.2	Unit	155	Unit	10	199	24	3	Positive
9	SubMix 1	JWH-018	FALSE	342.2	Unit	127	Unit	10	199	52	3	Positive
10	SubMix 1	JWH-250	FALSE	336.2	Unit	121	Unit	10	171	20	3	Positive
11	SubMix 1	JWH-250	FALSE	336.2	Unit	91.1	Unit	10	171	52	3	Positive
12	SubMix 1	CP 47,497-	FALSE	331.3	Unit	313.2	Unit	10	247	24	3	Negative
13	SubMix 1	CP 47,497-	FALSE	331.3	Unit	259.2	Unit	10	247	32	3	Negative
14	SubMix 1	JWH-073	FALSE	328.2	Unit	155	Unit	10	189	24	3	Positive
15	SubMix 1	JWH-073	FALSE	328.2	Unit	127	Unit	10	189	52	3	Positive
16	SubMix 1	CP 47,497	FALSE	317.2	Unit	299.2	Unit	10	232	24	3	Negative
17	SubMix 1	CP 47,497	FALSE	317.2	Unit	245.2	Unit	10	232	32	3	Negative
18	SubMix 1	CBN Cann	FALSE	311.2	Unit	293.2	Unit	10	126	16	3	Positive
19	SubMix 1	CBN Cann	FALSE	311.2	Unit	223	Unit	10	126	20	3	Positive
20	SubMix 1	CBN Cann	FALSE	309.2	Unit	279.1	Unit	10	209	32	3	Negative
21	SubMix 1	CBN Cann	FALSE	309.2	Unit	222	Unit	10	209	48	3	Negative
22	SubMix 1	Ketamine	FALSE	238.1	Unit	179.1	Unit	10	103	12	4	Positive
23	SubMix 1	Ketamine	FALSE	238.1	Unit	125	Unit	10	103	28	4	Positive
24	SubMix 1	HU-210	FALSE	387.3	Unit	71.1	Unit	10	151	24	3	Positive
25												
26												
27												
28												
29												
30												
31												
32												
33												
34												
35												

SubMix 1 | SubMix 2 | SubMix 3 | SubMix 4 | SubMix 5 | SubMix 6 | SubMix 7 | SubMix 8 | SubMi[]

Ready — Average: 127.0363636 Count: 277 Sum: 1

2 Open the MassHunter Data Acquisition program.

3 In the Method Editor window, click **QQQ > Acquisition**.

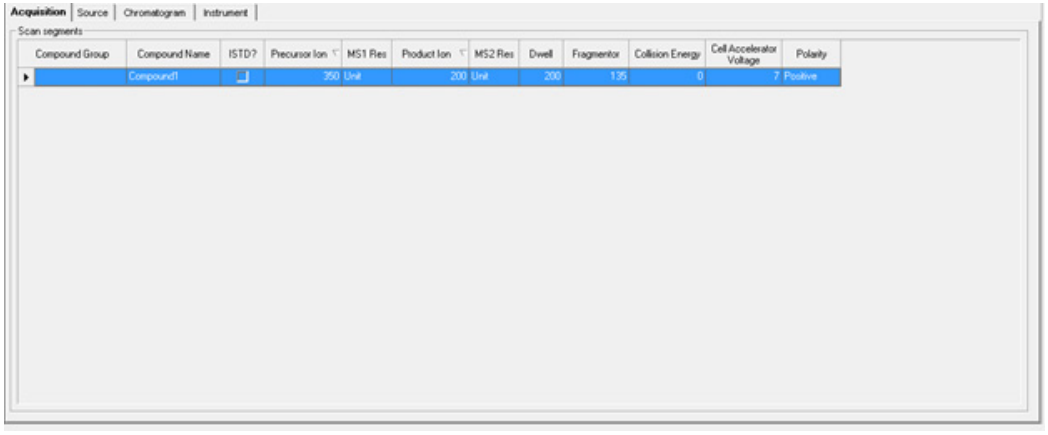


4 In the spreadsheet file, in the **SubMix 1** tab, select all of the cells that contain MRM information. Make sure that you select the two header rows. *Do not select the entire table!*

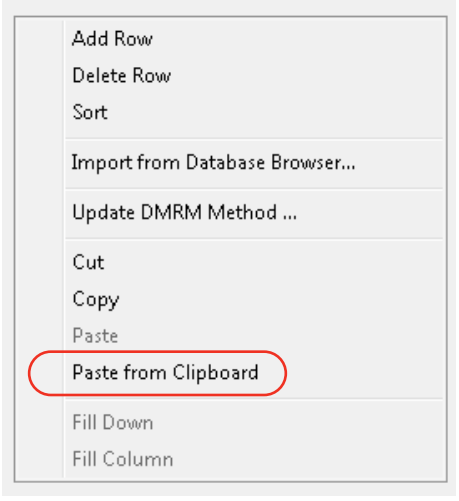
	A	B	C	D	E	F	G	H	I	J	K	L
1	MRM											
2	Compound Group	Compoun	ISTD?	Precursor	MS1 Res	Product Ic	MS2 Res	Dwell	Fragment	Collision E	Cell Accel	Polarity
3	SubMix 1	HU-210	FALSE	387.3	Unit	43.1	Unit	10	151	56	3	Positive
4	SubMix 1	HU-210	FALSE	385.3	Unit	367.2	Unit	10	285	24	3	Negative
5	SubMix 1	HU-210	FALSE	385.3	Unit	301.2	Unit	10	285	36	3	Negative
6	SubMix 1	JWH-200	FALSE	385.2	Unit	155	Unit	10	184	20	3	Positive
7	SubMix 1	JWH-200	FALSE	385.2	Unit	114.1	Unit	10	184	28	3	Positive
8	SubMix 1	JWH-018	FALSE	342.2	Unit	155	Unit	10	199	24	3	Positive
9	SubMix 1	JWH-018	FALSE	342.2	Unit	127	Unit	10	199	52	3	Positive
10	SubMix 1	JWH-250	FALSE	336.2	Unit	121	Unit	10	171	20	3	Positive
11	SubMix 1	JWH-250	FALSE	336.2	Unit	91.1	Unit	10	171	52	3	Positive
12	SubMix 1	CP 47,497-	FALSE	331.3	Unit	313.2	Unit	10	247	24	3	Negative
13	SubMix 1	CP 47,497-	FALSE	331.3	Unit	259.2	Unit	10	247	32	3	Negative
14	SubMix 1	JWH-073	FALSE	328.2	Unit	155	Unit	10	189	24	3	Positive
15	SubMix 1	JWH-073	FALSE	328.2	Unit	127	Unit	10	189	52	3	Positive
16	SubMix 1	CP 47,497	FALSE	317.2	Unit	299.2	Unit	10	232	24	3	Negative
17	SubMix 1	CP 47,497	FALSE	317.2	Unit	245.2	Unit	10	232	32	3	Negative
18	SubMix 1	CBN Cann	FALSE	311.2	Unit	293.2	Unit	10	126	16	3	Positive
19	SubMix 1	CBN Cann	FALSE	311.2	Unit	223	Unit	10	126	20	3	Positive
20	SubMix 1	CBN Cann	FALSE	309.2	Unit	279.1	Unit	10	209	32	3	Negative
21	SubMix 1	CBN Cann	FALSE	309.2	Unit	222	Unit	10	209	48	3	Negative
22	SubMix 1	Ketamine	FALSE	238.1	Unit	179.1	Unit	10	103	12	4	Positive
23	SubMix 1	Ketamine	FALSE	238.1	Unit	125	Unit	10	103	28	4	Positive
24	SubMix 1	HU-210	FALSE	387.3	Unit	71.1	Unit	10	151	24	3	Positive

5 Copy the selected cells. (Press **Ctrl+C** or use the Copy command).

6 In the MassHunter Data Acquisition program, in the first line of the **Scan segments** table, click the leftmost column to select the first line.



7 Right-click and click **Paste from Clipboard**.



The Scan segments table for instruments that are not equipped with iFunnel technology looks similar to the next figure.

Scan segments

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Compound1	☐	350	Unit	200	Unit	200	135	0	7	Positive
SubMix 1	HU-210	☐	387.3	Unit	43.1	Unit	10	151	56	3	Positive
SubMix 1	HU-210	☐	385.3	Unit	367.2	Unit	10	285	24	3	Negative
SubMix 1	HU-210	☐	385.3	Unit	301.2	Unit	10	285	36	3	Negative
SubMix 1	JWH-200	☐	385.2	Unit	155	Unit	10	184	20	3	Positive
SubMix 1	JWH-200	☐	385.2	Unit	114.1	Unit	10	184	28	3	Positive
SubMix 1	JWH-018	☐	342.2	Unit	155	Unit	10	199	24	3	Positive
SubMix 1	JWH-018	☐	342.2	Unit	127	Unit	10	199	52	3	Positive
SubMix 1	JWH-250	☐	336.2	Unit	121	Unit	10	171	20	3	Positive
SubMix 1	JWH-250	☐	336.2	Unit	91.1	Unit	10	171	52	3	Positive
SubMix 1	CP 47,497-C8 homolc	☐	331.3	Unit	313.2	Unit	10	247	24	3	Negative
SubMix 1	CP 47,497-C8 homolc	☐	331.3	Unit	259.2	Unit	10	247	32	3	Negative
SubMix 1	JWH-073	☐	328.2	Unit	155	Unit	10	189	24	3	Positive
SubMix 1	JWH-073	☐	328.2	Unit	127	Unit	10	189	52	3	Positive

The Scan segments table for instruments that are equipped with iFunnel technology looks similar to the next figure.

Scan segments

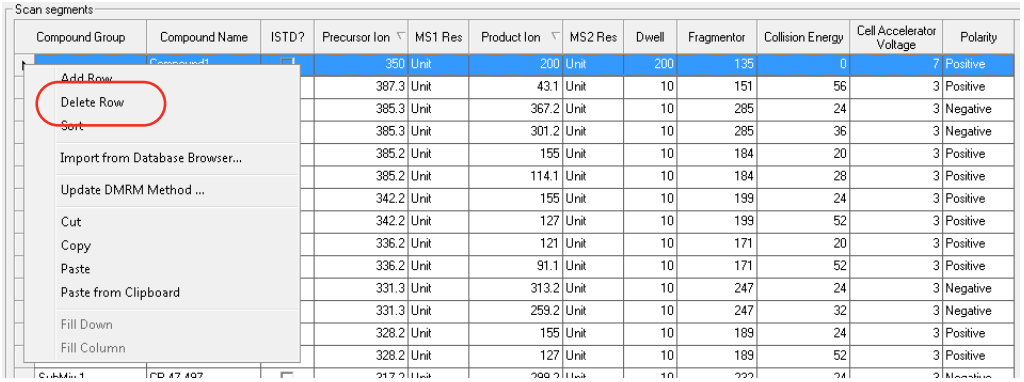
Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
SubMix 1	JWH-200	☐	385.2	Unit	114.1	Unit	10	380	28	3	Positive
SubMix 1	JWH-018	☐	342.2	Unit	155	Unit	10	380	24	3	Positive
SubMix 1	JWH-018	☐	342.2	Unit	127	Unit	10	380	52	3	Positive
SubMix 1	JWH-250	☐	336.2	Unit	121	Unit	10	380	20	3	Positive
SubMix 1	JWH-250	☐	336.2	Unit	91.1	Unit	10	380	52	3	Positive
SubMix 1	CP 47,497-C8 homolc	☐	331.3	Unit	313.2	Unit	10	380	24	3	Negative
SubMix 1	CP 47,497-C8 homolc	☐	331.3	Unit	259.2	Unit	10	380	32	3	Negative
SubMix 1	JWH-073	☐	328.2	Unit	155	Unit	10	380	24	3	Positive
SubMix 1	JWH-073	☐	328.2	Unit	127	Unit	10	380	52	3	Positive
SubMix 1	CP 47,497	☐	317.2	Unit	299.2	Unit	10	380	24	3	Negative
SubMix 1	CP 47,497	☐	317.2	Unit	245.2	Unit	10	380	32	3	Negative
SubMix 1	CBN Cannabinol	☐	311.2	Unit	293.2	Unit	10	380	16	3	Positive
SubMix 1	CBN Cannabinol	☐	311.2	Unit	223	Unit	10	380	20	3	Positive
SubMix 1	CBN Cannabinol	☐	309.2	Unit	279.1	Unit	10	380	32	3	Negative
SubMix 1	CBN Cannabinol	☐	309.2	Unit	222	Unit	10	380	48	3	Negative
SubMix 1	Ketamine	☐	238.1	Unit	179.1	Unit	10	380	12	4	Positive
SubMix 1	Ketamine	☐	238.1	Unit	125	Unit	10	380	28	4	Positive
▶ SubMix 1	HU-210	☐	387.3	Unit	71.1	Unit	10	380	24	3	Positive

Note that polarity switching is supported for MRM, but the transitions within each compound must have the same polarity. Polarity switching (positive and negative transitions within a compound) is not supported.

8 Remove the first compound from the Scan segments table:

a Select the first line. For the first method that you create, the line contains the compound **Compound1**. For the other methods, the line contains a compound from the previous submix.

b Right-click and click **Delete Row**. See the next figure.



The final method now looks like the next figure.

Scan segments

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
SubMix 1	HU-210		387.3	Unit	43.1	Unit	10	151	56	3	Positive
SubMix 1	HU-210		385.3	Unit	367.2	Unit	10	285	24	3	Negative
SubMix 1	HU-210		385.3	Unit	301.2	Unit	10	285	36	3	Negative
SubMix 1	JWH-200		385.2	Unit	155	Unit	10	184	20	3	Positive
SubMix 1	JWH-200		385.2	Unit	114.1	Unit	10	184	28	3	Positive
SubMix 1	JWH-018		342.2	Unit	155	Unit	10	199	24	3	Positive
SubMix 1	JWH-018		342.2	Unit	127	Unit	10	199	52	3	Positive
SubMix 1	JWH-250		336.2	Unit	121	Unit	10	171	20	3	Positive
SubMix 1	JWH-250		336.2	Unit	91.1	Unit	10	171	52	3	Positive
SubMix 1	CP 47,497-C8 homolc		331.3	Unit	313.2	Unit	10	247	24	3	Negative
SubMix 1	CP 47,497-C8 homolc		331.3	Unit	259.2	Unit	10	247	32	3	Negative
SubMix 1	JWH-073		328.2	Unit	155	Unit	10	189	24	3	Positive
SubMix 1	JWH-073		328.2	Unit	127	Unit	10	189	52	3	Positive

9 Click **Method > Save As** (or click  in the Method Editor toolbar) and save the method as **ForTox_MRM_Mix1.m**.

10 Delete all but one compound from the Scan segments table.

The Scan segments table cannot be empty. You must leave one compound in the table.

11 Repeat step 4 through step 10 for each of the submixes to be analyzed.

When you save each method, use a name that reflects the submix name, such as **ForTox_MRM_Mix2.m** for the values in the **SubMix 2** tab.

Step 4. Set up a worklist to run the submixes

- Set up the worklist as shown in Figure 2. Include all submixes. Inject the first standard twice to allow the system to come to equilibrium.

		Sample Name	Sample Position	Method	Data File	Sample Type	Level Name
1	✓	SubMix_01	P1-A1	ForTox_MRM_Mix_1.m	to_delete.d	Calibration	1
2	✓	SubMix_01	P1-A1	ForTox_MRM_Mix_1.m	SubMix_01.d	Calibration	1
3	✓	SubMix_02	P1-A2	ForTox_MRM_Mix_2.m	SubMix_02.d	Calibration	1
4	✓	SubMix_03	P1-A3	ForTox_MRM_Mix_3.m	SubMix_03.d	Calibration	1
5	✓	SubMix_04	P1-A4	ForTox_MRM_Mix_4.m	SubMix_04.d	Calibration	1
6	✓	SubMix_05	P1-A5	ForTox_MRM_Mix_5.m	SubMix_05.d	Calibration	1
7	✓	SubMix_06	P1-A6	ForTox_MRM_Mix_6.m	SubMix_06.d	Calibration	1
8	✓	SubMix_07	P1-A7	ForTox_MRM_Mix_7.m	SubMix_07.d	Calibration	1

Figure 2 Worklist

After you acquire the MRM data for each submix, you can create dMRM and tMRM methods and optimize the parameters. Follow the steps in *Quick Start Guide*.

Refer to the *Quick Start Guide* for this database, or the MassHunter Data Acquisition for 6400 Series Triple Quadrupole LC/MS *Familiarization Guide* or *online Help*.

NOTE

In a standard method development workflow, the trigger parameters in the tMRM methods such as Threshold, Trigger Entrance, Trigger Delay and Trigger Window are created for analysis of standards in solvent. These trigger parameters must be rechecked for matrix samples as signal abundances and noise characteristics of some MRM transitions can be different in samples with complex matrix compared to solvent standards.

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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 to create Dynamic MRM (dMRM) and Triggered MRM (tMRM) methods for the Comprehensive Test Mix.

Revision A, March 2015

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Agilent Technologies