MassHunter VistaFlux Software

Workflow Guide

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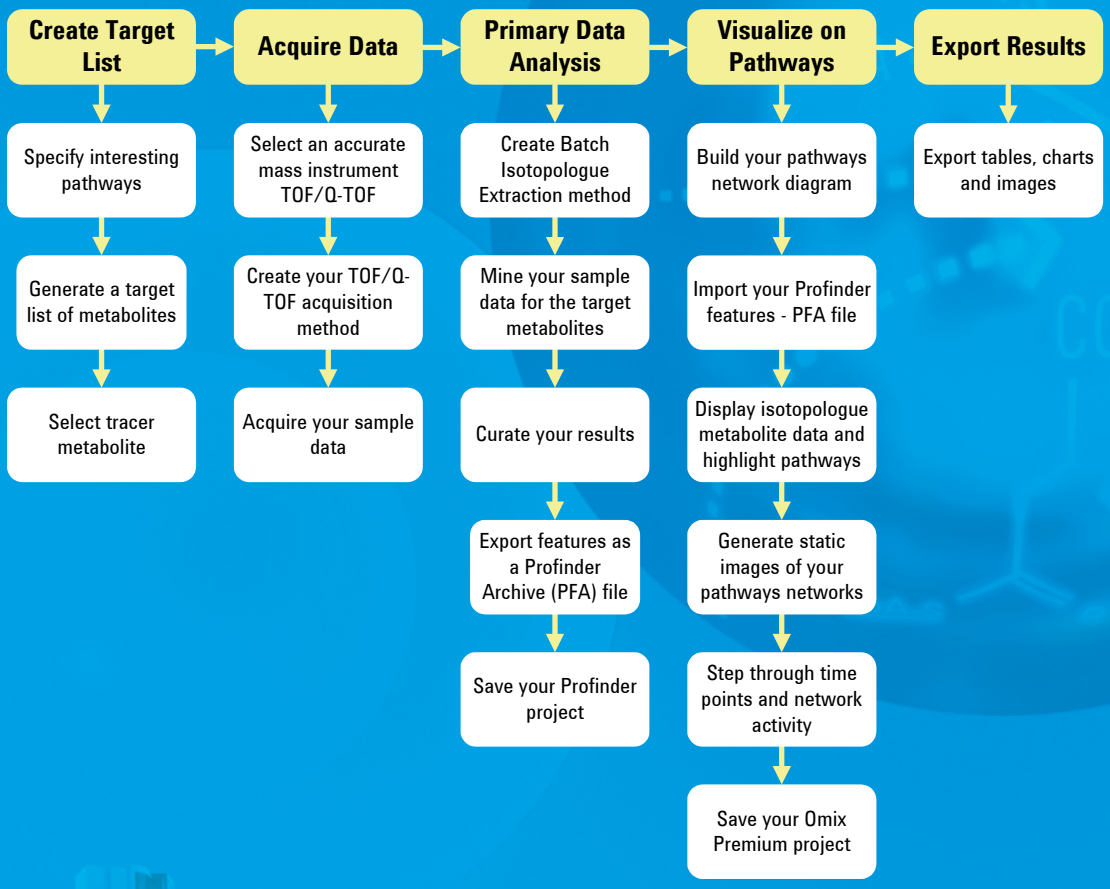

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Notices

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See "Acknowledgments and Citations" on page 145.

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About this guide

- This MassHunter VistaFlux Software workflow guide is part of a series of workflow guides developed to help you to analyze your sample data. Other workflow guides available in this series include:

  Agilent Metabolomics Workflow - Discovery Workflow Guide

  Integrated Biology with Agilent Mass Profiler Professional Workflow Guide

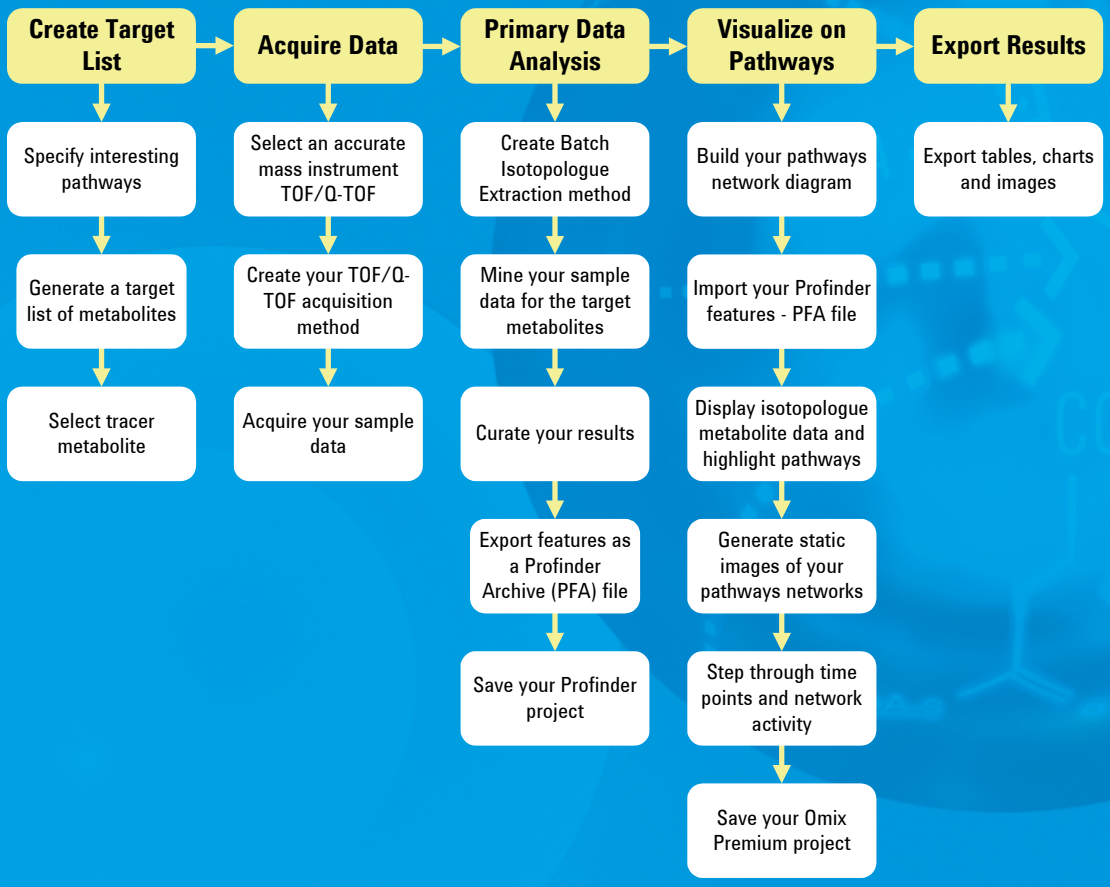
  Class Prediction with Agilent Mass Profiler Professional Workflow Guide

  Agilent Lipidomics Workflow Guide

- This workflow guide presents the steps involved in a qualitative flux analysis:
  - Create a target metabolite list using MassHunter Pathways to PCDL and MassHunter PCDL Manager
  - Extract the target metabolite features and isotopologues from your sample data using MassHunter Profinder.
  - View the results on pathways network diagrams using Omix Premium.
- Formatting of text that appears in the left-hand margin helps guide you through the operations.
- Operations are illustrated with flow charts that show you how the wizards are navigated based on your experiment and selections.

Before you begin

Make sure you read and understand the information in this chapter and have the necessary computer equipment and software before you start your qualitative flux analysis.



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Introduction

Qualitative flux analysis comprises the steps to identify target metabolites of interest for your experiment, create visual pathways networks for your experiment, acquire accurate mass data from your samples, extract accurate isotopic metabolite information from the sample data, and then view the results on network diagrams of the pathways involved in your experiment. The chapters of this workflow guide follow the flow illustrated in Figure 1.

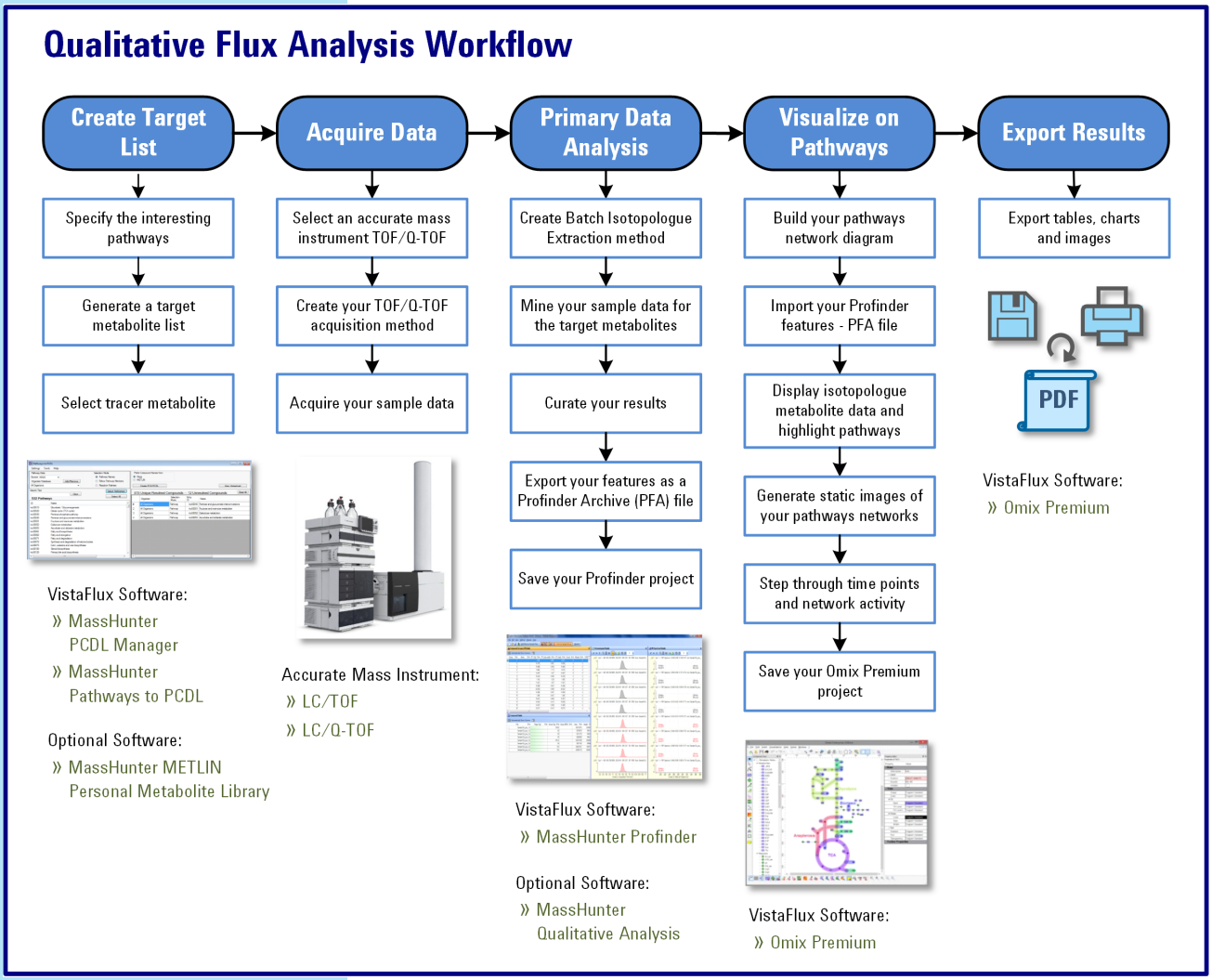

Figure 1 *Steps to perform a qualitative flux analysis.*

This workflow guide is part of a series of workflow guides developed to help you to analyze your sample data. Other workflow guides available in this series include *Agilent Metabolomics Workflow - Discovery Workflow Guide* (5990-7067EN), *Integrated Biology with Agilent Mass Profiler Professional Workflow Guide* (5991-1909EN), *Class Prediction with Agilent Mass Profiler Professional Workflow Guide* (5991-1911EN), and *Agilent Lipidomics Workflow Guide* (5991-1643EN).

To increase your confidence in obtaining reliable and statistically significant results, review the chapter "Prepare for an experiment" in the *Agilent Metabolomics Work-*

flow - Discovery Workflow Guide and make sure your analysis includes a carefully thought-out experimental design that includes the collection of replicate samples.

What is flux analysis?

Metabolomics is a powerful technique for understanding biological systems by measuring the abundance of metabolites; however, data interpretation is often complicated by a lack of dynamic information. For example, an increase in metabolite abundance can result from either increased production (pathway is up-regulated) or decreased consumption (pathway is down-regulated). Similarly, significant changes in flux through a pathway can possibly not result in altered abundance of metabolite intermediates at all. For example, the rate of metabolite production and turnover can have changed significantly, but the total metabolite pool abundances can have not changed due to homeostasis. Stable isotope tracing has tremendous potential to help address these situations and drive a deeper understanding of biological systems through contextualization of the interconversion of metabolites using pathways. Qualitative flux analysis highlights the relative rate at which these reactions take place.

In qualitative flux analysis, a stable isotope labeled tracer (typically containing ^{13}C, ^{15}N, or ^{2}H) is introduced into the biological system and results in changes in the natural isotopic pattern of downstream metabolites. Following analysis by LC/MS, local metabolic fluxes can be investigated by mining the data using a target list derived from known metabolic pathways. Isotopologues (metabolites differing only in isotopic composition) are measured for each target compound and this information is used to track metabolic flux.

Qualitative flux analysis presents multiple analytical challenges such as how to mine the target metabolites, account for isotopologues, correct for naturally occurring isotopes, and visualize the results in a biological context. MassHunter VistaFlux is designed to meet these challenges and is a qualitative flux analysis solution for MS-only data acquired on Agilent TOF-based high resolution LC/MS systems.

Metabolomics tells us which investigated pathways are active in your experiment:

- Results from samples are associated with your experimental conditions.
- Metabolite abundance, isotopologue incorporation, and fold change between conditions are calculated and visualized.
- Metabolites are often part of more than one pathway.

Qualitative flux analysis identifies active pathways and affected metabolic enzymes in your experiment:

- Introduction of an isotopically labeled sugar or amino acid leads to the gradual appearance of metabolite isotopologues.
- By monitoring the isotopologues of the metabolites in your experiment, you can identify the active pathways in your experiment.
- Isotopologue data that indicates a change in metabolic flux rate can suggest that the enzyme activity may have changed due to regulation or mutation in the gene product itself.

The Agilent MassHunter VistaFlux Software provides an intuitive workflow to perform your qualitative flux analysis and visualize your results on pathways network

diagrams. The MassHunter tools necessary to perform a qualitative flux analysis are referred to collectively as MassHunter VistaFlux.

The qualitative analysis workflow, see Figure 2, is enabled by isotopologue tracing from an experiment employing an isotopically labeled source, such as ^{13}C labeled glucose, glutamine, amino acid, or lipid. Data is acquired using a high resolution, accurate mass instrument that has good isotopic ratio fidelity, such as a TOF or Q-TOF mass spectrometer. After you have identified the compounds (metabolites) you want to search for, you can mine the data based on the isotope patterns of the most important compounds using batch isotopologue feature extraction available in Profinder. The results are viewed in Omix Premium ("Visualize on pathways" on page 67), a visualization platform used to view your data within the context of biochemical networks.

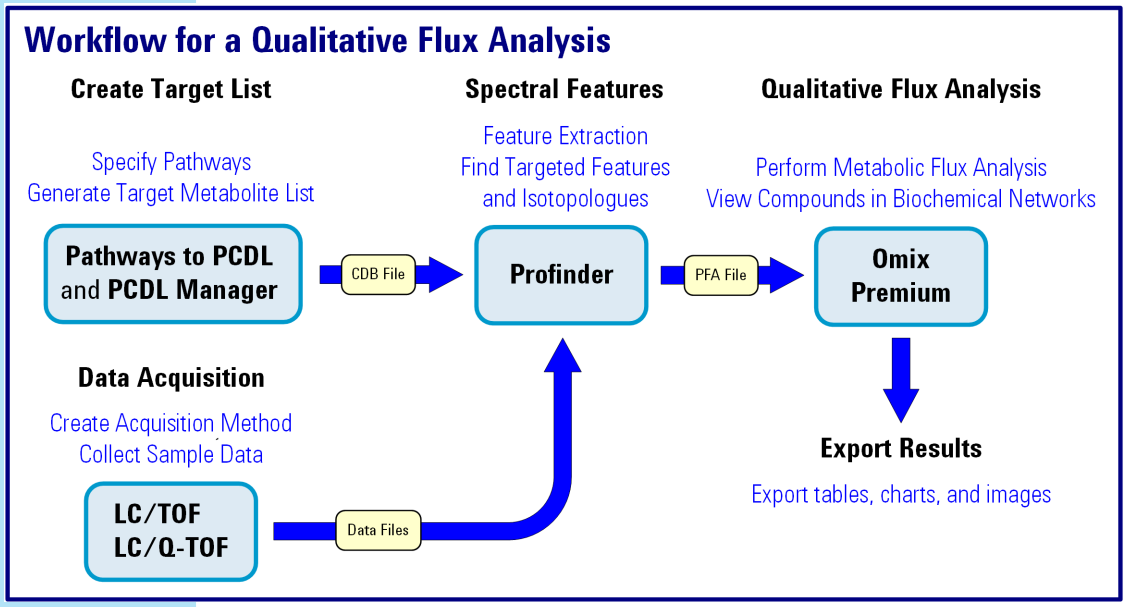

Figure 2 *Illustration of the qualitative flux analysis workflow.*

Isotopes, isotopologues, and isotopomers

Mass spectrometry is well known to use the presence of naturally occurring isotopes to improve feature quality and mass measuring accuracy. In a qualitative flux analysis isotopic measurements are important during an experiment employing an isotopically enriched compound. Samples are collected and analyzed to assess the effects of the experimental conditions on the isotopic enrichment of the metabolites. Isotopic enrichment during a qualitative flux analysis experiment leads to isotopomers and isotopologues. MassHunter VistaFlux Software mines your sample data for target metabolites and isotopologues, corrects the isotopologue results for the presence of naturally occurring isotopes, and helps you visualize the results in a biological context.

Isotopologues are molecules that contain the same molecular formula and structure but differ in their isotopic composition through the substitution of one or more atoms with a different isotope. The exact location of the isotope in the molecule,

while important chemically, is not important in flux analysis, just the number of isotopes in the molecule. Isotopologues can be identified using single-stage MS.

Isotopomers are molecules that contain the same molecular formula, structure, and number of isotopes but differ in the specific atomic location of the isotopes in the molecular structure. Isotopomers can be identified using advanced MS/MS techniques.

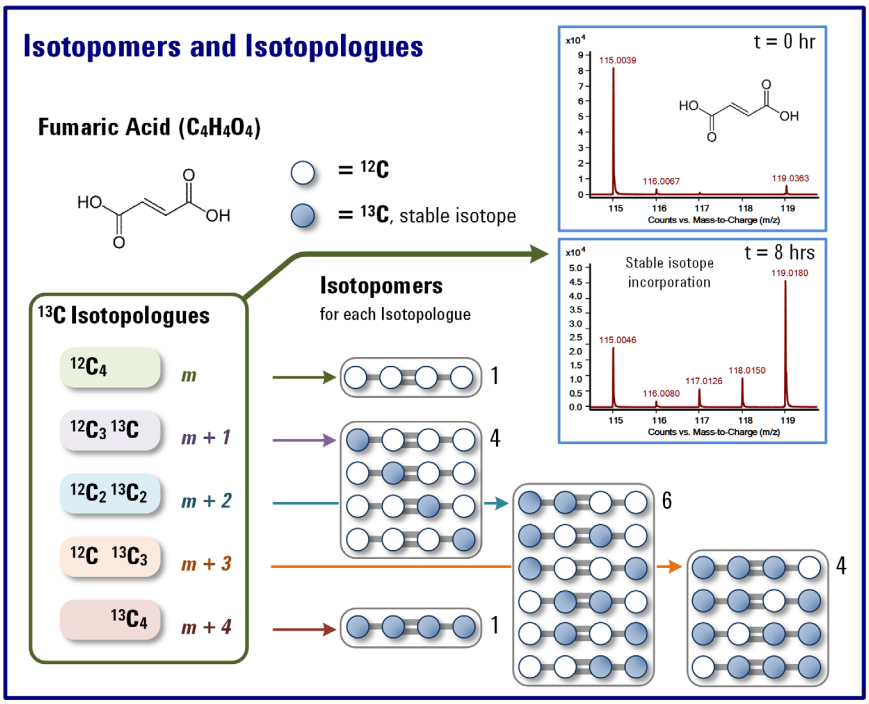

Figure 3 *An illustration of how two stable carbon isotopes in a four-carbon molecule relate to isotopomers and isotopologues. Isotopologues are viewed in mass spectra during flux analyses.*

A simple four carbon molecule, fumaric acid ($C_4H_4O_4$), is used to explain the relationship of isotopes to isotopomers, isotopologues, and mass spectra. The most abundant isotope of carbon is ^{12}C. However, ^{13}C, also stable, is not nearly as naturally abundant as ^{12}C; ^{13}C has a natural occurrence of 1.1% of ^{12}C. For simplicity, naturally occurring ^{13}C is considered to be negligible; therefore, the mass of the naturally occurring four-^{12}C molecule is m (represented as $^{12}C_4$), and there are no positional differences among the isotopes of the carbon atoms.

When a single ^{13}C atom is substituted for one ^{12}C atom, four locations are possible where the ^{13}C atom can be placed (isotopomers as shown in Figure 3), and each isotopomer has a mass of *m* + 1 ($^{12}C_3\,^{13}C$). When two of the ^{12}C atoms are replaced with ^{13}C atoms, six isotopomers are possible, and each of the doubly substituted molecules has a mass of *m* + 2 ($^{12}C_2\,^{13}C_2$). When three of the ^{12}C atoms are replaced with ^{13}C atoms, four isotopomers are possible, and each isotopomer has a mass of *m* + 3 ($^{12}C\,^{13}C_3$). Finally, when all four of the ^{12}C atoms are replaced with ^{13}C atoms, only a single arrangement with a mass of *m* + 4 ($^{13}C_4$) exists. The five different

masses *m*, *m* + 1, *m* + 2, *m* + 3, and *m* + 4 represent the masses of the five isotopologues visible in the resulting mass spectra.

The isotopologue data contained in the mass spectra from your samples, collected over time during your experiment (replicate samples collected for each time period constitute a group), can be visualized using Omix Premium within the context of the biochemical networks under study. Qualitative flux analysis in-turn helps you identify pathways whose changes in metabolic activity associate with conditions that are controlled in your experiment design.

Overview of the example experiment

The example experiment used in this workflow guide is based on a mutation of the isocitrate dehydrogenase 2 (IDH2) enzyme associated with chondrosarcoma, a bone cancer consisting of a malignant tumor from cartilage-producing cells. IDH2 catalyzes the conversion of isocitrate to α-ketoglutarate (α-KG). In some tumors, a mutant form of this enzyme, a neo-enzyme, gains a function and instead catalyzes a reaction consuming α-KG and producing 2-hydroxyglutarate. Three replicate samples were acquired for each of five time points within an experiment; therefore, the experiment consists of five sample groups with three replicates for a total of fifteen sample data files.

IDH2 catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate outside of the context of the tricarboxylic acid (TCA) cycle (Figure 4 on page 11). IDH2 uses the cofactor NADP to achieve its biological activity. During studies of cell lines that exhibit chondrosarcoma from the mutation of IDH2, a novel compound, 2-hydroxyglutarate, appears as a byproduct of the metabolism. IDH2 enzymes exhibiting the mutation produce 2-hydroxyglutarate from α-KG, thereby competing with the conversion of α-KG to succinyl-coenzyme A, and subsequently with succinic acid and additional downstream metabolites in the TCA cycle. When a chondrosarcoma tumor has been established, elevated levels of glutathione can be associated with cancerous activity.

Qualitative flux analysis begins by isotopologue monitoring in an experiment using a tracer. In the example experiment the tracer is labeled $^{13}C_5$-glutamine (U-^{13}C-Gln) that directly feeds the formation of α-KG in the TCA cycle. A list of the compounds that are part of this example experiment are presented in Table 1 on page 11.

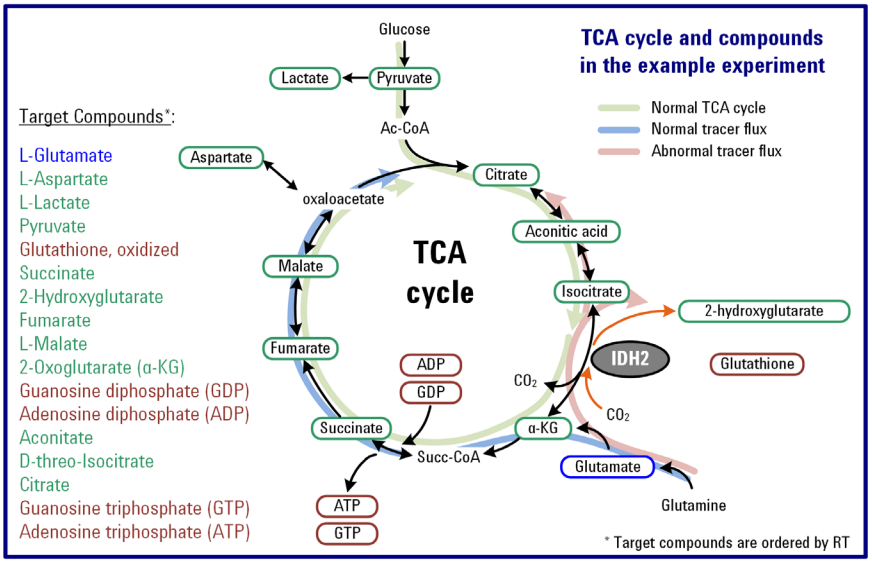

Figure 4 *Illustration of the TCA cycle with the target metabolites listed and highlighted in the network diagram*

	Target Compound Name	Formula	Mass	RT (min)▲	CAS	HMP	KEGG	LMP	Source[1]
1	L-Glutamate	$C_5H_9NO_4$	147.05316	2.26	56-86-0	HMDB00148	C00025		AMP
2	L-Aspartic Acid	$C_4H_7NO_4$	133.03751	2.29	56-84-8	HMDB00191	C00049		AMP
3	L-Lactic acid	$C_3H_6O_3$	90.03169	2.93	79-33-4		C00186	LMFA01050410	AMP
4	Pyruvate	$C_3H_4O_3$	88.01604	3.34	127-17-3	HMDB00243	C00022	LMFA01060077	AMP
5	Glutathione, oxidized	$C_{20}H_{32}N_6O_{12}S_2$	612.15196	4.84	27025-41-8		C00127		AMP
6	Succinic acid	$C_4H_6O_4$	118.02661	5.05	110-15-6	HMDB00254	C00042	LMFA01170043	TCA
7	2-Hydroxyglutarate	$C_5H_8O_5$	148.03717	5.11	2889-31-8		C02630		AMP
8	Fumaric acid	$C_4H_4O_4$	116.01096	5.13	110-17-8	HMDB00134	C00122		TCA
9	L-Malic acid	$C_4H_6O_5$	134.02152	5.13	97-67-6	HMDB00156	C00149		TCA
10	Oxoglutaric acid (α-KG)	$C_5H_6O_5$	146.02152	5.24	328-50-7	HMDB00208	C00026		TCA
11	Guanosine diphosphate (GDP)	$C_{10}H_{15}N_5O_{11}P_2$	443.02433	5.83	146-91-8	HMDB01201	C00035		TCA
12	Adenosine diphosphate (ADP)	$C_{10}H_{15}N_5O_{10}P_2$	427.02942	5.93	58-64-0	HMDB01341	C00008		TCA
13	Aconitic acid	$C_6H_6O_6$	174.01644	6.10	499-12-7	HMDB00072	C00417		TCA
14	D-threo-Isocitric acid	$C_6H_8O_7$	192.02700	6.10	6061-97-8	HMDB01874	C00451		TCA
15	Citric acid	$C_6H_8O_7$	192.02700	6.10	77-92-9	HMDB00094	C00158		TCA
16	Guanosine triphosphate (GTP)	$C_{10}H_{16}N_5O_{14}P_3$	522.99066	6.57	86-01-1	HMDB01273	C00044		TCA
17	Adenosine triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	506.99575	6.63	987-65-5	HMDB00538	C00002		TCA

▲ Table sorted by this column, increasing values

[1] Workflow source for each compound:

TCA - BioCyc/MetaCyc Tricarboxylic acid (TCA) cycle pathway

AMP - Agilent Metlin PCDL

Table 1 *Compounds that are part of the example experiment*

After administration of the tracer into the cell medium, samples are collected and analyzed over time to assess the isotopic enrichment of the metabolites downstream from the glutamine. Isotopic enrichment leads to isotopologues of the metabolites and are measurable in the acquired mass spectra. Viewing the isotopo-

logue results in Profinder and Omix Premium helps you identify the isotopic enrichments rates, flux.

More information

The *MassHunter VistaFlux Software Workflow Guide* is part of the collection of Agilent manuals, help, application notes, and videos. The current collection of manuals and help are valuable to users who understand flux analysis, class prediction, and metabolomics workflows and who requires familiarization with the Agilent software tools. Video tutorials are also available and provide step-by-step instructions to analyze example sample data files. This workflow provides a step-by-step overview of performing a qualitative flux analysis using MassHunter VistaFlux Software.

The following selection of publications provides materials related to MassHunter VistaFlux Software used to analyze your sample data:

- Manual: *MassHunter Pathways to PCDL Software Quick Start Guide* (G6825-90008, Revision B, May 2016)
- Manual: *MassHunter Personal Compound Database and Library Manager Quick Start Guide* (G3336-90022, Revision A, September 2014)
- Manual: *MassHunter PCDL for Qualitative Analysis Familiarization Guide* (G5883-90002, Revision B, February 2016)
- Manual: *MassHunter METLIN Metabolite PCD/PCDL Quick Start Guide* (G6825-90009, Revision A, October 2014)
- Manual: *MassHunter Profinder Software Quick Start Guide* (G3835-90027, Revision A, April 2016)
- Manual: *MassHunter Workstation Software Qualitative Analysis Familiarization Guide* (G3336-90023, Revision A, September 2014)

A complete list of references can be found in "References" on page 143.

NOTE

In this guide, some references are listed by publication numbers while others are listed by document name. You can easily download the documents from the Agilent literature library.

Go to the Agilent literature library at www.agilent.com/search and type the publication number or the publication title in the search box.

Then click the **Search** button.

"Definitions" on page 134 contains a list of terms and their definitions as used in this workflow.

Required items

This workflow performs best when using the hardware and software described in the "required" sections below.

Required hardware

See the *G4992-90005 Site Preparation Checklist for MassHunter VistaFlux Ver 1.0* for the required hardware for MassHunter VistaFlux Software.

Required software

VistaFlux Software and sample data:

- Agilent MassHunter Pathways to PCDL Software B.07.00
- Agilent MassHunter PCDL Manager B.07.00 SP2
- Agilent MassHunter Profinder B.08.00
- Omix Premium Version 1.9
- "Data" folder containing VistaFlux example data and project files:
  - > Folder - "CS Gln Flux" data folder containing 15 sample data files
  - > Folder - "Profinder-Gln-IDH2.m" Profinder method folder
  - > File - "BioCyc metabolites.cdb" PCDL for the example data
  - > File - "CS-Gln-IDH2.pfa" Profinder archive file
  - > File - "CS-Gln-IDH2.Profinder" Profinder project file
  - > File - "TCA-IDH2.omx" Omix Premium project file
  - > File - "TCA-BioCyc-IDH2.cdb" metabolite compound database

Optional software

Optional MassHunter software you can use with VistaFlux Software:

- Agilent MassHunter METLIN Personal Metabolite Library B.07.00
- Agilent MassHunter Qualitative Analysis B.07.00

Target metabolite discovery for your experiment can benefit from access to online pathways databases, such as BioCyc, KEGG, MetaCyc, and WikiPathways.

Next step...

You have now completed reviewing background materials for the VistaFlux Software workflow. In the next workflow step you create your target list.

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Create target list

This chapter presents a sequence of steps to create a custom personal compound database and library from metabolites present in pathway content contained within popular databases.

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Introduction to MassHunter PCDL Manager 17
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PCDL overview

Creating a personal compound database (PCD) or a personal compound database and library (PCDL) containing one or more compounds that participate in, or are associated with, metabolic reactions in selected pathways is the first step in the VistaFlux Software workflow. During this step you use the following MassHunter products that are part of the MassHunter VistaFlux Software:

MassHunter Pathways to PCDL: Create an Agilent metabolite compound database and library from popular databases such as BioCyc/MetaCyc, KEGG, and WikiPathways.

MassHunter PCDL Manager: Search, add, remove, and edit compounds within a PCD/PCDL database and create new databases from prior databases.

Terminology

Compound database: MassHunter PCD and PCDL files are also referred to as a compound database. Compound database file names have an extension .cdb.

Compound names: Many compounds are commonly known by their salts. The mass spectrometer, however, detects the anion or cation portion of the salt, rather than the neutral salt. Compound database entries may contain the familiar compound names, but the empirical formulae reflect the detectable cation or anion portion of the molecule rather than the formula of the neutral compound salt. For example, the full name Mg-ATP (magnesium adenosine 5′-triphosphate salt) and Ca-ATP (calcium adenosine 5′-triphosphate salt) may be used for identification in the compound database, even though the mass and formula only includes the ATP anion (adenosine triphosphate).

PCD: An accurate mass compound database. The database may or may not contain retention time data.

PCDL: An accurate mass compound database that also contains an MS/MS accurate mass spectral database (spectral library).

Introduction to MassHunter Pathways to PCDL

MassHunter Pathways to PCDL (Figure 5) facilitates the creation of custom Agilent PCDLs from metabolites present in pathway content culled from popular databases such as BioCyc/MetaCyc, KEGG (with the required license you provide), and WikiPathways. You can filter and select pathways based on database, organism, and/or custom text entries.

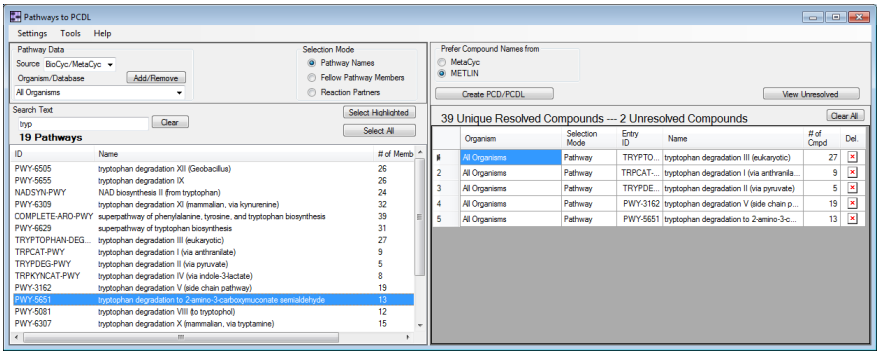


Figure 5 *MassHunter Pathways to PCDL*

The databases created with Pathways to PCDL can be modified using MassHunter PCDL Manager and used for targeted data acquisition and data analyses. Results from targeted analyses can be mapped onto biological pathways maps, as described in later steps of this workflow.

For more information

See the *MassHunter Pathways to PCDL Software Quick Start Guide* for more information.

Introduction to MassHunter PCDL Manager

The MassHunter PCDL Manager (Figure 6) helps you manage the compounds and compound content within PCDs and PCDLs. Some of the actions that can performed on a PCD/PCDL include:

- Search for compounds using text, formula, accurate mass, and retention time.
- Search and view MS/MS spectra in PCDLs.
- Create and edit custom PCDs and PCDLs, including adding proprietary compounds, retention times, and MS/MS spectra.
- Import mass lists with retention time in the form of a TXT or CSV file.
- Load spectra from either a CEF file or copy-and-paste of mass spectra from MassHunter Qualitative Analysis software.
- Link to websites for more information on compounds.

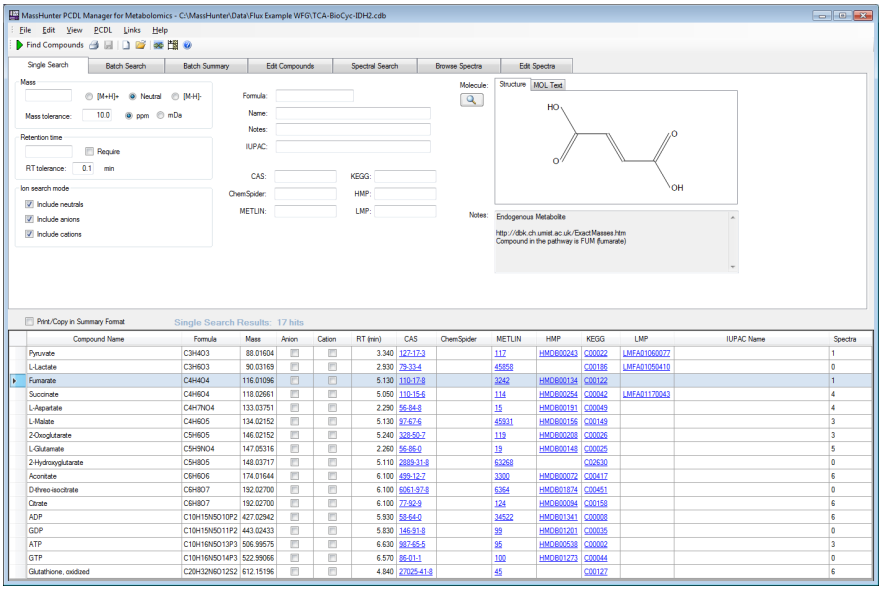


Figure 6 *MassHunter PCDL Manager*

For more information

See the *MassHunter Personal Compound Database and Library Manager Quick Start Guide* for more information.

Specify the interesting pathways

The first step in the VistaFlux Software workflow is to specify the pathways of interest and create a metabolite database using MassHunter Pathways to PCDL. While the initial metabolite database created in this section may contain more metabolites than really necessary to be targeted, "Generate a target metabolite list" on page 27 guides you through the steps to manage the metabolites in your database.

Launch Pathways to PCDL

A brief overview for Pathways to PCDL is presented in the section "Introduction to MassHunter Pathways to PCDL" on page 16.

1. Start Pathways to PCDL software.

   a Double-click the Pathways to PCDL icon located on your desktop, or click **Start > All Programs > Agilent > MassHunter Workstation > Pathways to PCDL > Pathways to PCDL**.

   b Skip to "Create a custom PCDL" on page 20 if you have previously used Pathways to PCDL and are familiar with the user interface and workflow.

2. Select the reference database (first-time use).

   The first time you run Pathways to PCDL on a PC you are prompted to set a reference METLIN database folder and filename. You are not required to have a METLIN database and can skip this step, click **Cancel**.

   a Click **Select** in the **Select Reference METLIN PCD/PCDL** dialog box (Figure 7).

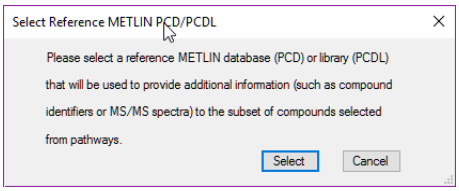


Figure 7 *Select Reference METLIN PCD/PCDL dialog box*

   b Navigate to the folder that contains your METLIN database, or any other personal compound database, in the **Please specify the reference METLIN PCD or PCDL (*.cdb) file** dialog box (Figure 8).

   c Click on the database file name to select your database.

   d Click **Open**.

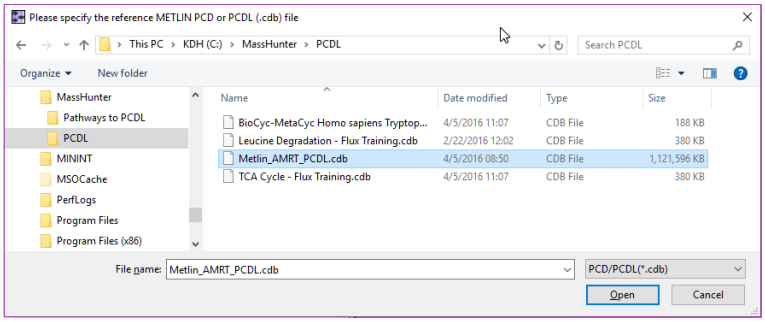


Figure 8 *Please specify the reference METLIN PCD or PCDL (*.cdb) file dialog box*

Review the Pathways to PCDL user interface

The main functional areas of Pathways to PCDL are shown in Figure 9.

The Pathways to PCDL window consists of two parts: *Menu Bar* and *Display Pane*. The *Display Pane* is divided into the *Pathways List* and the *Selected Pathways List* to help you visualize your progress as you select pathways to create your PCDL. The number of pathways and compounds that meet your criteria are shown above each table in the *Display Pane*.

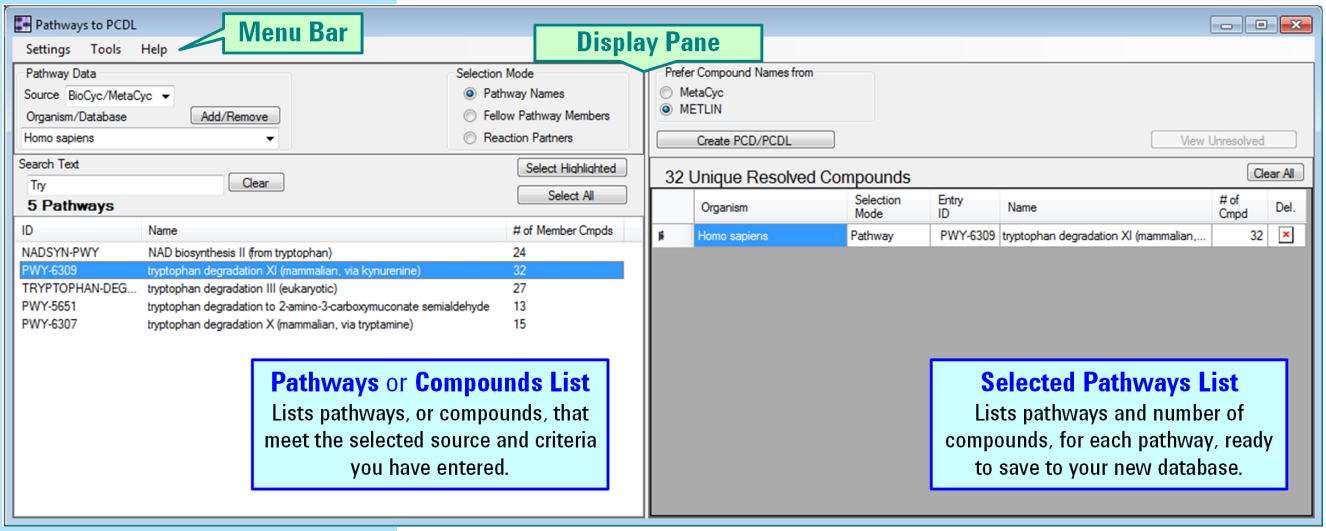


Figure 9 *Screen areas of MassHunter Pathways to PCDL*

Review the Pathways to PCDL workflow

The Pathways to PCDL Workflow shown in Figure 10 presents the order of a typical interaction with the Pathways to PCDL software to create a target metabolite database, described in section "Specify the interesting pathways" on page 18, from pathways that are interesting to your experiment.

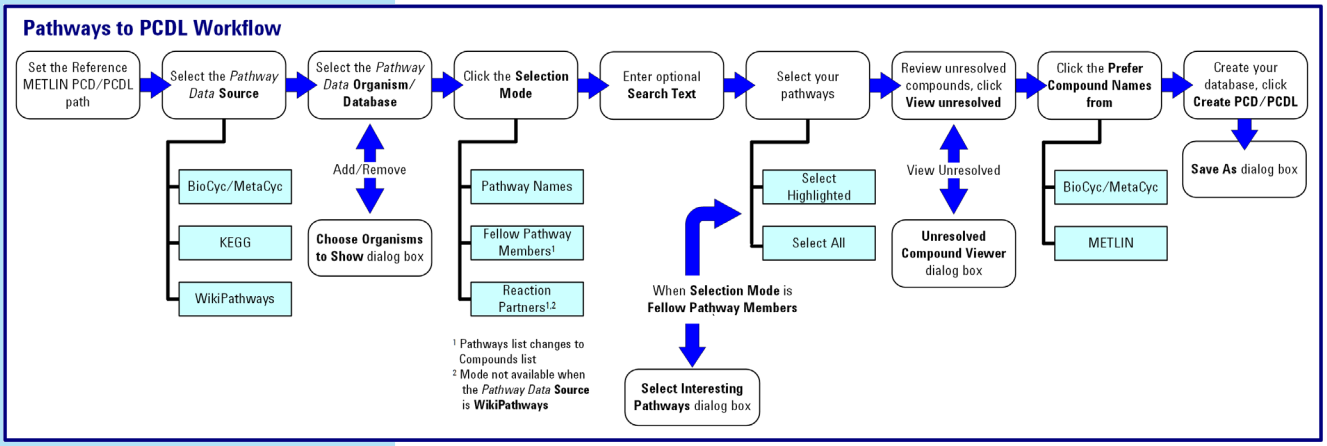


Figure 10 *Typical Pathways to PCDL workflow*

Create a custom PCDL

A custom compound database is created from the metabolites present in pathways content following the simple workflow as shown in Figure 10 on page 19. This example creates a database of the compounds in the tricarboxylic acid (TCA) cycle.

1. Select your pathway data source.

The Pathways to PCDL converter supports pathway content from BioCyc/MetaCyc, KEGG, and WikiPathways databases. A user name and password are required in order to obtain content from the KEGG database.

a Select **BioCyc/MetaCyc** for the **Source** under *Pathway Data*.

b Review the updated list of pathways in the display pane (Figure 11). If the pathways list is long, you may want to select an **Organism/Database** and/or enter **Search Text** to reduce the list of pathways.

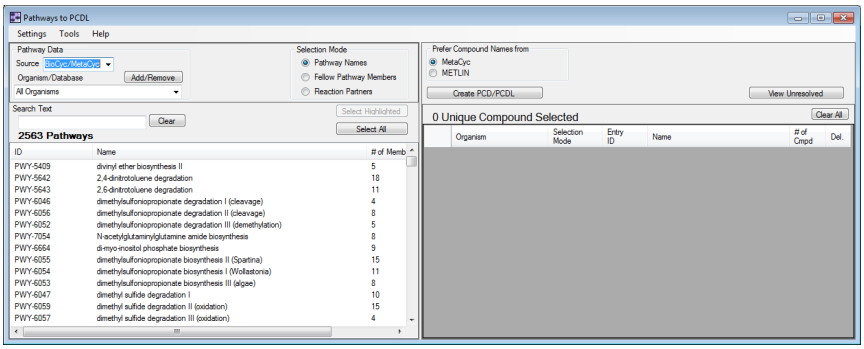


Figure 11 *Select pathways source*

2. Select an organism to filter the pathways from the data source.

You can add and remove organisms from the **Organism/Database** selection list. Click Add/Remove and mark or clear the organism check boxes in the **Choose Organisms to Show** dialog box.

a Select **Homo sapiens** from the **Organism/Database** list.

b Review the updated list of pathways in the display pane (Figure 12).

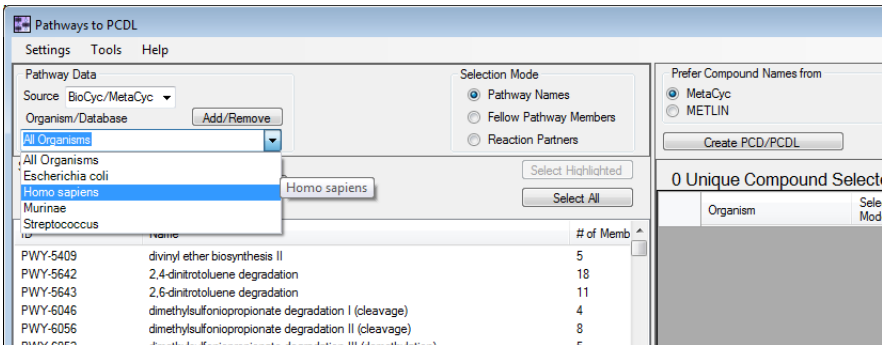


Figure 12 *Select an organism to filter the pathways*

3. Choose a selection mode.

The *Selection Mode* provides you with an option to select compounds (metabolites) for your personal compound database by association with a pathway name, pathways that have a common compound name, and pathway reactions that have a common compound name.

Pathway Name: This is a one-step selection process. Select compounds within a pathway that you select from the *Pathway List* in the display pane. The compounds associated with the pathway are moved the *Selected Pathways List* immediately after you click **Select Highlighted** or **Select All**.

Fellow Pathway Members: This is a two-step selection process. Select compounds within a pathway that you select from the **Select Interesting Pathways** dialog box - the list of pathways in this dialog box are related (a fellow) to the compound you previously select from the *Compound List* in the display pane. After you click **Select Highlighted** or **Select All**, the *Fellow Pathways* are displayed in the **Select Interesting Pathways** dialog box. The compounds associated with the pathway are moved to the *Selected Pathways List* immediately after you click **Select Highlighted** or **Select All** from the **Select Interesting Pathways** dialog box.

Reaction Partners: This is a one-step selection process. Select compounds associated with pathway reactions involving the compound you select from the *Compound List* in the display pane. The compounds associated with the associated reactions are moved the *Selected Pathways List* immediately after you click **Select Highlighted** or **Select All**. This selection mode is only available for the BioCyc/MetaCyc pathway data source.

a Click **Pathway Names** for the **Selection Mode**.

b Review the updated list of pathways in the display pane (Figure 13).

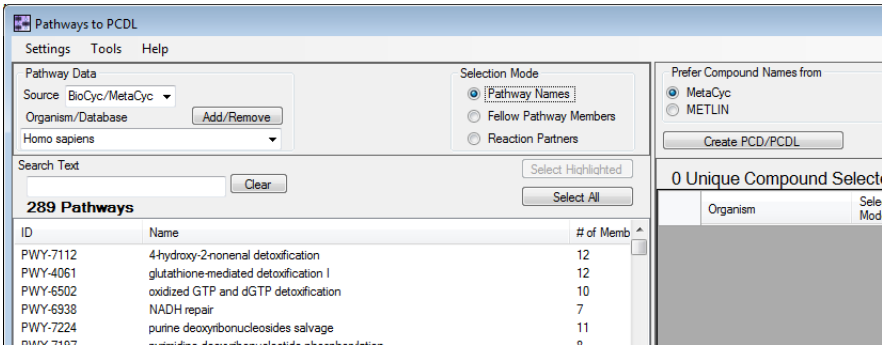


Figure 13 *Pathway Names Selection Mode*

4. Enter custom search text.

In cases when the number of pathways, or compounds, displayed in the *Pathways or Compounds List* in the display pane is large, you can reduce the number of pathways, or compounds, by using search text.

a Type `TCA` for the **Search Text**.

b Review the updated list of pathways in the display pane (Figure 14 on page 22). The number of pathways in this example is reduced to a single pathway.

Figure 14 *Reduction in the number of pathways using TCA for the search text*

5. Add one or more pathways to your *Selected Pathways List* using **Pathways Names**.

Build your personal compound database by selecting pathways individually (as shown in this step) or by clicking **Select All** from the *Compounds List*.

a Click the row containing the **TCA cycle** pathway.

b Click **Select Highlighted** to move the pathway to the *Selected Pathways List*.

The compounds associated with the pathway are moved to the *Selected Pathways List* immediately after you click **Select Highlighted,** or **Select All.**

c *(Optional)* Repeat steps a and b to continue adding pathways sources for your compound database; the example in this workflow does not require additional pathway sources.

d Review the updated list of pathways in the display pane (Figure 15). The number of pathways in this example is reduced to a single pathway.

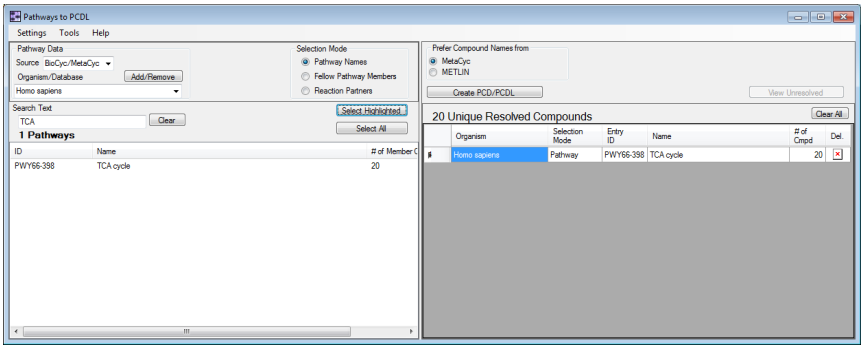


Figure 15 *TCA cycle pathway selected for the personal compound database*

6. Add one or more pathways to your *Selected Pathways List* using **Fellow Pathway Members**.

Build your database from the compounds within pathways that are related to each other (fellow pathways) by the compound you previously selected from the *Compounds List* in the *Display Pane*. Pathways can be added individually (as shown in this step) or by clicking **Select All**.

a Click **Fellow Pathway Members**.

Note: When you change **Selection Mode**, the **Search Text** is cleared and the *Pathways or Compounds List* is refreshed.

b Review the updated list of compounds in the *Pathways List*.

c Type `fu` for the **Search Text**. Nine compounds are available in the *Compounds List* as shown in Figure 16.

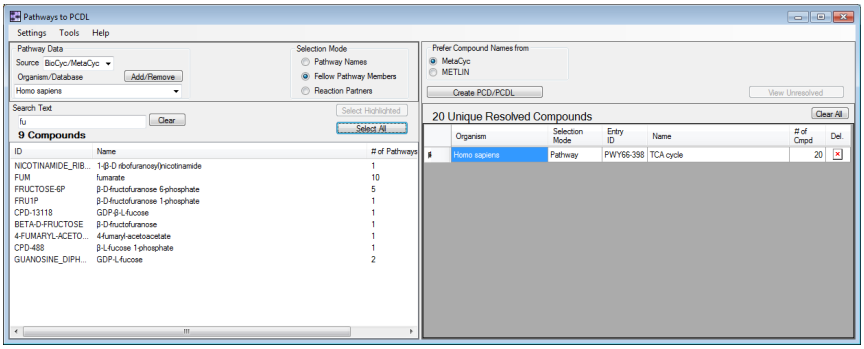


Figure 16 *Fellow Pathway Members Compounds List*

d Click **Select All**. Twenty pathways are shown in the **Show Interesting Pathways** dialog box that involve the nine compounds selected.

e Click **TCA cycle** pathway as shown in Figure 17.

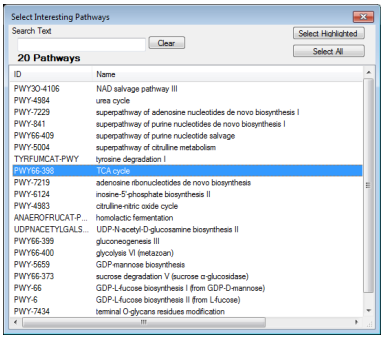


Figure 17 *Select Interesting Pathways dialog box*

f Click **Select Highlighted** to move the pathway to the *Selected Pathways List*. The **Show Interesting Pathways** dialog box closes automatically.

The compounds associated with the pathway are moved to the *Selected Pathways List* immediately after you click **Select Highlighted,** or **Select All.**

g *(Optional)* Repeat steps a through f to continue adding pathways sources for your compound database; the example in this workflow does not require additional pathway sources.

h Review the updated list of pathways in the display pane (Figure 18 on page 24).

Note: Because a selected pathway is described by the means that it was selected "TCA cycle" is not listed twice in the *Selected Pathways List*. You can tell the same pathway was added since the table heading remains unchanged, stating "20 Unique Resolved Compounds," compare Figure 15 on page 22 and Figure 18 on page 24.

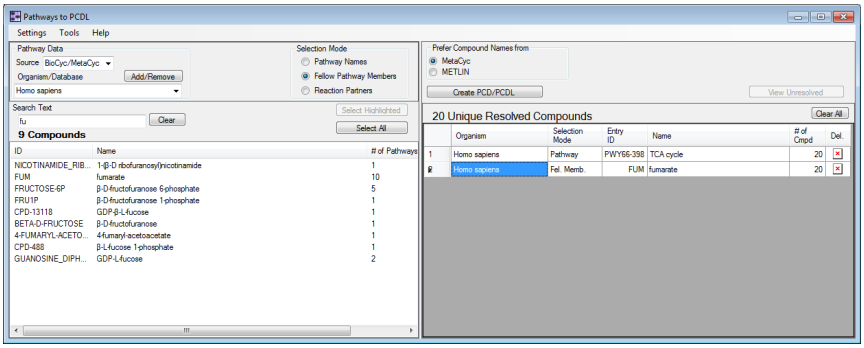


Figure 18 *TCA cycle pathway selected twice for the personal compound database*

7. Add one or more pathways to your *Selected Pathways List* using **Reaction Partners**.

Build your personal compound database by selecting compounds associated with pathway reactions involving the compound you select from the *Compounds List* in the display pane. Pathways can be added individually (as shown in this step) or by clicking **Select All**.

Note: This selection mode is only available for the **BioCyc/MetaCyc** pathway data source.

a Click **Reaction Partners**.

b Review the updated list of compounds in the *Compounds List*.

c Click **ATP** in the *Compounds List* - 118 reaction partners are associated with this compound.

d Click **Select Highlighted**.

e *(Optional)* Repeat steps a through f to continue adding pathways sources for your compound database; the example in this workflow does not require additional pathway sources.

f Review the updated list of pathways in the display pane (Figure 19).

Note: The number of unique resolved compounds increased significantly to 947 and there are 10 unresolved compounds.

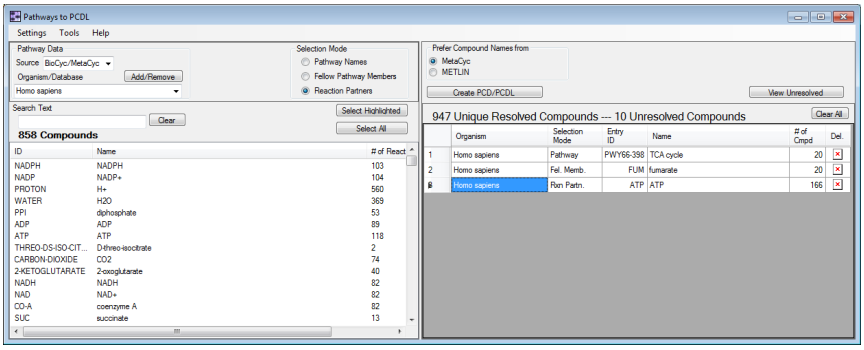


Figure 19 *ATP reaction partner added to the Selected Pathways List*

8. Review unresolved compounds in your *Selected Pathways List*.

Pathways to PCDL provides you with an opportunity to manually review the compounds in your *Selected Pathways List* that may not have a strong association with the pathways you have selected (unresolved compounds).

The **View Unresolved** button is available when you have unresolved compounds in your compound list.

a Click **View Unresolved**. The **View Unresolved** button is available when you have unresolved compounds in your compound list.

b Review the list of unresolved compounds in the **Unresolved Compound Viewer** dialog box (Figure 20).

c Click **OK**.

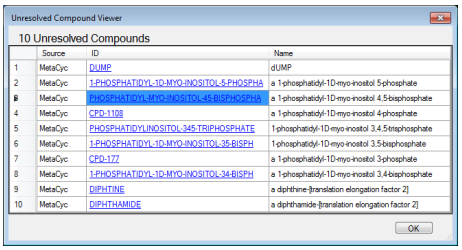


Figure 20 *Unresolved Compound Viewer dialog box.*

---

9. Remove pathways from your *Selected Pathways List*.

For the example experiment remove the pathway that was added using **Reaction Partners**.

a Mark ☒ under the *Del.* column for the row that corresponds to the **ATP** pathway in the *Selected Pathways List*.

Note: When you mark the pathway under the *Del.* column, it is immediately removed from the *Selected Pathways List*.

b The pathways to create your personal compound database reverted back to just the TCA cycle as shown in Figure 18 on page 24.

---

10. Create your custom PCD/PCDL.

Create your personal compound database from the compounds within the *Selected Pathways List*.

a Click **Create PCD/PCDL**.

b Select the folder to save your PCD/PCDL database (Figure 21). The default folder is `C:\MassHunter\PCDL`.

c Type the name for your PCD/PCDL database in File name, `Target_01`.

d Click **Save**.

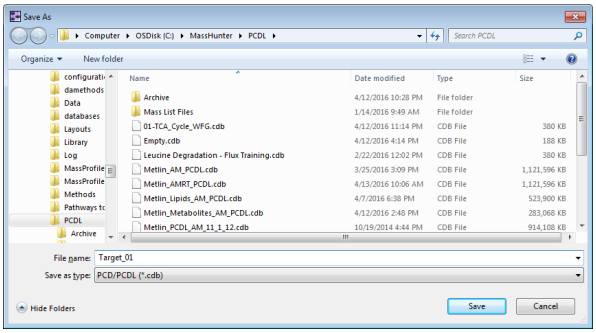


Figure 21 *Save As dialog box*

e Click **OK** after you have reviewed the summary information describing your custom PCD/PCDL (Figure 22).

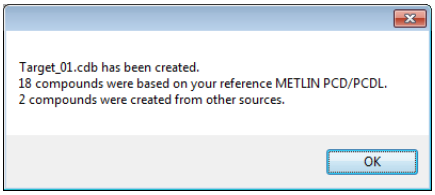


Figure 22 *Summary information*

Generate a target metabolite list

The second step in the VistaFlux Software workflow is to generate a target list of metabolites of interest to your experiment using MassHunter PCDL Manager. In this step you edit the compound list in the personal compound database you created in "Specify the interesting pathways" on page 18.

Launch PCDL Manager

A brief overview for PCDL Manager is presented in the section "Introduction to MassHunter PCDL Manager" on page 17.

1. Start PCDL Manager and open the PCDL created using Pathways to PCDL.

a Double-click the Pathways to PCDL icon located on your desktop, or click **Start > All Programs > Agilent > MassHunter Workstation > PCDL Manager *ver***, where *ver* is the version number for the current installation, such as "B.07.00".

b Select the personal compound database created in "Specify the interesting pathways", step "Create your custom PCD/PCDL." on page 25, `Target_01.cdb`. The default folder is `C:\MassHunter\PCDL` (Figure 23).

Note: The PCDL that was last used is displayed as the default PCDL to open.

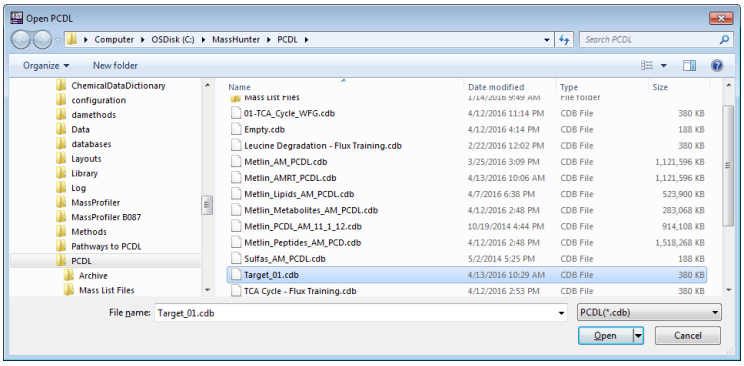


Figure 23 *Open PCDL dialog box*

2. Make a new copy of your TCA cycle personal compound database.

This step makes a new copy of your compound database before you begin making changes to the original PCDL. Edits made to a personal compound database using PCDL Manager are saved in real-time to the open database and cannot be undone.

a Click **File > New PCDL**. The **Create new PCDL** dialog box immediately opens and has information entered for the open compound database.

b Select **General** for **Select the PCDL type**.

c Type `Target_02` for **Enter the new PCDL name**.

d Enter `TCA Cycle after removing uninteresting compounds` for **Enter a description of this PCDL.**

e Click **Create** to make a copy of the compound database (Figure 24 on page 28).

Note: When a new PCDL is created, it is automatically opened in PCDL Manager. *Target_02.cdb* is opened for editing and the original TCA cycle compound database, *Target_01.cdb*, is retained for reference.

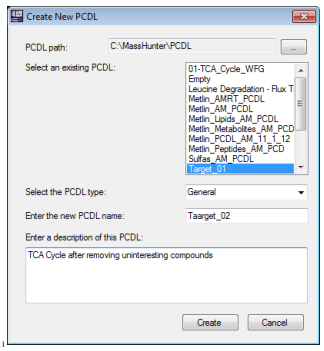


Figure 24 *Create New PCDL dialog box*

3. View the compounds in your personal compound database.

When a compound database is opened, the compounds can be viewed after you **Find Compounds** based on your search criteria.

a Click the **Single Search** tab.

b Click Find Compounds on the toolbar. Twenty compounds, that are part of the TCA cycle pathway, are viewed in the *Compound Results Table*.

Note: If the compound database is large, searching the database without narrowing the search criteria can take a long time.

Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Spectra
Hydrogen Ion	H	1.00783							0
Water	H2O	18.01057				7732-18-5			0
Carbon dioxide	CO2	43.98983				124-38-9			0
Fumaric acid	C4H4O4	116.01096			0.634	110-17-8			1
Succinic acid	C4H6O4	118.02661				110-15-6			4
Oxaloacetate	C4H4O5	132.00587				328-42-7			1
L-Malic acid	C4H6O5	134.02152							3
Oxoglutaric acid	C5H6O5	146.02152			0.423	328-50-7			3
Aconitic acid	C6H6O6	174.01644				499-12-7			6
threo-Isocitric acid	C6H8O7	192.02700							0
Citric acid	C6H8O7	192.02700			0.394	77-92-9			6
ADP	C10H15N5O10P2	427.02942			1.015	58-64-0			6
5'-guanylate diphosphate (guanosine diphosphate)	C10H15N5O11P2	443.02433				146-91-8			0
Adenosine triphosphate (ATP)	C10H16N5O13P3	506.99575				987-65-5			3
Guanosine triphosphate (GTP)	C10H16N5O14P3	522.99066				86-01-1			0
Nicotinamide adenine dinucleotide (NAD)	C21H28N7O14P2	664.11695		☑		53-84-9	5682		0
NADH	C21H29N7O14P2	665.12477			2.622	58-68-4	9425919		6
Coenzyme A (CoA)	C21H36N7O16P3S	767.11521				85-61-0			0
Acetyl-CoA	C23H38N7O17P3S	809.12577							3
Succinyl-CoA	C25H40N7O19P3S	867.13125				604-98-8			3

Figure 25 *All twenty compounds in the Target_02.cdb PCDL*

c Skip to "Review and modify your custom PCDL" on page 30 if you have previous experience with PCDL Manager to create a personal compound database.

Review the PCDL Manager user interface

The main functional areas of PCDL Manager are shown in Figure 26 on page 29.

The PCDL Manager window consists of five parts: *Menu Bar*, *Toolbar*, *Action Tabs*, *Action Pane*, and *Compound Results*. The number of compounds that meet your search criteria are shown above the table in the compounds results.

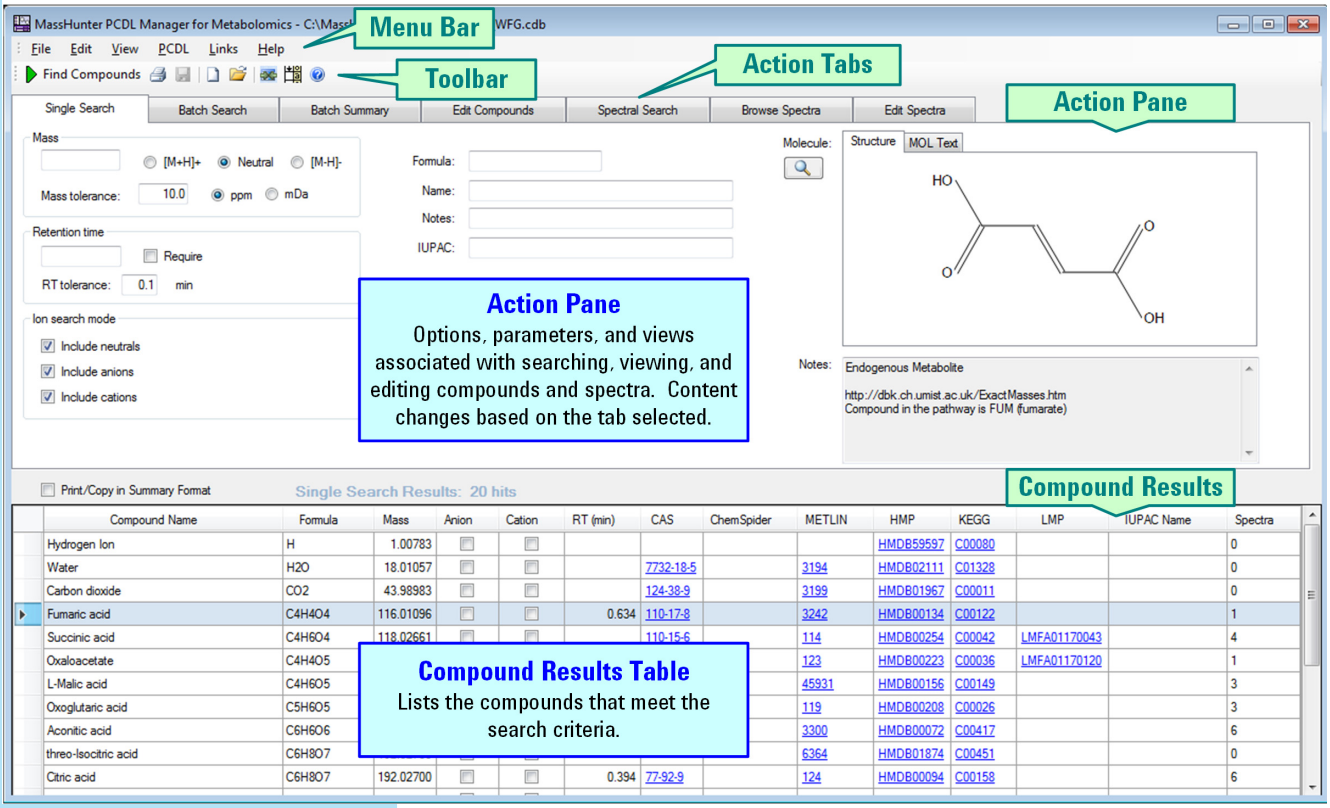


Figure 26 *Screen areas of MassHunter PCDL Manager*

Review the PCDL Manger workflow

The Pathways to PCDL Workflow shown in Figure 27 presents the steps used to create the target metabolite compound database for the example experiment.

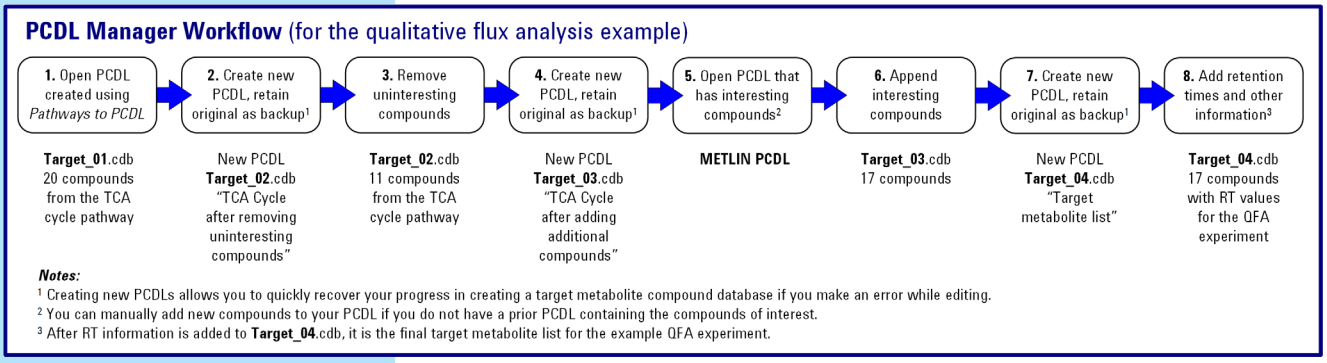


Figure 27 *PCDL Manager workflow for the example experiment*

Review and modify your custom PCDL

A custom compound database is created from the metabolites present in pathways content extracted using Pathways to PCDL following the workflow as shown in Figure 10 on page 19. This example modifies the compound database created from the tricarboxylic acid (TCA) cycle pathway.

Note: Edits made to the compound database using PCDL Manager are saved in real-time to your open database and cannot be undone. It is recommended to make a copy of your compound database before editing (see "Make a new copy of your TCA cycle personal compound database." on page 27).

---

1. Remove uninteresting compounds.

Since the database was created from all of the BioCyc/MetaCyc compounds associated with the TCA cycle, compounds that are not interesting your experiment can be removed; eleven compounds are interesting and nine compounds are removed.

a Click **PCDL > Allow Editing** to enable editing of the personal compound database. Compounds in the database can be added and removed using the *Edit actions* only when **Allowing Editing** is marked in the menu.

b Click the **Edit Compounds** tab.

Hydrogen ion, water, and carbon dioxide

c Click **Hydrogen Ion** in the *Compounds Results Table*.

d Shift-click **Carbon dioxide** in the *Compounds Results Table* to select the compounds between, and including, the selected compounds in the table. Selected compounds are highlighted with a background color.

e Click **Delete Selected** from the *Edit actions* (Figure 28).

Note: While these compounds are metabolically significant, they are not observable in the mass spectrometer used to acquire this sample data set; therefore, hydrogen, water, and carbon dioxide are uninteresting target compounds.

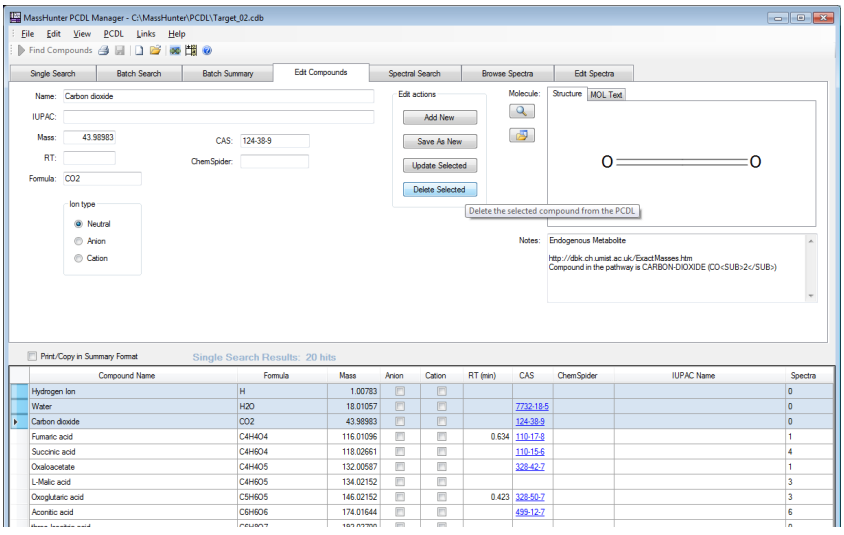


Figure 28 *Delete selected compounds*

f Click **Yes** in the **Delete Compound** dialog box. This dialog box verifies the action to delete the compounds from your open database. The compound database now contains 17 compounds.

Note: Edits made to the compound database while **Allowing Editing** is enabled are saved in real-time to your open database and cannot be undone.

g Click the **Single Search** tab to refresh the search results value above the *Compound Results Table*.

h Click the **Edit Compounds** tab to return to editing the compounds.

Oxaloacetate

i Click **Oxaloacetate** in the *Compounds Results Table.*

j Click **Delete Selected** from the *Edit actions.*

k Click **Yes** in the **Delete Compound** dialog box. The compound database now contains 16 compounds.

NAD, NADH, Coenzyme A (CoA), Acetyl-CoA, and Succinyl-CoA

l Click **Nicotinamide adenine dinucleotide (NAD)** in the *Compounds Results Table.*

m Shift-click **Succinyl-CoA** in the *Compounds Results Table.*

n Click **Delete Selected** from the *Edit actions.*

o Click **Yes** in the **Delete Compound** dialog box. The compound database now contains 11 compounds.

p Click **PCDL > Allow Editing** to disable editing of the personal compound database.

---

2. Make a new copy of your edited personal compound database.

This step makes a new copy of your edited personal compound database before you begin adding new compounds.

a Click **File > New PCDL**. The **Create new PCDL** dialog box immediately opens and has information completed for the open compound database.

b Select **General** for **Select the PCDL type**.

c Type `Target_03` for **Enter the new PCDL name**.

d Enter `TCA Cycle after adding additional compounds` for **Enter a description of this PCDL.**

e Click **Create** to make a copy of the compound database.

Note: When a new PCDL is created, it is automatically opened in PCDL Manager. *Target_03.cdb* is opened for editing and the original edited TCA cycle compound database, *Target_02.cdb*, is retained for reference.

---

3. Add new compounds from another PCDL.

While you can manually add new compounds to your compound database, a simple method is to append compounds to your compound database from another PCDL. In this example, six compounds are added from the Agilent METLIN PCDL.

a Click **File > Open PCDL**.

b Click the file name for the *METLIN PCDL* in the **Open PCDL** dialog box.

c Click **Open**.

d Click the **Batch Search** tab.

e Enter the masses for the new compounds into the *Masses* table (Figure 29). The list of the masses is shown in Table 2.

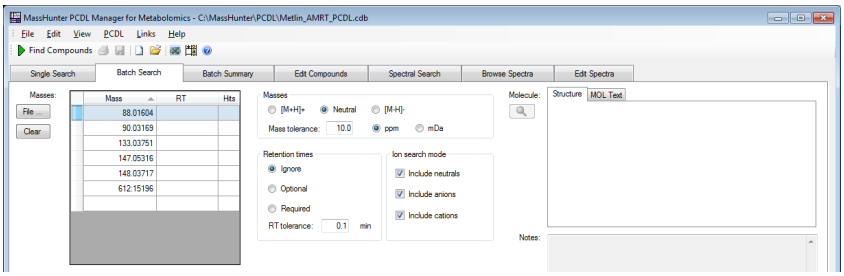


Figure 29 *Masses added to the Batch Search tab*

	Target Compound Name	Formula	CAS	Mass
1	Pyruvate	$C_3H_4O_3$	127-17-3	88.01604
2	L-Lactic acid	$C_3H_6O_3$	79-33-4	90.03169
3	L-Aspartic Acid	$C_4H_7NO_4$	56-84-8	133.03751
4	L-Glutamate	$C_5H_9NO_4$	56-86-0	147.05316
5	2-Hydroxyglutarate	$C_5H_8O_5$	2889-31-8	148.03717
6	Glutathione, oxidized	$C_{20}H_{32}N_6O_{12}S_2$	27025-41-8	612.15196

Table 2 *Six compounds to add from the METLIN PCDL, sorted by mass*

f Mark **Ignore** for *Retention times*.

g Click Find Compounds on the toolbar.

When the search is complete, the results are displayed in the *Compound Results Table*. The compounds results are shown for a single mass at a time; the *Compound Results Table* is refreshed after you click on a mass in the *Masses* table in the *Action Pane*. You can view all of the results in the *Batch Summary* tab.

For each mass you entered, PCDL Manager marks the row for the compound that is the best match for your search criteria. Since the search criteria in this example is minimal, the compound selected to append to our target compound database is selected based on the *Compound Name* and *CAS* ID.

h Right-click the row containing **2-Hydroxyglutarate** for mass **148.03717** or, if using the *Batch Summary* tab, highlight one search result for each mass and right-click on a highlighted row.

i Click **Append to PCDL** (Figure 30 on page 33).

j Select **Target_03**, the compound database containing the interesting compounds from the TCA cycle pathway, for **Select the PCDL to append to** in the **Append to PCDL** dialog box (Figure 30 on page 33).

k Click **Append**.

l Click **No** in the **Compounds Appended** dialog box since there are additional compounds to append to the **Target_03** compound database (Figure 31 on page 33). If this is the last compound go to step o.

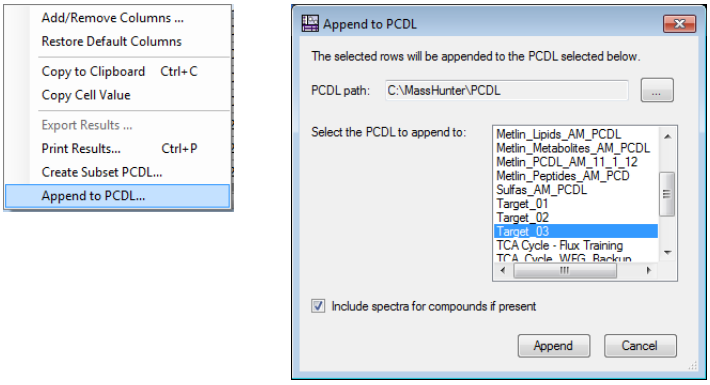


Figure 30 *Append to PCDL from the Compound Results Table and the Append to PCDL dialog box.*

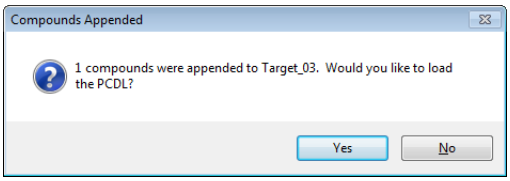


Figure 31 *Compounds Appended dialog box.*

m Click the next mass in the *Masses* table in the *Action Pane,* if you are adding one mass at a time using the *Batch Search* tab. Otherwise, skip to step 5 on page 34.

n Repeat steps h through m and select the appropriate compound for each mass from the compounds listed in Table 2 on page 32.

o Click **Yes** in the **Compounds Appended** dialog box after you appended the last of the six compounds to the **Target_03** compound database (Figure 31).

p Click the **Single Search** tab.

q Click Find Compounds on the toolbar. The compound database now contains 17 target compounds.

---

4. *(Optional)* Add new compounds manually.

If a compound, or a few compounds, are missing you can add them manually to the database. This example adds 2-oxoglutarate to a different compound database.

a Click **PCDL > Allow Editing** to enable editing of the personal compound database.

b Click **Edit > Add New Compound**.

c Click **Links > PubChem** to PubChem Compound search in your browser.

d Type `2-oxoglutarate` in the search box in PubChem.

e Click **Search**.

f Click the first of the results.

g Enter as much compound information as needed for pyruvate.

h Click **Update Selected** after you have entered the compound information, and before you click on any other compound in the *Compound Results Table* (Figure 32).

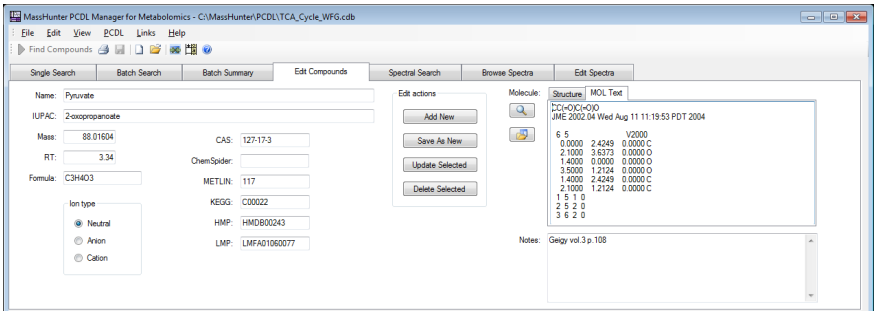


Figure 32 *Add new compound 2-oxoglutarate*

i Click **PCDL > Allow Editing** to disable editing of the personal compound database.

---

5. Make a new copy of your target personal compound database.

This step makes a new copy of your target personal compound database before you begin adding additional information, including retention times.

a Click **File > New PCDL**. The **Create new PCDL** dialog box immediately opens and has information completed for the open compound database.

b Select **General** for **Select the PCDL type**.

c Type `Target_04` for **Enter the new PCDL name**.

d Enter `Target metabolite list` for **Enter a description of this PCDL.**

e Click **Create** to make a copy of the compound database.

Note: When a new PCDL is created, it is automatically opened in PCDL Manager. *Target_04.cdb* is opened for editing and the original appended TCA cycle compound database, *Target_03.cdb*, is retained for reference.

---

6. Add retention times and additional identifiers to your target metabolite list.

This step adds retention times, and any additional information, to the target metabolite list. The retention time values are obtained either from sample data acquired during your acquisition method development or from the method used to acquire your sample data if it has already been acquired. Inclusion of compound identifiers, such as CAS, HMP, and KEGG, improves the data matching accuracy when you import your Profinder project into Omix Premium.

Note: As an alternative, this step can be done using the *Batch Search* tab with a CSV file from MassHunter Qualitative Analysis or Profinder. When the results are displayed in the *Batch Summary* tab you can click **Apply Retention Times**.

a Click the **Edit Compounds** tab.

b Click **PCDL > Allow Editing** to enable editing of the personal compound database.

c Click on a compound row in the *Compound Results Table.* This is the compound that can be edited in the *Action Pane*.

d Add the retention time from Table 3 on page 35 to **RT**, in addition to and any additional information to complete the compound data.

Note: You can add retention times using a mass list file. See "Exercise 8. Add retention times to a PCDL" in the *MassHunter Personal Compound Database and Library Manager Quick Start Guide*.

e Click **Update Selected** to write the new information to the compound data and before you click on any other compound in the *Compound Results Table.*

	Target Compound Name	Formula	CAS	Mass▲	RT (min)	Source[1]
4	Pyruvate	$C_3H_4O_3$	127-17-3	88.01604	3.34	AMP
3	L-Lactic acid	$C_3H_6O_3$	79-33-4	90.03169	2.93	AMP
8	Fumaric acid	$C_4H_4O_4$	110-17-8	116.01096	5.13	TCA
6	Succinic acid	$C_4H_6O_4$	110-15-6	118.02661	5.05	TCA
2	L-Aspartic Acid	$C_4H_7NO_4$	56-84-8	133.03751	2.29	AMP
9	L-Malic acid	$C_4H_6O_5$	97-67-6	134.02152	5.13	TCA
10	Oxoglutaric acid (a-KG)	$C_5H_6O_5$	328-50-7	146.02152	5.24	TCA
1	L-Glutamate	$C_5H_9NO_4$	56-86-0	147.05316	2.26	AMP
7	2-Hydroxyglutarate	$C_5H_8O_5$	2889-31-8	148.03717	5.11	AMP
13	Aconitic acid	$C_6H_6O_6$	499-12-7	174.01644	6.10	TCA
14	D-threo-Isocitric acid (not resolved from citric acid)	$C_6H_8O_7$	6061-97-8	192.02700	6.10	TCA
15	Citric acid (not resolved from threo-isocitric acid)	$C_6H_8O_7$	77-92-9	192.02700	6.10	TCA
12	Adenosine diphosphate (ADP)	$C_{10}H_{15}N_5O_{10}P_2$	58-64-0	427.02942	5.93	TCA
11	Guanosine diphosphate (GDP)	$C_{10}H_{15}N_5O_{11}P_2$	146-91-8	443.02433	5.83	TCA
17	Adenosine triphosphate (ATP)	$C_{10}H_{16}N_5O_{13}P_3$	987-65-5	506.99575	6.63	TCA
16	Guanosine triphosphate (GTP)	$C_{10}H_{16}N_5O_{14}P_3$	86-01-1	522.99066	6.57	TCA
5	Glutathione, oxidized	$C_{20}H_{32}N_6O_{12}S_2$	27025-41-8	612.15196	4.84	AMP

▲ Table sorted by this column, increasing values

[1] Source for each compound:

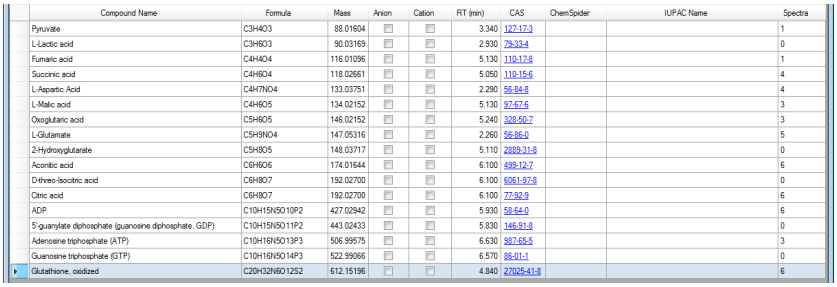
TCA - BioCyc/MetaCyc Tricarboxylic acid (TCA) cycle pathway

AMP - Agilent Metlin PCDL

Table 3 *Target Compound list with retention times, sorted by mass*

f Repeat steps c through e to update the retention time for each compound in the target metabolite list, as noted in Table 3.

g Click **PCDL > Allow Editing** to disable editing of the personal compound database. Your target metabolite list may appear as shown in Figure 33.



	Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Spectra
	Pyruvate	C3H4O3	88.01604			3.340	127-17-3			1
	L-Lactic acid	C3H6O3	90.03169			2.930	79-33-4			0
	Fumaric acid	C4H4O4	116.01096			5.130	110-17-8			1
	Succinic acid	C4H6O4	118.02661			5.050	110-15-6			4
	L-Aspartic Acid	C4H7NO4	133.03751			2.290	56-84-8			4
	L-Malic acid	C4H6O5	134.02152			5.130	97-67-6			3
	Oxoglutaric acid	C5H6O5	146.02152			5.240	328-50-7			3
	L-Glutamate	C5H9NO4	147.05316			2.260	56-86-0			5
	2-Hydroxyglutarate	C5H8O5	148.03717			5.110	2889-31-8			0
	Aconitic acid	C6H6O6	174.01644			6.100	499-12-7			6
	D-threo-Isocitric acid	C6H8O7	192.02700			6.100	6061-97-8			0
	Citric acid	C6H8O7	192.02700			6.100	77-92-9			6
	ADP	C10H15N5O10P2	427.02942			5.930	58-64-0			6
	5'-guanylate diphosphate (guanosine diphosphate, GDP)	C10H15N5O11P2	443.02433			5.830	146-91-8			0
	Adenosine triphosphate (ATP)	C10H16N5O13P3	506.99575			6.630	987-65-5			3
	Guanosine triphosphate (GTP)	C10H16N5O14P3	522.99066			6.570	86-01-1			0
▸	Glutathione, oxidized	C20H32N6O12S2	612.15196			4.840	27025-41-8			6

Figure 33 *Final target metabolite list*

7. Save your target metabolite compound list.

All of the edits made while **Allowing Editing** is enabled are saved in real-time to your open database.

a Click the **Single Search** tab.

b Click Find Compounds on the toolbar to view the 17 compounds that are part of the target metabolite list (Figure 33).

c Your target metabolite list (Target_04.cdb) is now ready to be used as the **Target Source** as part of the MassHunter Profinder *Batch Isotopologue Extraction* method ("Create your Batch Isotopologue Extraction method" on page 51).

d Click **File > Exit** to close PCDL Manager. Alternatively, open a different PCDL or perform other actions using PCDL Manager.

Select your tracer metabolite

Metabolomics studies measure the relative abundance of metabolites to better understand biological systems. However, this does not provide insight into the dynamics of metabolism since significant changes in flux through a pathway may not result in altered abundances of metabolite intermediates. Qualitative flux analysis addresses this issue and allows researchers to obtain information about selected pathways and fluxes. In qualitative flux analysis a stable isotope labeled compound, referred to as a tracer, is introduced into the biological system. The tracer typically contains multiple atoms of ^{13}C, ^{15}N, or ^{2}H. As the tracer is utilized by the organism, or metabolized, changes in the natural isotopic pattern occur in downstream metabolites. The isotopic composition of the downstream metabolites change over time leading to isotopologues whose mass signatures can be measured via a mass spectrometry.

An isotopically labeled source of cellular energy, such as ^{13}C labeled glucose and glutamine, was used as the tracer in the example data set for this workflow guide. Agilent LC/TOF and LC/Q-TOF MS systems provide the high level of mass accuracy and isotopic fidelity required to measure isotopologues of the metabolites downstream from the point of origin for this example qualitative flux analysis.

Example data

The example data in this workflow guide demonstrates qualitative flux analysis in a chondrosarcoma cell line, using $^{13}C_5$-glutamine (U-^{13}C-Gln) as the tracer. The example data was acquired on an Agilent LC/TOF system. The example qualitative flux analysis demonstrates the effect of an isocitrate dehydrogenase 2 (IDH2) mutation on TCA cycle metabolism; in particular the diversion of glutamine to the production of 2-hydroxyglutarate (2-HG) by the mutant IDH2 isoform.

Next step...

You have now completed the first step of the VistaFlux Software workflow. In the next workflow step you acquire your sample data.

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Acquire data

The quality of your results are influenced by your mass spectrometer, acquisition parameters, and sampling approach.

Select an accurate mass instrument 40
Create your acquisition method 41
Acquire your sample data 42

Agilent Technologies

Select an accurate mass instrument

MassHunter VistaFlux software is designed to help scientists perform qualitative flux analysis, and facilitate the processing and visualization of high-resolution accurate mass TOF and Q-TOF LC/MS data. Time-of-flight mass spectrometers are especially well suited for flux analyses due to the combination of fast scan speed, high resolution, and high dynamic range.

Instrument resolution

The resolution observed in TOF instruments is a function of the actual resolving power of the analyzer, and the ability of the detector circuitry to respond to the signal and measure the output at the necessary scan speed. Since there is a natural spread in the ion arrival times at the detector, improvements in instrument resolution improve the accuracy of the instrument in measuring the actual time of flight of the ions and therefore improve the mass accuracy.

For time-of-flight mass spectrometers (TOF-MS), higher resolution is achieved through improvements in the following parameters:

- Ion pulse formation at the inlet to the flight tube
- Energy focus of the pulsed ions
- Flight time
- Vacuum
- Gain and speed of the ion detector
- Digitization rate of the detector electronics

Within limits, higher resolution narrows the mass spectral peak width and in-turn produces a more accurate *m/z* value.

Instrument dynamic range

Dynamic range is significant because accurate *m/z* values are difficult to assign to saturated signals and signals with a very low signal to noise. While a single qualitative flux analysis employs many target metabolites that have a wide range of concentrations in the biological system under study, it is the tracer and propagation of the isotope labeling that places a very high demand on the dynamic range of the instrument.

Small molecule tuning (LC/MS)

Qualitative flux analyses involve the analysis of small molecules, molecules nominally with a molecular weight from 50 to 6000 Da. Since the best results for flux analysis studies involve the identification of the exact mass of the molecular ion, LC/MS instrument tunes should be adjusted to (1) improve the sensitivity for intact molecular ions and (2) improve the overall sensitivity for small, low-mass, compounds. A typical automated instrument tune optimizes the instrument sensitivity across the entire mass range and may result in lower sensitivity for small molecules combined with higher fragmentation than desired for metabolomics.

Create your acquisition method

While the samples for your experiment may be considerably different from this example, the acquisition method used in the example is presented.

Cell extracts were dried, resuspended, incubated, and centrifuged to remove particulates.100 µL volumes of the final resuspension was transferred to reduced-volume autosampler vials.10 µL was removed from every sample to assemble a pooled quality control vial that was injected six times at the start of the analytical run, then regularly throughout the analysis to ensure chromatographic stability.

Data were acquired on an Agilent LC/MS system consisting of:

- Agilent 6230 time-of-flight mass spectrometer
- Agilent 1290 Infinity Binary UHPLC pump for the chromatography
- Agilent 1290 Infinity thermostatted column compartment
- Agilent 1290 Infinity HTS autosampler
- Agilent 1260 Infinity isocratic pump with a 100:1 fl ow splitter was used to introduce the reference mass solution

The LC was interfaced to the MS with a dual electrospray ionization source, and data were acquired in negative ion mode using the 4 GHz high resolution mode at 1.5 spectra per second. Table 1 shows the analytical method settings.

As with this example, the analytical method must be designed for your target metabolites (Table 1 on page 11. Because the TCA cycle is composed of organic acids that are poorly retained by reversed-phase chromatography, ion-pair reversed-phase chromatography was used. Negative mode electrospray ionization provided the optimal means to introduce the sample for detection of the target compounds using time-of-flight mass spectrometry.

LC Parameters	Value
Column	C18+ 150 mm × 2.1 mm, 2.7 mm
Mobile phase	A) 5 mM N,N-dimethyloctylamine in water, pH 5.5
	B) 5 mM N,N-dimethyloctylamine in 90 % methanol in water, pH 5.5
Flow rate	0.4 mL/min from 0–8 minutes, then 0.6 mL/min from 8.1–10 minutes
Gradient	10 %B at 0 minutes
	100 %B at 8.0 minutes
Stop time	10 minutes
Post time	5 minutes
Column temperature	30 °C
Injection volume	15 µL

MS Parameters	Value
Ionization mode	Dual ESI
Ionization polarity	Negative
Gas temperature	250 °C
Drying gas flow rate	13 L/min
Nebulizer pressure	45 psi
Capillary voltage	3,500 V
Octopole RF Voltage	600 V
Acquisition range	*m/z* 50–1,700
Reference masses	*m/z* 119.0363 and 980.01638

Table 4 *Example LC/MS analysis parameters*

Acquire your sample data

Statistical significance of your qualitative flux analysis depends on understanding natural variation and how replicate sampling can influence your results. Acquiring an adequate number of samples for each experimental condition, or time observation, (sample groups) improves the statistical significance of your qualitative flux analysis.

Natural variability

Not all data analysis requires an assumption that the samples represent a population with a normal distribution. In Omix Premium a Welch's t-test does not assume a normal distribution. However, the following discussion on natural variability is based on a normal distribution.

Before any statistical analysis is begun, it is important to understand how a sample represents the population as a whole, and how increasing the sample size improves the accuracy of the sample set in describing characteristics of the population.

Under identical conditions, all life systems produce a range of results. Samples from the population may show one of the following characteristics:

(1) Results comparable to the mean of the population (i.e., characteristics shown by the majority of the population), for example results within ±1 standard deviation (~68%) from the mean.

(2) Results that differ significantly from those shown by the majority of the population (i.e., characteristics that are not shown by the majority of the population), for example results beyond ±3 standard deviations (~99.7%) from the mean.

(3) Results anywhere in between ±1 to ±3 standard deviations from the mean.

Metabolite abundances in many biological and biochemical systems are found to show a probability of variation referred to as a normal distribution. Figure 34 on page 43, for instance, shows a normal distribution of a characteristic within a population where 68% of the sampled population is shown to have the mean characteristic plus or minus one standard deviation (σ). This natural variation of the population response to identical conditions is referred to as natural variability. Natural variability thus means that any single sample taken from a population is not guaranteed to reflect the mean characteristics of the population.

Natural variability is found to occur from inherent randomness and unpredictability in the natural world. Natural variability is found in all life and natural sciences and in all forms of engineering. For example, a population of plants grown under identical conditions of illumination, precipitation, and nutrient availability shows a range in growth mass. This range of variable growth mass may be expressed as a mean where 95% of the population is expected to show a natural range of variability within two standard deviations of the mean (Figure 34 on page 43).

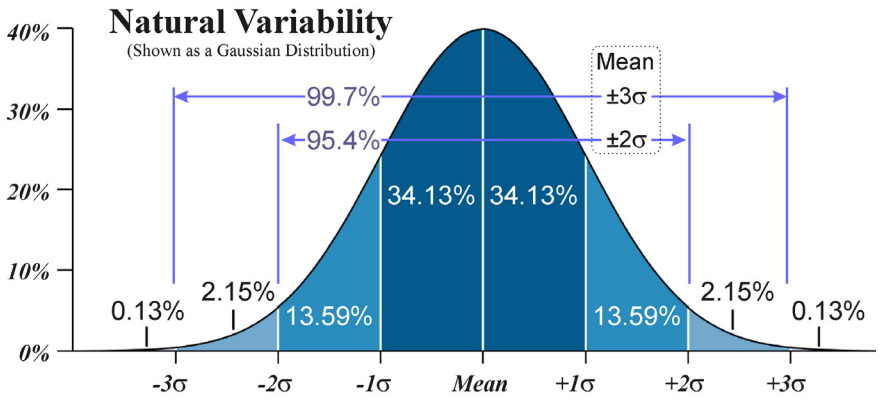

Figure 34 *Natural variability shown as a Gaussian (normal) distribution. Depending on the predefined requirement for significance, if the mean of a sample set is beyond ±2σ from the natural variation there may be a significant effect. Similarly, if a particular observation routinely falls beyond ±2σ from the natural variability of the data the change producing the effect may be considered significant.*

In other words, for a set of fixed attributes (independent variables), a representative set of samples taken from the population of plants shows a natural variability in the dependent variable growth mass. When an experiment is undertaken where plants from the same population are subject to variations in one ore more controlled conditions, the plants response shows a change in growth mass in addition to their natural variability in growth mass. Thus if the entire population is sampled, two adjacent normal distributions with means reflecting the plant growth mass under the two conditions results (Figure 35).

Likewise, an cell line population subject to controlled exposures shows a natural variability of that exposure as expressed through the chemical makeup of samples taken from the population. Such unpredictability in the measurable variability of any biochemical expression must not be mistakenly correlated with deliberate variations of an controlled condition (independent variable).

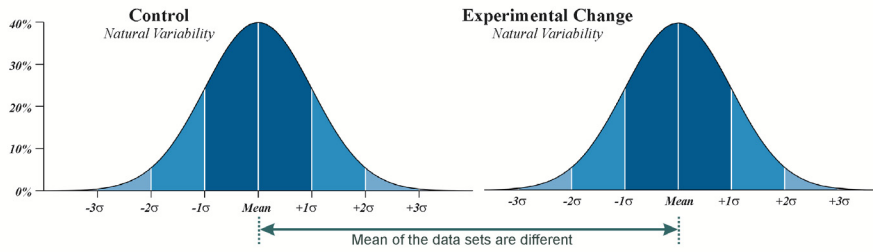

Figure 35 *Natural variability of populations with subject to two different experimental conditions where the means of each data set falls outside of the mean ±3σ of the other data set.*

During your experiment, the natural variability of the data representing a population must be understood in order to confidently express any experimental correlation. The investment of time and resources in performing a statistical analysis requires that the natural variability of the subject specimen be known or reasonably estimated so that the results of the analysis may be conclusively shown to be either

within the natural variability (no correlation) or outside of the natural variability and therefore provide for a degree of correlation to your controlled condition.

Experimental data collection that does not incorporate a consideration of the natural variability of the data does not yield meaningful results. Thus, as with all statistical data treatments, an understanding and well planned collection of the data is required from your qualitative flux analysis; without an understanding of natural variability the results follow the adage "garbage in, garbage out."

Replicate data

Replicate sampling and measurement from the population is the only way to estimate the natural variability of your data. No guarantee exists that a single sample specimen from a population represents the mean of the population. Any single sample from a population with a natural variability shown in Figure 34 on page 43 has a 99.99% chance that it lies within four standard deviations ($\pm 4\sigma$) of the mean of the true population, but in fact that single sample may on a rare occasion fall even further from the population mean.

However, if ten samples are taken from the population, the mean of these samples produces a statistically more accurate approximation of the true mean of the population than fewer samples. The accuracy of the approximation of the true population mean proportionally improves with more samples. The true value of the population mean is achieved only if the entire population is sampled. However, sampling the entire population is not typically feasible because of constraints imposed by time, resources, and finances. On the other hand, evaluating fewer samples increases the chance of false negative and false positive correlations from the analysis of sample data from your experiment.

Too few samples may lead to an incorrect conclusion

Figure 36 on page 45 shows that if too few samples are evaluated, and if these samples just happen to be samples lying far from the mean because of natural variability, an incorrect conclusion may be drawn that the change in the independent variable produced no significant change in the response. The estimate of the standard deviation of the sample mean estimate of a population mean (standard error) is equal to the standard deviation of the samples divided by the square root of the number of samples (Equation 1).

$$SE = \sigma/(\sqrt{N}) \quad \textbf{(1)}$$

where SE = standard error of the population; σ = standard deviation; and N = number of samples.

Large sample sizes lead to more confident conclusions

As the sample size increases, the likelihood that the data approximates the true response of the population increases. The standard deviation of the sample may become smaller, and the likelihood of making a correct correlation between cause and effect is improved (Equation 2).

$$\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \bar{X})} \quad \textbf{(2)}$$

where σ = standard deviation; N = number of samples; x_i = the value of an individual sample; and $\bar{X}$ = mean (or average) of all N samples.

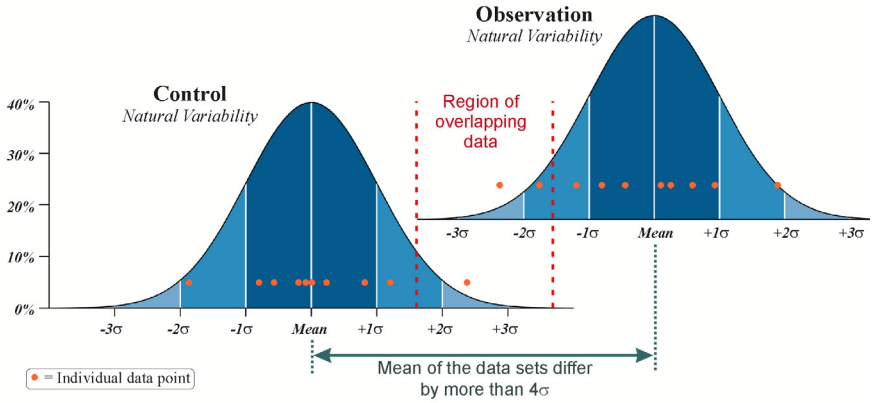

Figure 36 *Replicate data are necessary to distinguish whether the represented populations actually show significant differences. The three data points in the region of overlap of the natural variability may, if too few replicates are selected, lead to a result suggesting a less significant difference in the populations.*

Successful application of qualitative flux analysis depends on the availability of sufficient replicate samples and specimens. Coupled with an understanding of the systems under study and a well planned collection of the samples and concomitant data, the statistical data treatment of the replicate samples is the backbone of your qualitative flux analysis. A sufficient set of replicate data, ten or more replicates, may provide a significant answer to the hypothesis and prevent a total loss of time and resources invested in performing your analyses.

Next step...

You have now completed the second step of the VistaFlux Software workflow. In the next workflow step you perform your primary data analysis using MassHunter Profinder.

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Primary data analysis

MassHunter Profinder is used to perform batch isotopologue feature extraction using the target metabolite compound database.

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Create your Batch Isotopologue Extraction method 51
Mine your sample data for the target metabolites 58
Curate your results 60
Export your results as a Profinder Archive 65
Save your Profinder project 66

Isotopologue extraction overview

Performing a primary data analysis requires a basic understanding of isotopes, isotopomers, and isotopologues, and MassHunter Profinder software. MassHunter Profinder is used to perform batch isotopologue feature extraction from your sample data files using the target metabolite compound database you created in the chapter "Create target list" on page 15.

See "Isotopes, isotopologues, and isotopomers" on page 8 for a brief overview on isotopes, isotopomers, and isotopologues.

Introduction to MassHunter Profinder

MassHunter Profinder is part of the MassHunter VistaFlux software suite. MassHunter Profinder software, optimized for batch feature extraction from TOF, Q-TOF, and MSD data files, is the primary data analysis software for your qualitative flux analysis sample data acquired using Agilent LC/TOF and LC/Q-TOF.

Profinder is optimized to extract features from large data sets and provides you with an intuitive user interface to inspect and review each feature across the files associated with your data set. With Profinder, you can review and compare extracted ion chromatograms, mass spectral data, and isotopologues associated with each feature. You can also save your feature extraction progress to a project to review, adjust, and reprocess at a later time. Profinder archive (PFA) files contain extracted compound features and sample group information. PFA files are imported into Omix Premium to view your data in the context of biochemical networks, including isotopologue results, as part of a qualitative metabolic flux analysis described in this workflow.

Review the Profinder user interface

The main functional areas of Profinder are shown in Figure 37 on page 49. The main Profinder window consists of three parts: (1) the Menu Bar, (2) the Toolbar, and (3) the Main Window.

The main window is further divided into up to five windows — (3a) Compound Groups, (3b) Compound Details, (3c) Chromatogram Results, (3d) MS Spectrum Results, and (3e) Isotopologue Results that are used to review the results from applying a feature extraction method to your data set. Each window can be floated independently to any location and size on your computer display or arranged to your preference within the main window.

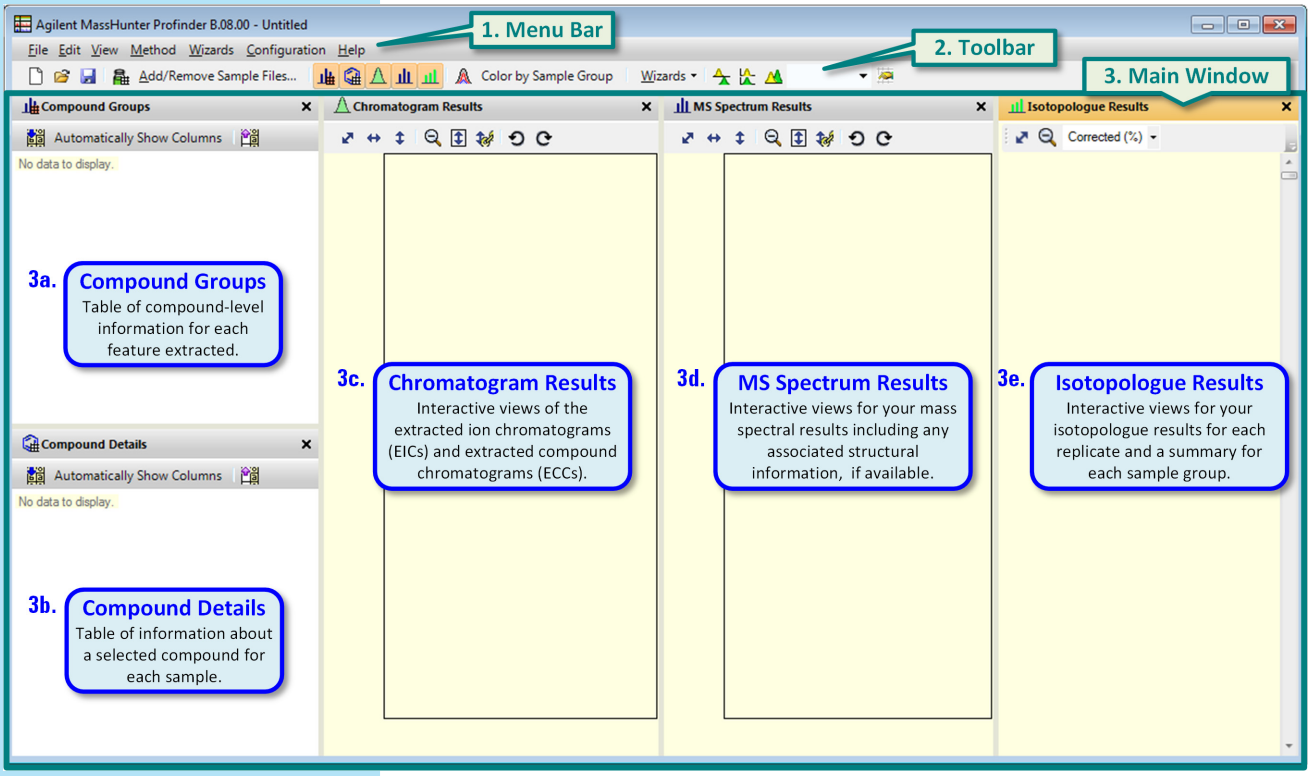


Figure 37 *Screen areas of MassHunter Profinder*

Review the Profinder batch isotopologue extraction

Batch isotopologue extraction, one of five feature extraction wizards within Profinder, uses an input CSV file or personal compound database file, PCD/PCDL, containing the target feature molecular formulas, mass, and retention time information. Batch isotopologue extraction anticipates that the target compounds may have undergone some degree of isotope labeling, to extract features from your data using a process referred to as Find Compounds by Formula. After feature extraction is performed, the extraction algorithm determines which of the possible isotopologues are actually present, measures the raw abundances of the isotopologues, and corrects the isotopologues abundances for the natural occurrence of the unlabeled ions.

Batch isotopologue extraction supports only LC/MS acquired data. Unlike the other batch feature extraction wizards, target ***retention times are required*** for this workflow. Profinder performs isotopologue mining in two stages, an initial screening followed by refinement. The initial screening stage extracts isotopologue EICs around the target retention time range and then evaluates peak mass spectral data to find ions that match the predicted list of possible isotopologues. The refinement stage uses a self-optimizing peak finder to refine the *m/z* assignment from the profile data. During refinement, Profinder re-extracts the EICs using the new isotopologue *m/z* values, refines the start and end retention time bounds on the newly extracted EICs, and then reports both EIC peak area and summed isotopologue peak heights as the compound abundances.

Note: Profinder finds and extracts isotopologues in your sample data; it does not find or extract isotopomers.

The batch isotopologue extraction wizard is shown in Figure 38.

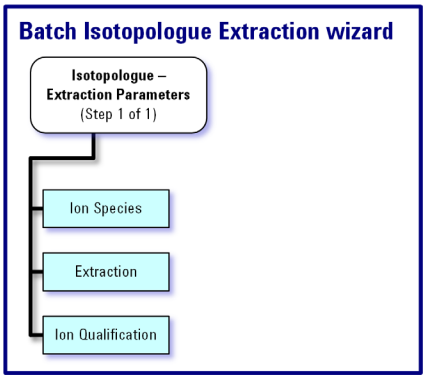


Figure 38 *One step is presented in the Batch Isotopologue Extraction wizard*

Retention times

As a reminder, batch isotopologue extraction requires that your **Target Source** contain accurate retention times for each target in your sample data files.See "Add retention times and additional identifiers to your target metabolite list." on page 34

Note: If your **Target Source** database does not have retention times, or requires retention times to be updated, you can use Profinder to generate the new retention times. Run the **Batch Targeted Extraction** on one group, or other subset of your sample data, to find the features in your sample data. Then export the features as a CSV file using **File > Export as CSV**. The CSV output from Profinder can then be used as a mass list file in the *Batch Search* tab of PCDL Manager as described in the ***note*** in "Add retention times and additional identifiers to your target metabolite list." on page 34.

For more information

See the *MassHunter Profinder Software Quick Start Guide* for more information.

Create your Batch Isotopologue Extraction method

In this task, you launch Profinder, select your sample files, add the sample files to your Profinder project and create your Batch Isotopologue Extraction method.

Launch Profinder

A brief overview of Profinder is presented in "Introduction to MassHunter Profinder" on page 48.

Start the Profinder software.

Double-click the Profinder icon located on your desktop, or click **Start > All Programs > Agilent > MassHunter Workstation > Profinder B.08.00**.

Create your Profinder project

When Profinder opens, you can begin a new project or open a saved project. This section guides you through the steps to create a new project.

1. Add sample files to your new project.

   a Click **File > Add/Remove Sample Files**, click Add/Remove Sample Files... on the toolbar, click **File > New Project**, or click **New Project** on the toolbar.

   b Click **Add files** in the **Add/Remove Sample Files** dialog box.

   c Navigate to the folder containing your sample data in the **Open file** dialog box.

   d Click on your first sample data file. The selected sample data file is highlighted in a background color.

   e Shift-click on the last sample data file to select the entire set of sample data. All of the selected sample data files are highlighted in a background color.

   f Click **Open** in the **Open file** dialog box (Figure 39).

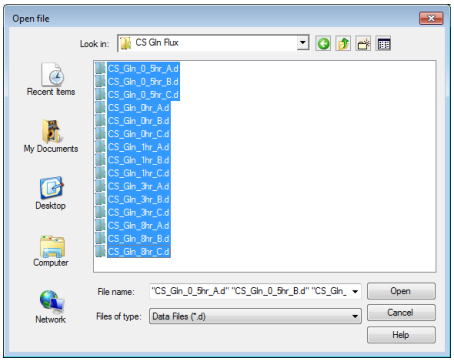


Figure 39 *Select sample data files in the Open file dialog box*

2. Enter sample group values.

When entering *Sample Group* values, the entries must use identical letters, numbers, punctuation, and case in order for the grouping functions to perform properly.

a Click the data entry cell under the *Sample Group* column, next to the sample file name.

b Enter the sample group identification text.

c Right-click the last replicate sample to open the entry shortcut menu.

d Click **Fill Down** (Figure 40).

The operations available in the shortcut menu help you ensure that replicate samples have identical group values.

e Repeat these steps for each sample file.

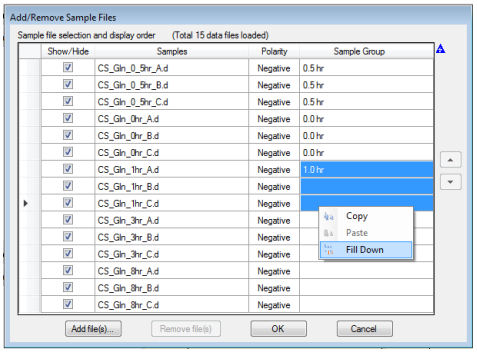


Figure 40 *Use Fill Down to add Sample Group values in the Add/Remove Sample Files dialog box*

3. Re-order your samples into a logical order.

The order of the appearance of the samples and sample groups you set in the **Add/Remove Sample Files** dialog box determines the order of the results viewed in the *Isotopologues Results* area of the *Main Window* (Figure 37 on page 49).

a Click the first sample row you want to re-order.

b Shift-click the last sample row that contains two or more samples that you want to re-order.

c Click the **Move Up** and **Move Down** arrows, along the sight side of the **Add/Remove Sample Files** dialog box, to sort your samples in a logical order and to place replicate samples in sequential rows (Figure 41).

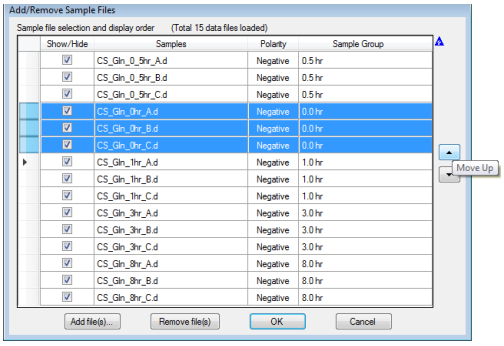


Figure 41 *Use the Move Up arrow re-order samples in the Add/Remove Sample Files dialog box*

d Repeat these steps until your samples and sample groups are in the order you want them to appear in *Isotopologues Results* (Figure 42).

e Click **OK** in the **Add/Remove Sample Files** dialog box.

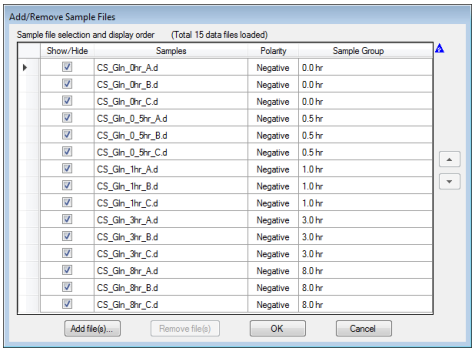


Figure 42 *Sample group values and order for the example Profinder project*

Create your Batch Isotopologue Extraction method.

After you add your sample data files to your Profinder project, you select a feature extraction workflow to set up your primary data analysis method.

When you make a change to a parameter in Profinder, the software automatically places a change icon  (a blue triangle shape) in the wizard tab and next to the value containing the parameter where you made a change. This icon indicates that you have unsaved parameters changes and serves as a reminder to save the changes you have made to the method. The original parameter value may be viewed by placing your pointer over the change icon. After you save your method, the change icon(s) disappear.

1. Select your feature extraction workflow.

a Select **Batch Isotopologue Extraction**.

b Click **Next** to select and enter the isotopologue extraction method parameters.

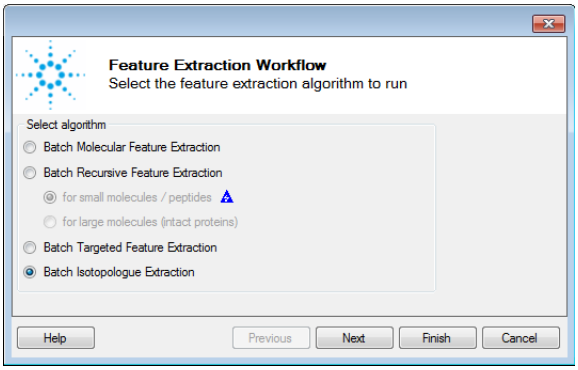


Figure 43 *Feature Extraction Workflow selection dialog box*

2. Enter the extraction parameters in **Ion Species** tab.

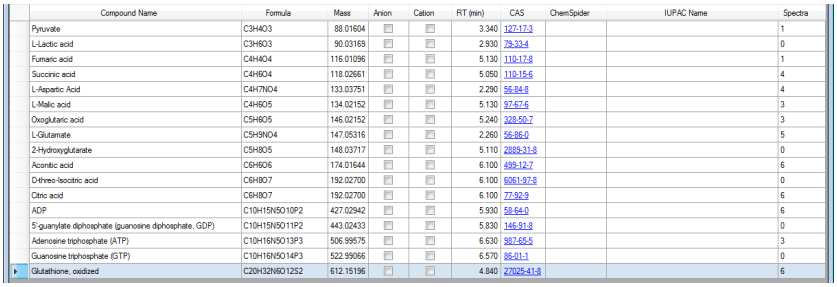
a Click the **Ion Species** tab.

b Click the **Select** ... button to select your target source.

c Navigate to the folder that contains your target metabolite database in the **Open database file** dialog box (Figure 45).

d Select the target metabolite database, Target_04.cdb, you created in chapter "Create target list" on page 15 in the **Open database file** dialog box.

The final target metabolite database, after adding additional compound identifiers, appears as shown in Figure 44. By adding compound identifiers to your target metabolite database the compound matching performed during visualization in Omix Premium is less sensitive to precise compound spelling; compound identifiers are matched with higher priority, when present, than compound names.



Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Spectra
Pyruvate	C3H4O3	88.01604			3.340	127-17-3			1
L-Lactic acid	C3H6O3	90.03169			2.930	79-33-4			0
Fumaric acid	C4H4O4	116.01096			5.130	110-17-8			1
Succinic acid	C4H6O4	118.02661			5.050	110-15-6			4
L-Aspartic Acid	C4H7NO4	133.03751			2.290	56-84-8			4
L-Malic acid	C4H6O5	134.02152			5.130	97-67-6			3
Oxoglutaric acid	C5H6O5	146.02152			5.240	328-50-7			3
L-Glutamate	C5H9NO4	147.05316			2.260	56-86-0			5
2-Hydroxyglutarate	C5H8O5	148.03717			5.110	2889-31-8			0
Aconitic acid	C6H6O6	174.01644			6.100	499-12-7			6
D-threo-Isocitric acid	C6H8O7	192.02700			6.100	6061-97-8			0
Citric acid	C6H8O7	192.02700			6.100	77-92-9			6
ADP	C10H15N5O10P2	427.02942			5.930	58-64-0			6
5'-guanylate diphosphate (guanosine diphosphate, GDP)	C10H15N5O11P2	443.02433			5.830	146-91-8			0
Adenosine triphosphate (ATP)	C10H16N5O13P3	506.99575			6.630	987-65-5			3
Guanosine triphosphate (GTP)	C10H16N5O14P3	522.99066			6.570	86-01-1			0
Glutathione, oxidized	C20H32N6O12S2	612.15196			4.840	27025-41-8			6

Figure 44 *Final target metabolite list with additional compound identifiers*

e Click **Open**.

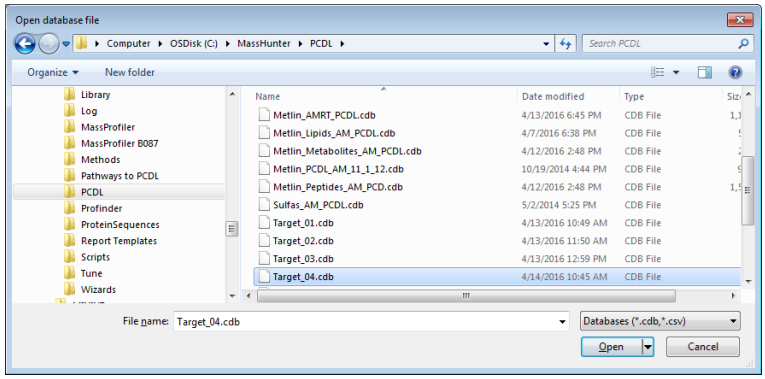


Figure 45 *Use the Move Up arrow re-order samples in the Add/Remove Sample Files dialog box*

f Select **+H** under the *Default ion species*, *Positive* group headings.

g Select **-H** under the *Default ion species*, *Negative* group headings.

h Enter `1` for **Charge state** under the *Charge state* group heading.

i Select **^{13}C** under the *Labeling* group heading.

j Enter `99` for **Isotope purity** under the *Labeling* group heading.

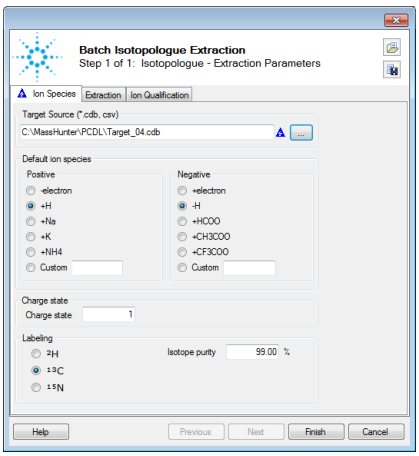


Figure 46 *Extraction Parameters - Ion Species tab*

3. Enter the extraction parameters in **Extraction** tab.

a Click the **Extraction** tab.

b Clear **Smooth EIC before integration** under the *Chromatogram smoothing* group heading.

c Select **Gaussian** for the **Smoothing function** under the *Chromatogram smoothing* group heading.

d Enter 5 points for **Function width** under the *Chromatogram smoothing* group heading.

e Enter 5 points for **Gaussian width** under the *Chromatogram smoothing* group heading.

f Select **Use peak core area** under the *Ion abundance criterion* group heading.

g Enter 20 for **% Peak height** under the *Ion abundance criterion* group heading.

h Mark **Prefer profile data** under the *Extraction data format* group heading.

i Select **Display isotopologue spectrum plots** under the *Extraction data format* group heading.

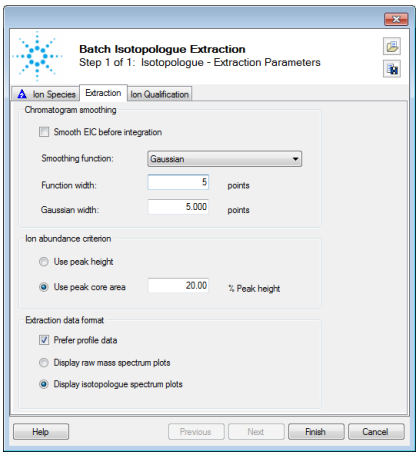


Figure 47 *Extraction Parameters - Extraction tab*

4. Enter the extraction parameters in **Ion Qualification** tab.

a Click the **Ion Qualification** tab.

b Enter `40.00` ppm for **Masses +/-** under the *Match tolerance* group heading.

c Enter `0.00` mDa for **Masses +** under the *Match tolerance* group heading.

d Enter `0.05` minutes for **Retention times** under the *Match tolerance* group heading.

e Enter `250` counts for **Anchor ion height >=** under the *Isotopologue ion thresholds* group heading.

f Enter `1000` counts for **Sum of ion heights >=** under the *Isotopologue ion thresholds* group heading.

g Enter `0.50` for **Correlation coefficient** under the *Coelution threshold* group heading.

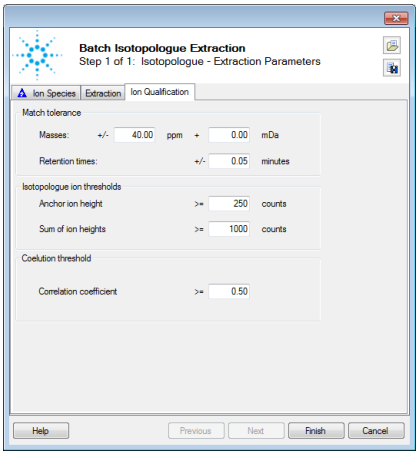


Figure 48 *Extraction Parameters - Ion Qualification tab*

Run your Batch Isotopologue Extraction method.

Click **Finish**. Isotopologue feature extraction, target metabolite mining, begins immediately and an operation progress dialog box is displayed (Figure 49).

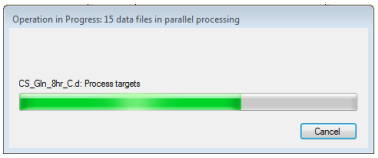


Figure 49 *Operation in progress dialog box*

Save your Batch Isotopologue Extraction method.

a Click the **Method > Save As**.

b Navigate to the folder to save your method in the **Save Method** dialog box.

c Enter `VistaFlux_WFG` for the **File name**.

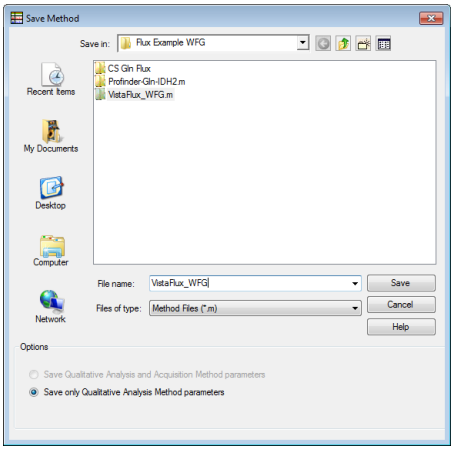


Figure 50 *Save Method dialog box used to save your Profinder method*

d Click **Save**.

Mine your sample data for the target metabolites

After you have created a Profinder project ("Create your Profinder project" on page 51), mining your target metabolites can be performed in two ways: (1) create a new Batch Isotopologue Extraction method as described in "Create your Batch Isotopologue Extraction method" on page 51, or (2) create a new project, load a previously saved method, and run Batch Isotopologue Extraction as described in this section.

Launch Profinder

Double-click the Profinder icon located on your desktop, or click **Start > All Programs > Agilent > MassHunter Workstation > Profinder B.08.00**.

Create your Profinder project

When Profinder opens you can begin a new project or open a saved project. Go to "Create your Profinder project" on page 51 to begin a new project. This section guides you through the steps to open and process a previously saved project.

1. Open a Profinder project.

   a Click **File > Open Project**, or click **Open Project** on the toolbar.

   b Adjust the main window layout and display options as necessary.

2. Review your sample data files and grouping.

   a Click **File > Add/Remove Sample Files**, click Add/Remove Sample Files... on the toolbar to review the sample data files and sample group assignments.

   b Add and remove samples files and/or designate specific files to include in this mining session using the *Show/Hide* column.

   c Click **OK** in the **Add/Remove Sample Files** dialog box.

Load your Batch Isotopologue Extraction method.

After you open your Profinder project, open your previously saved Batch Isotopologue Extraction method.

a Click **Method > Open**.

b Navigate to the folder that contains your method in the **Open Method** dialog box (Figure 51 on page 59).

c Select your previously saved method.

d Click **Open.**

e Click the appropriate response, Yes, No, or Cancel, in the **Save** dialog box to continue.

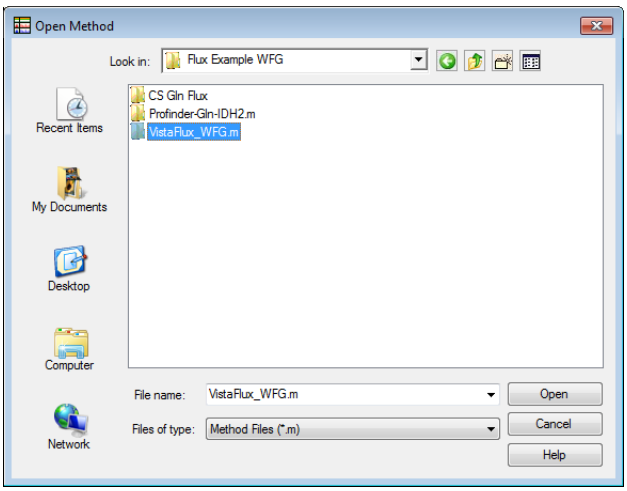


Figure 51 *Open Method dialog box*

Run your Batch Isotopologue Extraction method.

a Click **Wizards > Batch Isotopologue Extraction**.

b Review your isotopologue extraction parameters. See "Create your Batch Isotopologue Extraction method." on page 53 for an overview of the parameters.

c Click **Finish**.

d Click **Yes** in the **Warning** dialog box (Figure 52). Isotopologue feature extraction, target metabolite mining, begins immediately and an operation progress dialog box is displayed (Figure 49 on page 56)

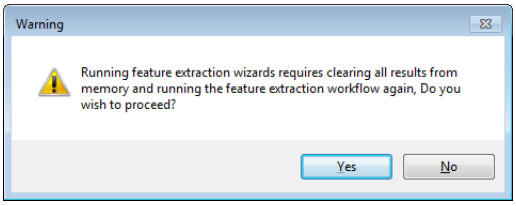


Figure 52 *Warning dialog box indicating that continuing the operation clears current results from Profinder memory*

Curate your results

The data mining results are displayed in the views available the main window. The main window, see Figure 37 on page 49, is divided into up to five windows - (a) Compound Groups, (b) Compound Details, (c) Chromatogram Results, (d) MS Spectrum Results, and (e) Isotopologue Results that are used to review the results from applying the Batch Isotopologue Extraction method to your data set. Each window can be floated independently to any location and size on your computer display or arranged to your preference within the main window.

Compound Groups

The data presented in the Compound Groups window (Figure 53) is organized as a list of all of your target compounds averaged across all of the data files in your project - averaged feature information.

The Compound Groups window shows a table of compound-level information for all of the targeted features. Semi-quantitative information is shown as the average value for the feature across all of the files where the feature was found. A *Group* row contains summary information for a single compound target as feature-extracted across your data set.

Information regarding the available columns are found in the online Help in the topic "Compound Groups Columns." A list of the available columns is displayed when you right-click within the Compound Groups table, and then click Add/Remove Columns.

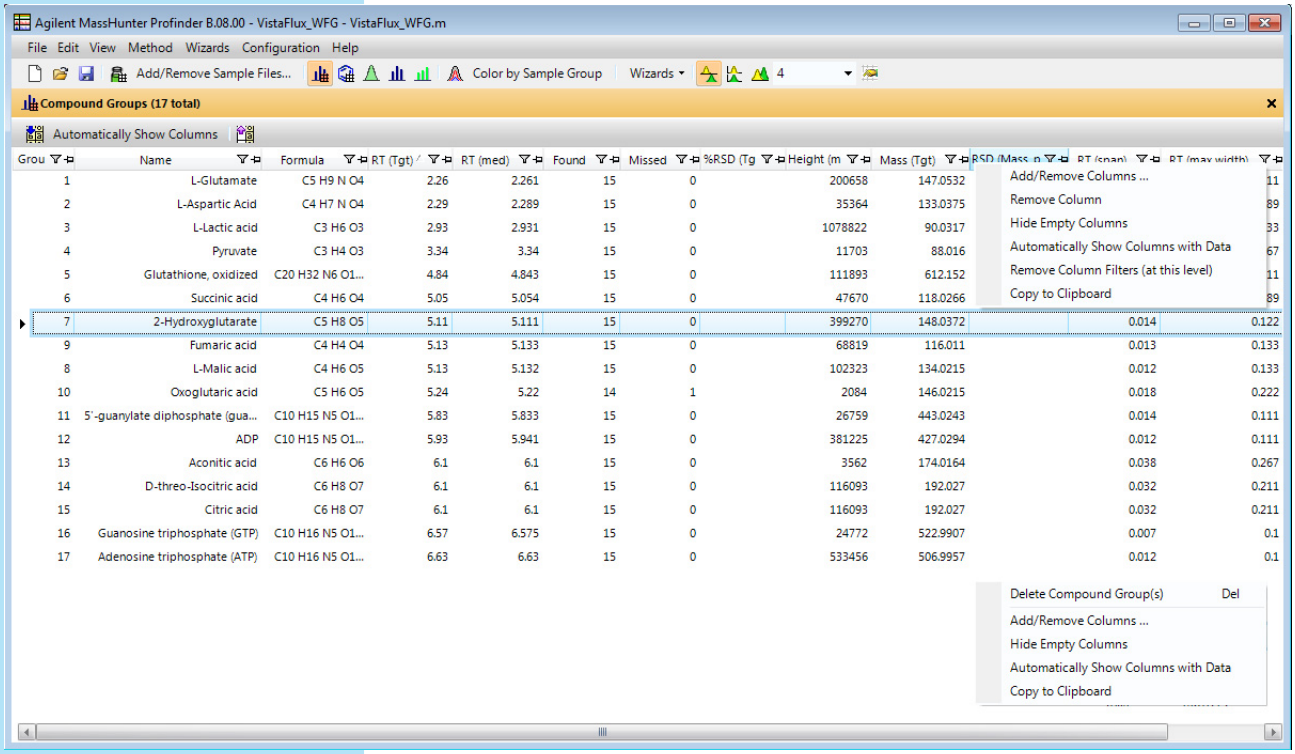


Figure 53 *Compound Groups window and shortcut menus*

Compound Details

The data presented in Compound Details window (Figure 54) is organized as a list of the appearance of a selected compound in all of the data files in your project - feature information by data file.

The Compound Details window shows a table of compound-level information for a single feature selected in the Compound Groups window. The quantitative information is shown for the selected feature as it is found in each data file in your project.

Information regarding the available columns are found in the online Help "Compound Details Columns." A list of the available columns is displayed when you right-click within the Compound Details table, and then click Add/Remove Columns.

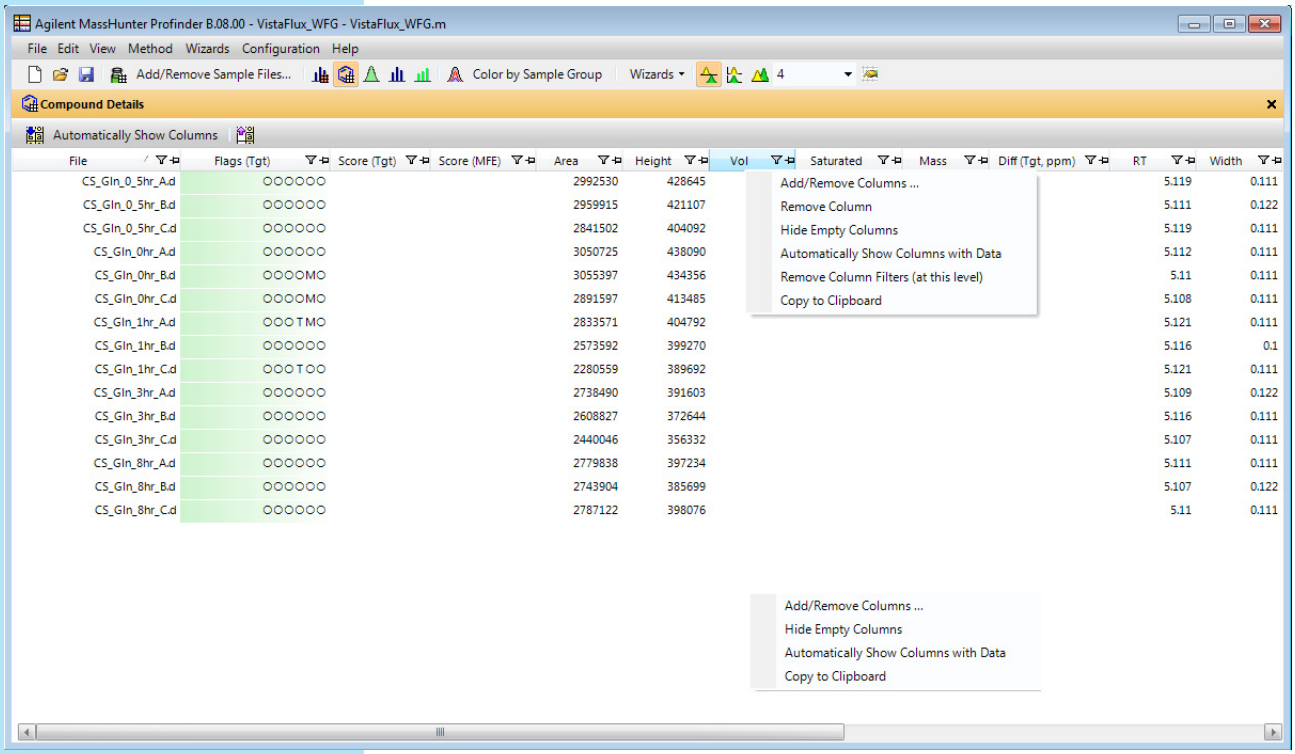


Figure 54 *Compound Details window and shortcut menus*

The *Flags (Tgt)* column, highlighted, provides a feature extraction summary for each isotopologue for the selected compound, for each sample data, file based on warning, or error flags, reported from the Find by Formula algorithm.

○ = no errors (qualified)
The isotopologue qualified and was used in the calculation.

M = *m/z* tolerance (disqualified)
The isotopologue was disqualified for not meeting the *m/z* tolerance.

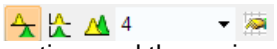
T = time correlation (disqualified)
The isotopologue was disqualified for not coeluting.

_ = no peak (disqualified)
The isotopologue was disqualified because its EIC did not have enough contiguous non-zero points.

Chromatogram Results

Chromatogram Results (Figure 55) presents the extracted ion chromatograms (EIC), or extracted compound chromatograms (ECC), for each feature. For isotopologue extraction results, the sum of the EICs for all of the qualified isotopologues are used to create ECCs for the for the target feature of the feature selected in the Compound Groups window. An ECC is displayed for each data file.

The Chromatogram Results windows provides an opportunity to quickly review the chromatograms to determine if you want to remove the compound, extract a compound manually, and adjust your Batch Isotopologue Extraction method.

The **Results Modes** buttons  on the toolbar allow you to set the display mode, display options, and the maximum number of chromatograms to display in the window. By default the chromatograms are displayed in an alternating cycle of ten colors to help you review the data for a particular data file as you select different features. **Color by Sample Group** displays the samples in an alternating cycle of colors based on the sample group assignment, time sequence for qualitative flux analyses. The order of the appearance of the samples and sample groups are set in the **Add/Remove Sample Files** dialog box.

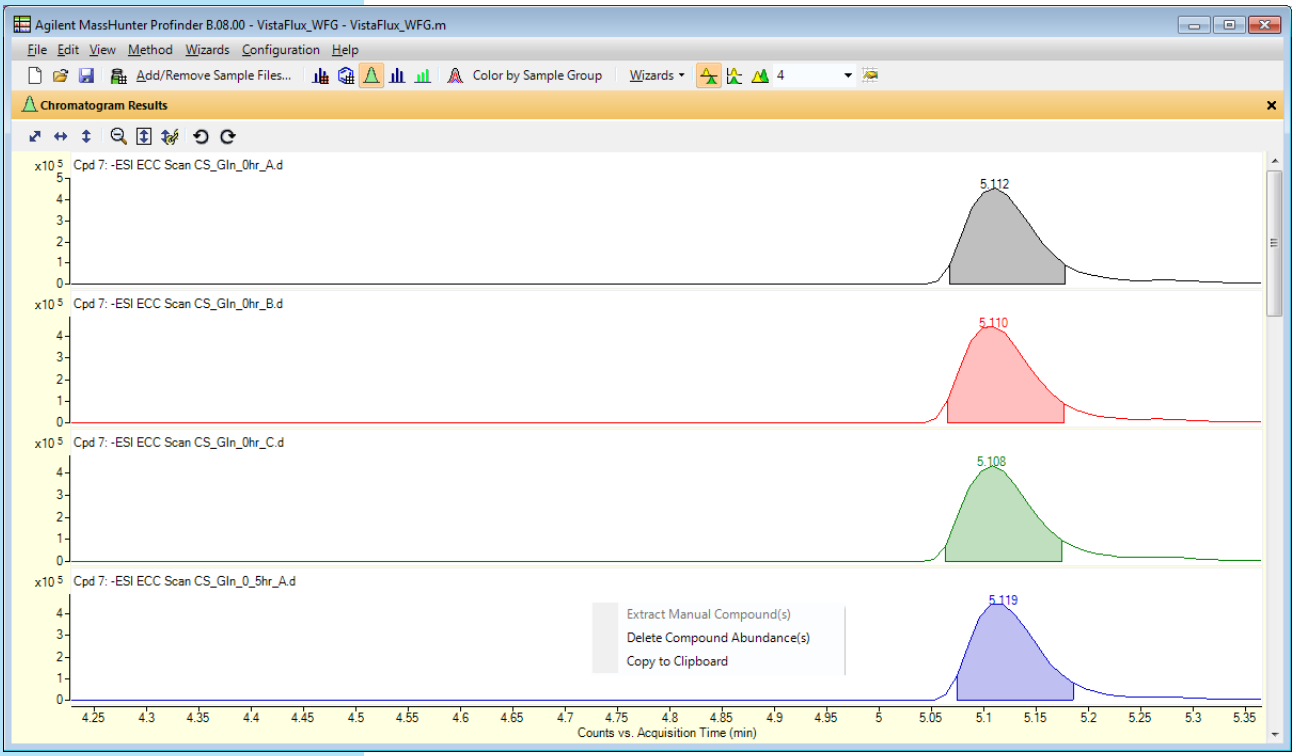
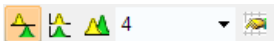


Figure 55 *Chromatogram Results window and shortcut menu*

MS Spectrum Results

MS Spectrum Results (Figure 56) presents the averaged mass spectrum (MS) across the integrated ECC for the feature selected in the Compound Groups window for each data file. For isotopologue extraction results the mass spectra are presented across the isotopologue extraction region.

The MS Spectrum Results windows provides an opportunity to quickly review the mass spectra to determine if you want to remove the compound or adjust your Batch Isotopologue Extraction method.

The **Results Modes** buttons  on the toolbar allow you to set the display mode, display options, and the maximum number of mass spectra to display in the window. By default the spectra are displayed in an alternating cycle of ten colors, matched with the Chromatogram Results, to help you review the MS data for a particular data file as you select different features. **Color by Sample Group** displays the samples in an alternating cycle of colors based on the sample group assignment, time sequence for qualitative flux analyses. The order of the appearance of the samples and sample groups are set in the **Add/Remove Sample Files** dialog box.

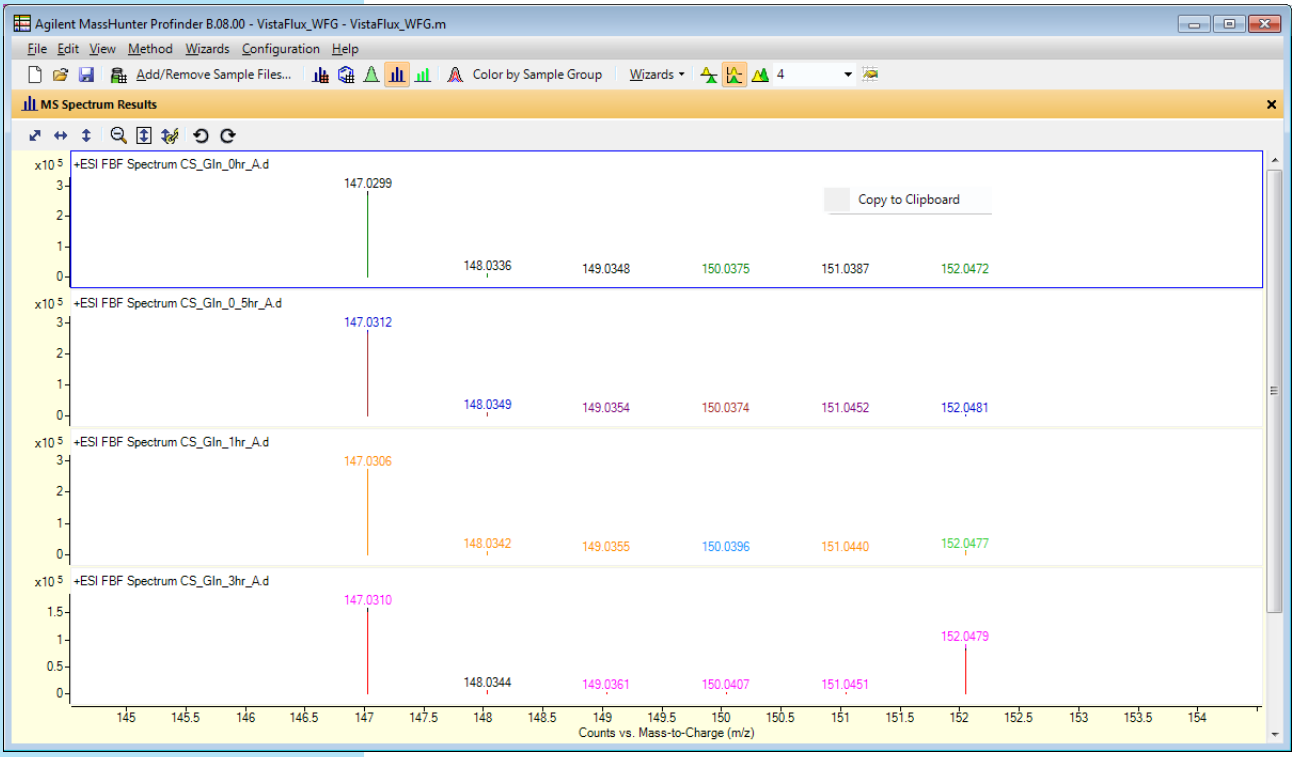


Figure 56 *MS Spectrum Results window and shortcut menu*

Isotopologue Results

Isotopologue Results presents a sequence of charts, or a single chart for the feature selected in the Compound Groups window. The display of the isotopologue results depends on the results mode  selected from the toolbar.

This view provides you with an opportunity to quickly review the flux results to determine if you want to adjust your Batch Isotopologue Extraction method.

List mode displays the isotopologue results for each sample file. The isotopologue charts are arranged in the order of your sample groups with each sample replicate displayed in an alternating cycle of ten colors, matched with the Chromatogram Results and MS Spectrum Results. The coloring can be changed to represent the sample groups by selecting **Color by Sample Group** from the toolbar.

Sample group mode (Figure 56)displays the isotopologue results for each sample group. Each sample replicate is displayed within each group chart in an alternating cycle of ten colors, matched with the Chromatogram Results and MS Spectrum Results. The coloring can be changed to represent the sample groups by selecting **Color by Sample Group** from the toolbar.

Overlaid mode displays a single summary chart of the isotopologue results. The summary chart contains the average and standard error for each isotopologue per sample group presented in gray scale. The sample groups can be viewed in color by selecting **Color by Sample Group** from the toolbar.

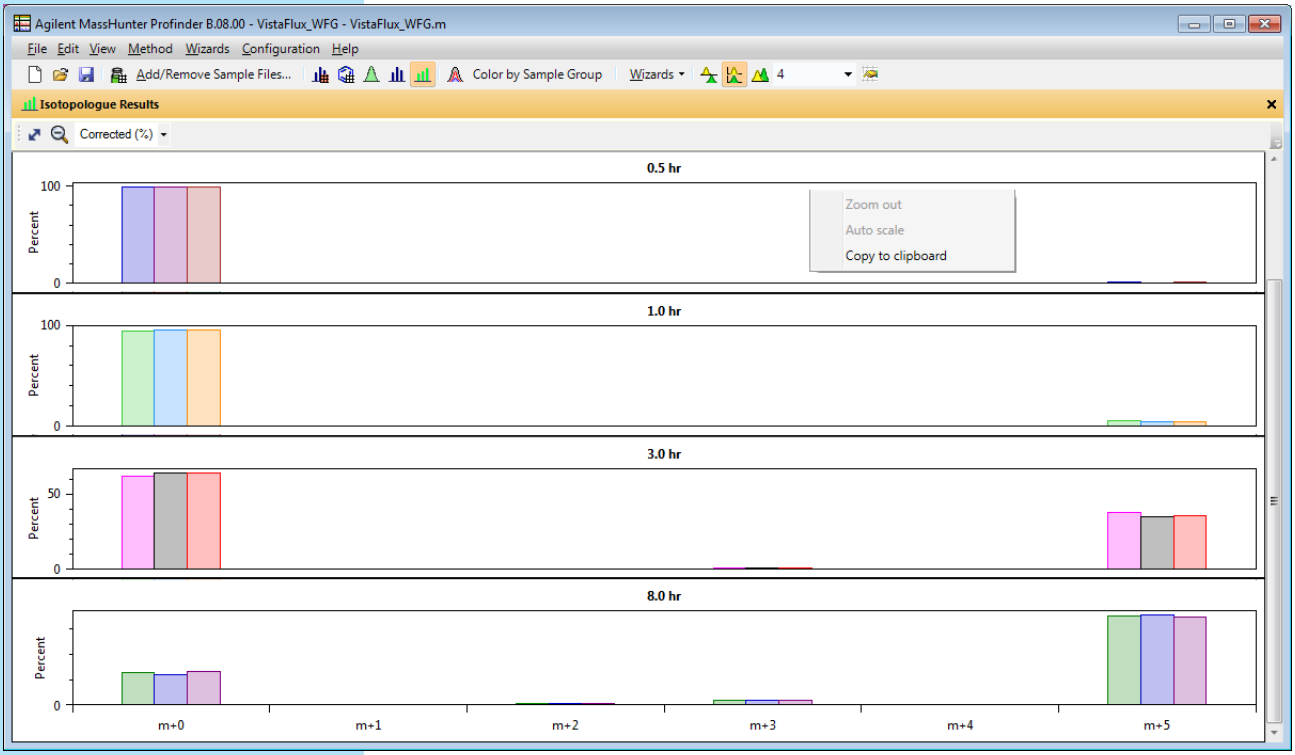


Figure 57 *Isotopologue Results window and shortcut menu*

The chart y-axes can be scaled to raw abundances (Raw), raw abundances normalized to 100% (Raw (%)), natural isotope abundance corrected abundances (Corrected), and natural isotope abundance corrected and normalized to 100% (Corrected (%)).

Export your results as a Profinder Archive

Save your Profinder results as a Profinder Archive (PFA) file to visualize your results in the context of biochemical networks in Omix Premium in chapter "Visualize on pathways" on page 67.

a Click the **File > Export as Profinder Archive**.

b Select **All compounds** under the *Export contents* heading in the **Export to Profinder Archive (.PFA)** dialog box.

c Click the **Select** ... button to select your export destination folder, and enter your file name.

d Navigate to the folder to save your project in the **Save Export File to** dialog box (Figure 58).

e Enter `VistaFlux_WFG` for the **File name**.

f Click **Save** to return to the **Export to Profinder Archive (.PFA)** dialog box. The Profinder Archive is not saved at this time; it is saved from the **Export to Profinder Archive (.PFA)** dialog box.

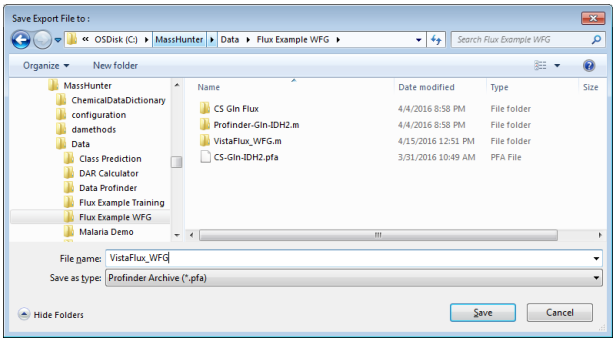


Figure 58 *Save Export File to dialog box used to save your Profinder Archive*

g Click **OK** to save your Profinder Archive file (Figure 59).

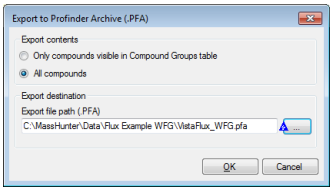


Figure 59 *Export to Profinder Archive dialog box*

Save your Profinder project

Save your Profinder project, method, and the current sample data file extraction results so that you can review your results and extraction method at a later time.

a Click the **File > Save Project as**.

b Navigate to the folder to save your project in the **Save As** dialog box.

c Enter `VistaFlux_WFG` for the **File name**.

d Click **Save**.

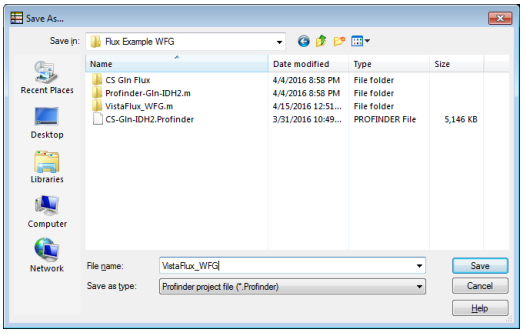


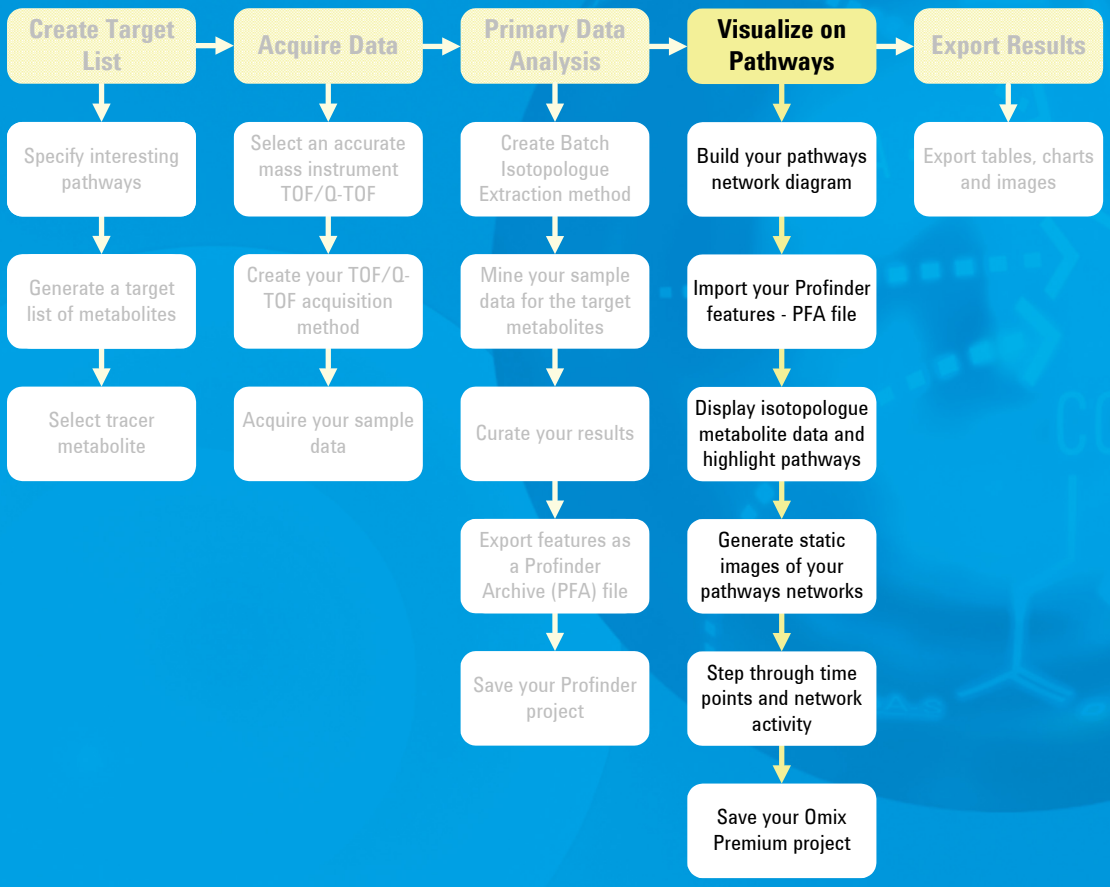
Figure 60 *Save As dialog box used to save your Profinder project*

Next step...

You have now completed the third step of the VistaFlux Software workflow. In the next workflow step you visualize your results on pathways.

Visualize on pathways

Omix Premium provides you with an intuitive, graphical, and powerful platform to build a pathways network diagram, import your Profinder results, and visualize your qualitative flux analysis in the context of the network diagram.



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Agilent Technologies

Network diagrams and Omix Premium overview

A pathways network diagram is created using Omix Premium to facilitate the visual relationship of the target compounds in the experiment. Isotopologue compositions of the target compounds, superimposed on the pathways network diagram, help you visualize the metabolic flux of the pathways and possible association with experimental conditions. The pathways network diagram in the example experiment includes target compounds from each of the following elements:

- Pathways
- Tracer compounds
- Novel metabolites
- Indirect metabolites
- Nucleosides

Example experiment

The example experiment used in this workflow guide is based on a mutation of the isocitrate dehydrogenase 2 (IDH2) enzyme associated with chondrosarcoma, a bone cancer consisting of a malignant tumor from cartilage-producing cells. IDH2 participates in the regulation of the metabolites D-*threo*-isocitrate and α-ketoglutarate in the TCA cycle. Mutation of the IDH2 results in the formation of a novel metabolite from α-ketoglutarate and perturbs energy production in the cell. To study the effect of the IDH2 mutation, the experiment monitors a set of target compounds that are directly and indirectly associated with pathways that are part of the central carbon metabolism, the primary energy producing mechanism for all aerobic biology. The following biological pathways are part of the network created for the example experiment:

- Tricarboxylic acid cycle
- Lactate fermentation
- Aspartate biosynthesis
- Glutamate biosynthesis/degradation

Description of the elements in the network diagram

Create a network diagram for each of the elements involved in the experiment with particular emphasis on the appearance of each of the seventeen (17) target compounds. The first appearance of each target metabolite in the LC/MS sample data is emphasized in the following overview of the diagram elements.

Pathways

Four BioCyc pathways are part of the example network diagram.

Lactate fermentation pathway: *Pyruvate*, a three carbon molecule formed from the glycolysis of glucose, is metabolized aerobically or anaerobically. The aerobic pathway leads to the TCA cycle. The anaerobic pathway leads to lactate (*L-lactic acid*) known for accumulating during intense muscle use.

TCA cycle: The TCA cycle is a high-output energy cycle. Entry into the TCA cycle from glycolysis is via the dehydrogenation of pyruvate to acetyl coenzyme A (acetyl-CoA). Travel around the TCA cycle leads to the following metabolite order:

START with acetyl coenzyme A (acetyl-CoA) > citrate (*citric acid*) > *cis*-aconitate (*aconitic acid*) > D-threo-isocitrate (*D-threo-isocitric acid*) > 2-oxoglutarate or α-ketoglutarate (*oxoglutaric acid* or α-KG) > succinyl coenzyme A (succinyl-CoA) > succinate (*succinic acid*) > fumarate (*fumaric acid*) > (S)-malate (*L-malic acid*) > oxaloacetate (oxalacetic acid) > RETURN to citrate.

Aspartate biosynthesis: The non-essential amino acid *L-aspartic acid* is formed from oxaloacetate, a metabolite that is part of the TCA cycle.

Glutamate biosynthesis/degradation: α-ketoglutaric acid is formed through the deamination of *glutamate*, a tracer in this study.

Tracers

The tracer is labeled glutamate, $^{13}C_5$- glutamate (U-^{13}C-Glu), that directly feeds the formation of 2-oxoglutarate in the TCA cycle. 2-oxoglutarate is in-turn acted on by the mutated enzyme IDH2 in the experimental cell line.

Novel metabolites

Novel metabolites are metabolites that form as a result of pathway perturbation, such as an enzyme mutation, and are sometimes referred to as an oncometabolite if observed in cancer cells. IDH2 normally catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate. During studies of cell lines that exhibit chondrosarcoma from the mutation of IDH2 a novel compound, *2-hydroxyglutarate*, appears as a byproduct of metabolism. IDH2 exhibiting the mutation produce 2-hydroxyglutarate from 2-oxoglutarate, thereby competing with the conversion of 2-oxoglutarate to succinyl-coenzyme A, succinic acid and additional downstream metabolites in the TCA cycle.

Indirect metabolites

Indirect metabolites show a commensurate increase, or decrease, in abundance as a result of the perturbation of a pathway. When a chondrosarcoma tumor has been established, elevated levels of *glutathione* may appear in samples.

Nucleosides

Nucleosides participate in general cellular metabolism and can be used for a variety of reasons in a flux analysis experiment. The nucleosides in this study include adenosine triphosphate (*ATP*), adenosine diphosphate (*ADP*), guanosine triphosphate (*GTP*), and guanosine diphosphate (*GDP*).

Introduction to Omix Premium

Omix Premium is a customizable visualization and modeling tool for biochemical network diagrams. Omix Premium brings together systems biology and metabolic engineering, two scientific disciplines that are involved with the understanding of complex biochemical networks, to produce high quality visualizations for qualitative flux analyses. Vector graphics generated with Omix Premium are ideally suited for scientific presentations and publications.

Based on human systems analysis activities, network diagrams generated with Omix Premium are structured using concepts such as metabolic pathways, regulatory motifs, genetic operons/regulons, signal transduction sequences, hierarchical organization, and spatial compartmentalization. Omix Premium has addressed the challenge of designing information-rich visual representations of biological network diagrams. Network diagrams created using Omix Premium are capable of displaying quantitative data from simulation runs or experimental data associated with the network nodes and edges. Omix Premium is therefore ideally suited to display your qualitative flux analysis data processed using MassHunter Profinder.

Review the Omix Premium user interface

The main functional areas of Omix Premium are shown in Figure 61. The Omix Premium window consists of four parts: (1) the Menu Bar, (2) the Toolbar, (3) the Document Area, and (4) the Status bar.

The document area can be further divided into up to five windows — (3a) Drawing Area, (3b) Component View window, (3c) Property Editor window, (3d) Data Manager window, and (3e) Log Messages window. Your visualization, and most of your interaction with Omix Premium, takes place in the Drawing Area. Each window can be floated independently to any location and size on your computer display or arranged to your preference within the Document Area, as shown in Figure 61.

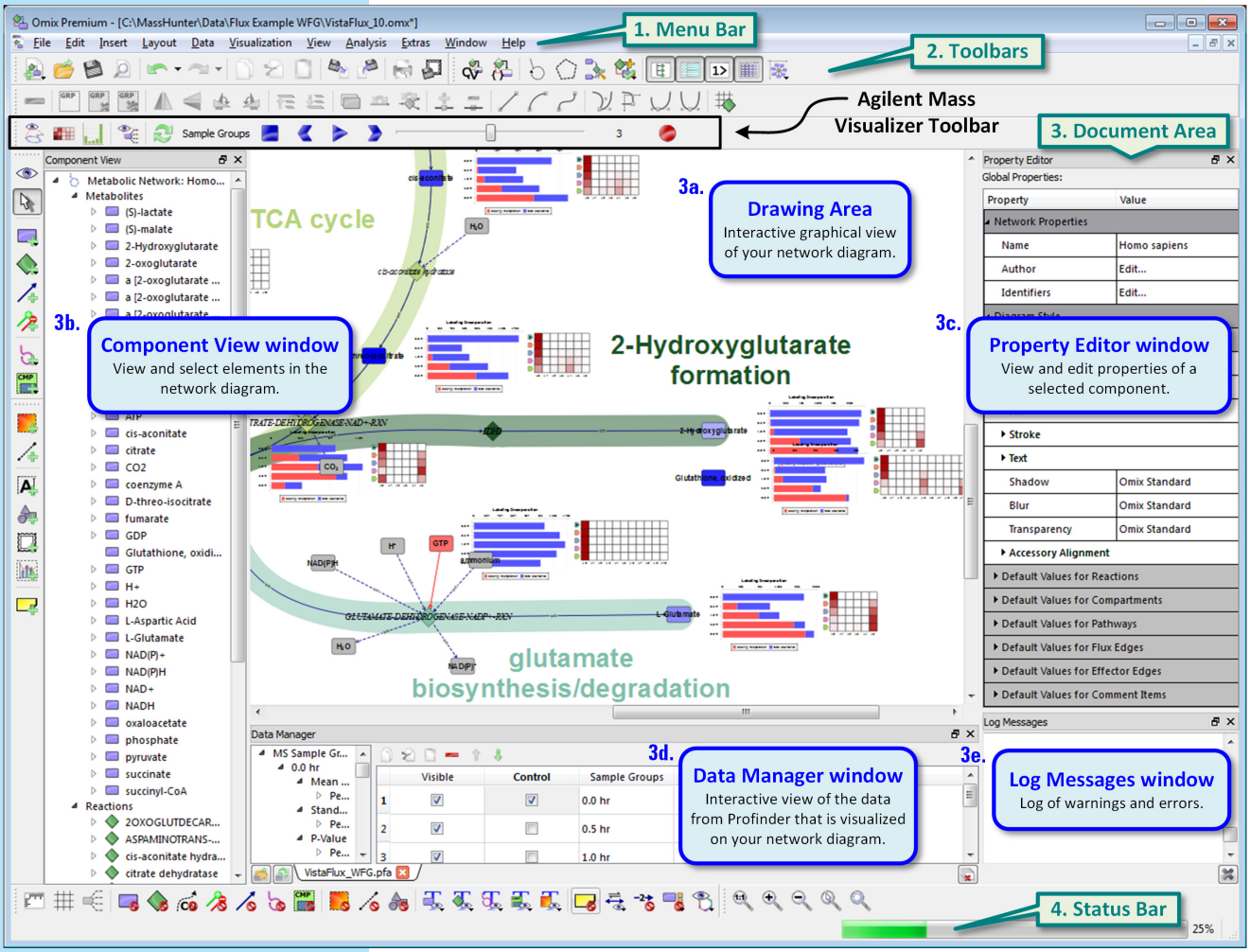


Figure 61 *Screen areas of Omix Premium with the MassVisualizer toolbar*

Review the workflow for creating a network diagram

The workflow to create your initial pathways network diagram is shown in Figure 62. The objective is to become familiar with several basic steps to create a new network pathways document.

Note: No specific sequence of steps must be followed to create your pathways network diagram. As you are learning, follow this workflow to become familiar with more than one approach to add pathways, add metabolites, and arrange elements using Omix Premium.

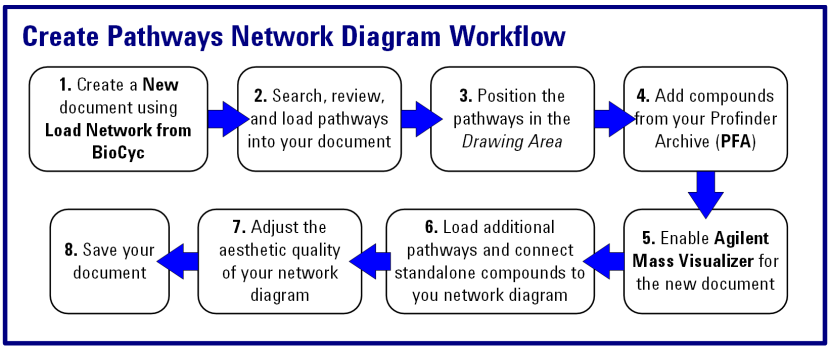


Figure 62 *Workflow to create a pathways network diagram for the data contained in your Profinder Archive*

Step 1. Create a new document using pathways content available on the BioCyc database. See section "1. Launch Omix Premium" on page 73.

Step 2. Use the Omix Premium interface to search, review, and select reactions from pathways for a specified organism. Once you have selected the target reactions for your project, import the target reactions into your new document. See section "2. Search, review, and select pathways reactions" on page 73.

Step 3. Arrange and position the components of the pathways reactions you selected into a basic order within your new pathways network diagram. During this initial exercise to arrange the imported pathways reactions you develop a familiarization with the various toolbars and working in the *Drawing Area*. See section "3. Arrange and position your pathways reactions" on page 75.

Step 4. Add the compounds from your Profinder Archive file to your pathways network diagram. Some compounds in the PFA file, generated in "Export your results as a Profinder Archive" on page 65, are not part of the initial pathways network diagram; you can create the new compounds automatically within Omix Premium. See section "4. Add compounds from your Profinder Archive file" on page 78.

Step 5. Enable the Agilent MassVisualizer plug-in for your new document. MassVisualizer enables the **MassVisualizer** Toolbar that is necessary to add the qualitative flux analysis visual elements to your network diagram. See section "5. Enable Agilent MassVisualizer" on page 80.

Step 6. Find and add additional BioCyc pathway reactions to connect and complete your pathways network diagram. Duplicate compounds created as a result of adding new pathway content can help you to become familiar with merging duplicates and linking pathways together. For compounds that are unique to your experiment, such as novel and indirect metabolites, reactions are created to link

them to the pathways network diagram. See section "6. Link new compounds and add new pathway" on page 80.

Step 7. Adjust your complete pathways network diagram using pattern tools, geometry tools for edge segments, and general visual placement in preparation for viewing your Profinder data within the *Drawing Area*. See section "7. Adjust your network diagram using pattern and geometry tools" on page 88.

Step 8. Save your pathways network diagram as an Omix Premium (OMX) document. See section "8. Save your network diagram" on page 98.

Omix Premium Plug-ins

In this workflow guide the plug-ins Agilent MassVisualizer, Atomic Layer, and Chemical Structures are enabled.

Click **Extras > Manage Plug-Ins** to launch the **Plug-in Manager** dialog box (Figure 63) to install or update plug-ins for your Omix Premium Installation.

You can also update your Omix Premium and Plug-ins using **Help > Check for Update** or **Help > Update Plug-ins**; the **Check for Update** menu is replaced with **Update Plug-ins** when the software is aware of an available plug-in update.

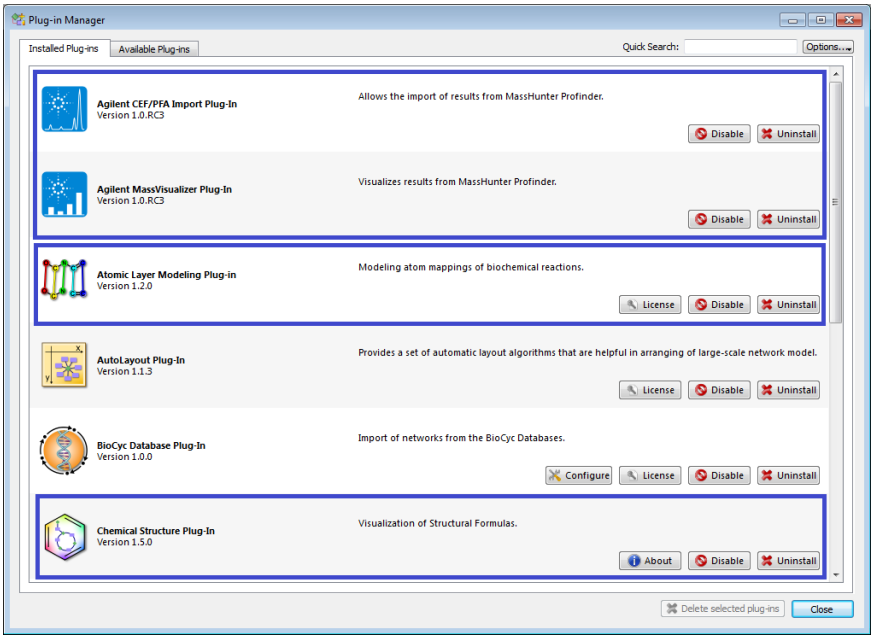


Figure 63 *Plug-in Manager dialog box*

Build your pathways network diagrams

Create your initial pathways network diagram using the basic steps described in "Review the workflow for creating a network diagram" on page 71. Similar to the creation of your target metabolite list, the pathways network diagram is based on the reactions and metabolites associated with the TCA cycle shown in Figure 4 on page 11.

1. Launch Omix Premium

A brief overview of Omix Premium is presented in "Introduction to Omix Premium" on page 69. This is *Step 1* of "Review the workflow for creating a network diagram" on page 71.

a Double-click the Omix Premium icon located on your desktop, or click **Start > All Programs > Omix Premium > Omix Premium**. When Omix Premium opens the document area is arranged to facilitate a quick means to review the software features, create a new document, and open a recent document.

Create a new document

b Click **New** in the *Document Area* (Figure 64).

c Click **Load Network from BioCyc**. The **Select BioCyc Database** dialog box is immediately opened.

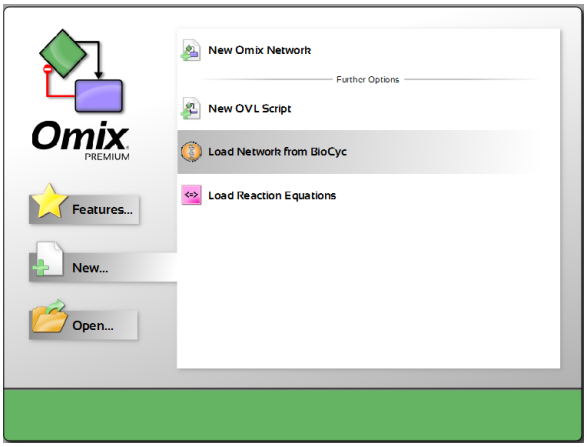


Figure 64 *Create a new document in Omix Premium*

2. Search, review, and select pathways reactions

Use the Omix Premium interface to search, review, and select reactions from pathways for a specified organism. Once you have selected the target reactions for your project, import the target reactions into your new document. This is *Step 2* of "Review the workflow for creating a network diagram" on page 71.

Select organism using **Select BioCyc Database** dialog box

a Click **Homo sapiens** for your search organism in the **Select BioCyc Database** dialog box (Figure 65 on page 74). The upper pane lists your *favorite organisms* and the lower pane lists *all BioCyc organisms*; you can mark and clear your favorite organisms from the lower pane.

b Click **OK**.

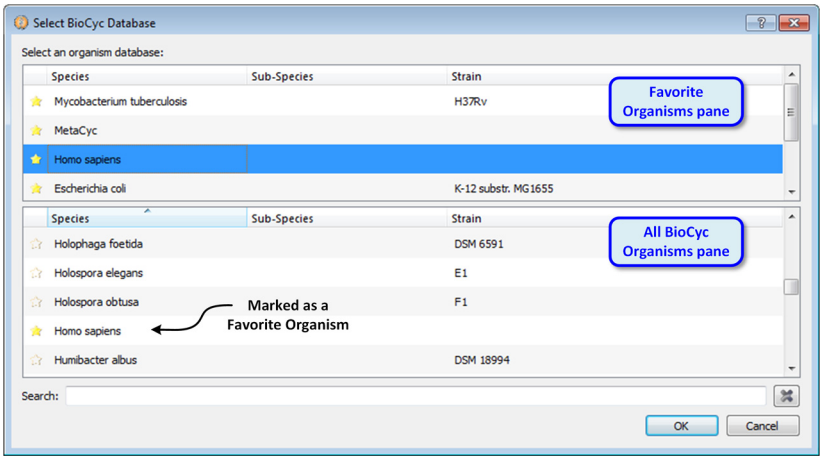


Figure 65 *Select BioCyc Database dialog box*

Select pathways using **Load from BioCyc** dialog box

a Type `TCA` in **Quick Search** to reduce the number of pathways listed in the **Browse Pathways** section of the *Database Content* in the **Load from BioCyc** dialog box (Figure 66).

b Click **TCA cycle** in the *Database Content* area.

c Click the **Show pathway on BioCyc website** button to view the pathway in your default browser.

d Click the **Add all Reactions of the selected Pathway(s) into your selected network** button to copy all of the reactions in the TCA cycle pathway to the *Selected Network* area.

Note: Double-click **TCA cycle** to view all of the TCA cycle pathway reactions.

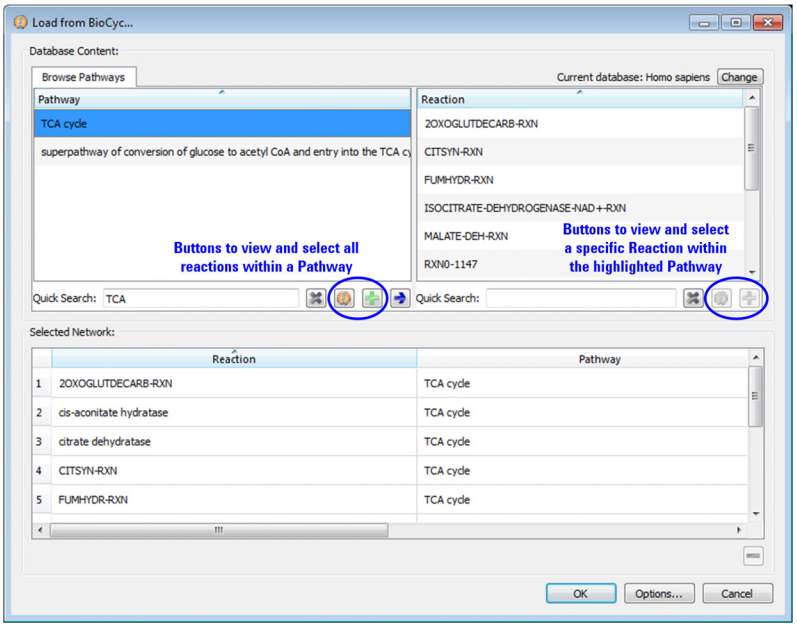


Figure 66 *Load from BioCyc dialog box*

e Type `lactate` in **Quick Search**.

f Click **lactate fermentation (reoxidation of cytosolic NADH)** in the *Database Content*.

g Click the **Show pathway on BioCyc website** button to view the pathway in your default browser.

h Click the **Add all Reactions of the selected Pathway(s) into your selected network** button to copy all of the reactions in the lactate fermentation pathway to the *Selected Network* area.

i Review the *Selected Network* table; you can remove and restore reactions.

Select one or more reactions that are not interesting. Use click, Shift-click, and Ctrl-click to select more than one reaction. Selected reactions are highlighted using a background color.

Click the **Remove all selected Reactions out of your selected network** button to remove the highlighted reactions from the *Selected Network*. If you remove a reaction you want to retain, click the source pathway in the *Database Content* and then click to restore the reactions in the selected network. Duplicate reactions are not created, Omix Premium restores the missing reaction to the *Selected Network* area.

j Click **OK** to create your new document. The two pathways are placed in the *Drawing Area*. The *lactate fermentation (reoxidation of cytosolic NADH)* pathway was moved from its initial position produce the view shown in Figure 67.

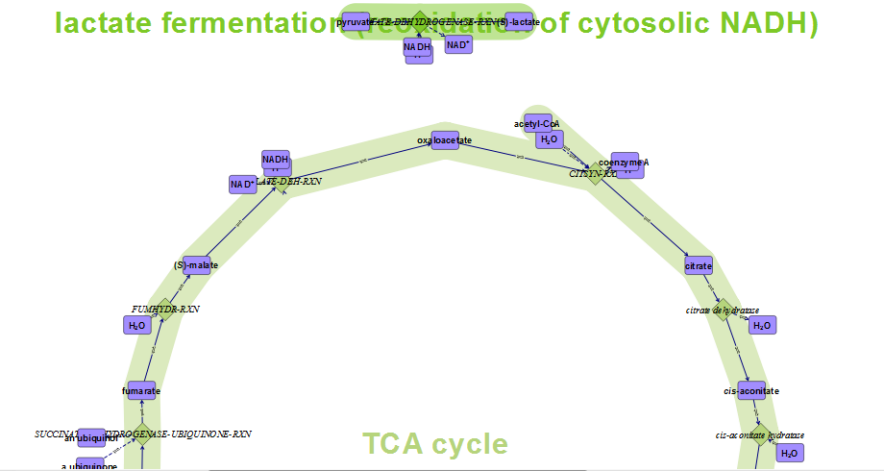


Figure 67 *Drawing Area showing an initial view of two BioCyc pathways. During this workflow you will move, edit, and align elements to improve the view.*

3. Arrange and position your pathways reactions

Use the Omix Premium *Drawing Area* interface to arrange and position the pathways reactions you selected into a basic order within your new pathways network diagram. This is *Step 3* of "Review the workflow for creating a network diagram" on page 71.

Edit pathway name

a Double-click the text **lactate fermentation (reoxidation of cytosolic NADH)** to edit the pathway name.

b Select the text **lactate fermentation (reoxidation of cytosolic NADH)**. Selected text is highlighted in a background color.

c Type `Lactate fermentation` for the pathway name, and then press Enter.

Move and arrange the pathway

a Click the pathway line associated with the *Lactate fermentation* pathway. The pathway line changes to a lighter shade of the same color indicating that the pathway is selected.

b Drag the entire pathway so that *pyruvate* (Lactate fermentation) is positioned near and above *acetyl-CoA* (TCA cycle).

c Right-click on the L-LACTATE-DEHYDROGENASE-RXN node, and then click **Automatically arrange single neighbors** to organize the nodes as shown in Figure 68.

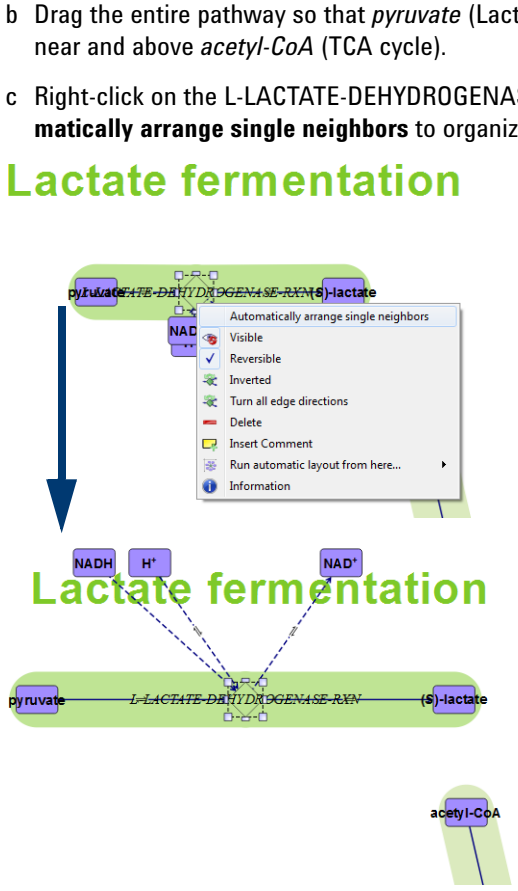


Figure 68 *Automatically arrange single neighbors to arrange cofactors*

Insert a new reaction

This set of steps creates a pathway to link the lactate fermentation pathway to the TCA cycle pathway.

a Click and drag the *Lactate fermentation* pathway so the pyruvate metabolite is above the acetyl-CoA metabolite (Figure 69 on page 77).

b Click **Insert Reaction** > **Insert Reaction** on the toolbar (or type Ctrl+Shift+R) to insert the new pyruvate decarboxylation reaction that links pyruvate to acetyl-CoA.

c Drag the new reaction to a location between the pyruvate to acetyl-CoA metabolites as shown in Figure 69.

d Type `PYR-DEC-RXN` for the name of the new reaction, and then press Enter.

Note: You must enter a reaction name when a new reaction is inserted into your pathways network diagram. Your new reaction is immediately added to the list of *Reactions* in the *Component View* window, Each reaction must have a unique name.

Insert flux edges

a Click **Insert Flux Edge** on the toolbar to insert a flux edge from pyruvate to the pyruvate decarboxylation reaction.

b Click on one of the nine control points around the pyruvate metabolite. The center control point provides the smoothest network appearance.

c Drag the flux edge to one of the nine control points around the pyruvate dehydrogenase reaction and release the flux edge. The center control point provides the smoothest network appearance.

d Click on one of the nine control points around the pyruvate decarboxylation reaction to insert a second flux edge from the pyruvate decarboxylation reaction to the acetyl-CoA metabolite. Since the Insert Flux Edge is still active, it is not necessary to click **Insert Flux Edge** a second time. The center control point provides the smoothest network appearance.

e Drag the flux edge to one of the nine control points around the acetyl-CoA metabolite and release the flux edge. The center control point provides the smoothest network appearance.

f Click **Select** on the toolbar, or press Esc, to exit the process of inserting flux edges.

Note: The pyruvate decarboxylation reaction joins the lactate fermentation reaction to the TCA cycle. The *Drawing Area* view in Figure 69 illustrates changes made to the lactate fermentation pathway name and movement of the relative positions of the metabolites and reactions to improve the pathway clarity.

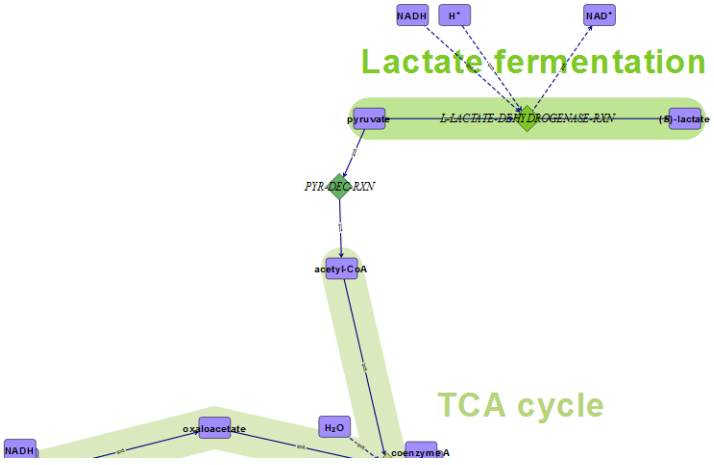


Figure 69 *Drawing Area showing the inserted pyruvate decarboxylation reaction*

4. Add compounds from your Profinder Archive file

Add the compounds from the PFA file you saved in "Export your results as a Profinder Archive" on page 65. This is *Step 4* of "Review the workflow for creating a network diagram" on page 71.

Note: You can import your isotopologue results into an Omix Premium document without a pathway ("Import Profinder results without a pathway" on page 100). When you import a PFA file from Profinder, you are notified when one or more compound in your PFA file does not match any compounds in your Omix Premium document; when you choose to insert the unmatched compounds from your PFA you can use all of the MassVisualizer tools (see "Display isotopologue metabolite data and highlight pathways" on page 103) to review any PFA file without first creating a pathways network diagram.

Open Profinder data

a Click **Data > Open Data Table**.

b Navigate to the folder and select file VistaFlux_WFG.pfa (Figure 70) in the **Open Data Source** dialog box.

c Click **Open**. The *Data Manager* window is automatically opened.

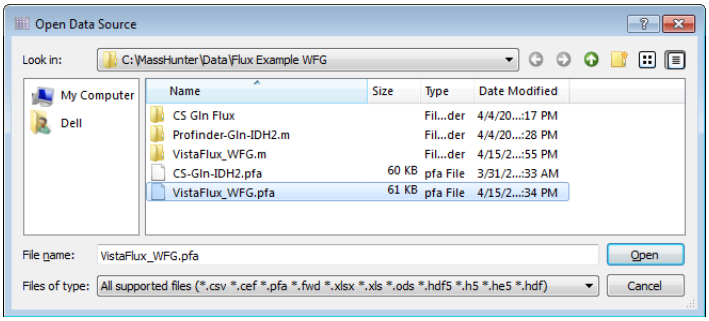


Figure 70 *Open Data Source dialog box*

Activate Agilent MassVisualizer plug-in

d Click **Yes** in the new **Open Data Source** dialog box to activate Agilent MassVisualizer (Figure 71). You can chose to activate the Agilent MassVisualizer plug-in later; however, MassVisualizer must be activated at this time in this workflow.

Note: If you do not activate the MassVisualizer plug-in you cannot add missing compounds in the next step. Click **Extras > Document Extensions > Agilent MassVisualizer** to manually enable MassVisualizer and continue to the next step.

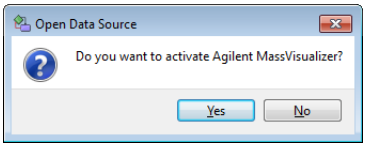


Figure 71 *Open Data Source dialog box*

e Click **Data > Show Data Manager** to close the *Data Manager* window. The purpose for opening your Profinder project is to obtain the compounds that are not present in the TCA cycle and lactate fermentation pathways; the *Data Manager* window is not necessary to import the missing compounds.

Add compounds to the network diagram

a Click **Visualization > Agilent MassVisualizer > Check for missing compounds**. If you have not previously checked for missing compounds, when you next click the **Show Abundance Changes** button Omix Premium will automatically check for missing compounds.

Note: When there are compounds in the PFA file that do not match compounds in the open document, the **Compound Availability** dialog box is opened after you click the **Show Abundance Changes** button. This provides an opportunity to import metabolites that have mass spectral data to a pathway diagram that does not yet contain nodes for them. Otherwise the **Abundance Change Visualization** dialog box is opened.

b Review the list of metabolites in the Profinder Archive file that do not match to a compound in the open document in the **Compound Availability** dialog box. Four compounds are not available in the network diagram (Figure 72).

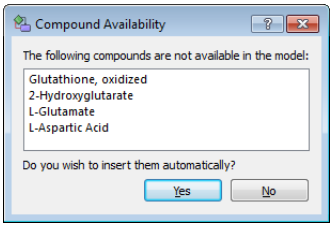


Figure 72 *Compound Availability dialog box*

c Click **Yes** to add the four missing compounds to the network document: Glutathione, oxidized, 2-Hydroxyglutarate, L-Glutamate, and L-Aspartic acid.

Note: Even though compound names in the Profinder Archive are not the same as the names used on by the imported BioCyc pathways, the compounds are successfully matched. Inclusion of compound identifiers, such as CAS, HMP, and KEGG (see "Add retention times and additional identifiers to your target metabolite list." on page 34) improved the data matching accuracy when you imported your Profinder project into Omix Premium.

d Drag the group of four metabolites to a position next to the TCA cycle.

e Click on the *Drawing Area* to place the four metabolites. Exact placement is not important; the metabolites are moved to new locations as they are linked to your network diagram (Figure 73).

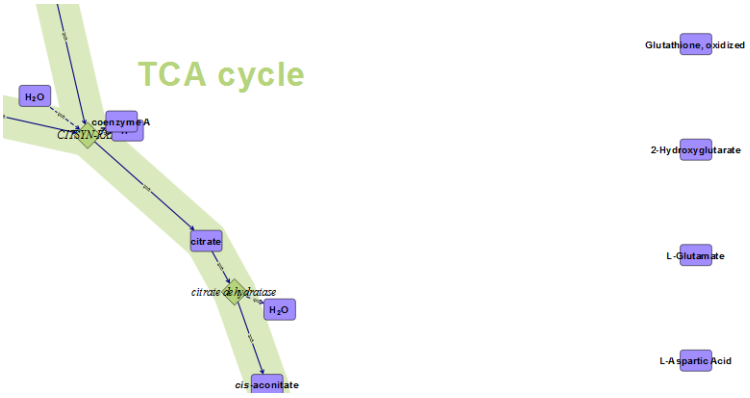


Figure 73 *Drawing Area showing the four missing metabolites*

5. Enable Agilent MassVisualizer

Choose the plug-ins to enable for your document. This is *Step 5* of "Review the workflow for creating a network diagram" on page 71.

a Click **Extras > Document Extensions > Agilent MassVisualizer** (Figure 74).

Note: You must enable the **Agilent MassVisualizer** plug-in to enable visualization of your isotopologue results from Profinder. If the **Agilent MassVisualizer** plug-in is not enabled when you open a PFA file Omix Premium will automatically prompt you (Figure 71).

b Click **Extras > Document Extensions > Atomic Layer**.

c Click **Extras > Document Extensions > Chemical Structures**.

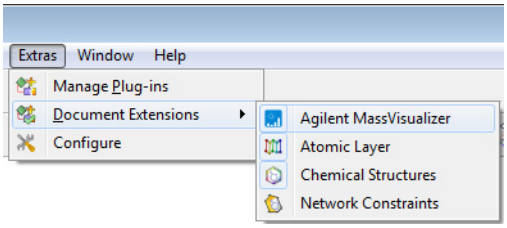


Figure 74 *Enable Document Extensions from the menu bar*

Enable the **Agilent Mass Visualizer** toolbar

a Right-click anywhere on the toolbar to view the status of the available toolbars.

b Click **MassVisualizer Toolbar** (Figure 75).

c Drag the **MassVisualizer Toolbar** group to a convenient location among the open toolbars.

Figure 75 *Initial MassVisualizer toolbar*

6. Link new compounds and add new pathway

Find and add BioCyc pathway reactions to complete your pathways network diagram. For compounds that are unique to your experiment, such as novel and indirect metabolites, reactions are created to link them to the pathways network diagram. This is *Step 6* of "Review the workflow for creating a network diagram" on page 71.

1. Link the novel metabolite 2-hydroxyglutarate to the TCA cycle.

The novel metabolite 2-hydroxyglutarate is linked to 2-oxoglutarate via a new reaction labeled IDH2.

a Drag the 2-hydroxyglutarate metabolite to a position near and to the right of 2-oxoglutarate on the TCA cycle.

Insert a new reaction

b Click **Insert Reaction** **> Insert Reaction** on the toolbar (or type Ctrl+Shift+R) to insert the new IDH2 reaction that links 2-hydroxyglutarate to 2-oxoglutarate.

c Drag the new reaction to a location between the 2-hydroxyglutarate and 2-oxoglutarate metabolites.

d Type `IDH2` for the name of the new reaction, and then press Enter (Figure 76).

Note: You must enter a reaction name when a new reaction is inserted into your pathways network diagram. Your new reaction is immediately added to the list of *Reactions* in the *Component View* window. Each reaction must have a unique name.

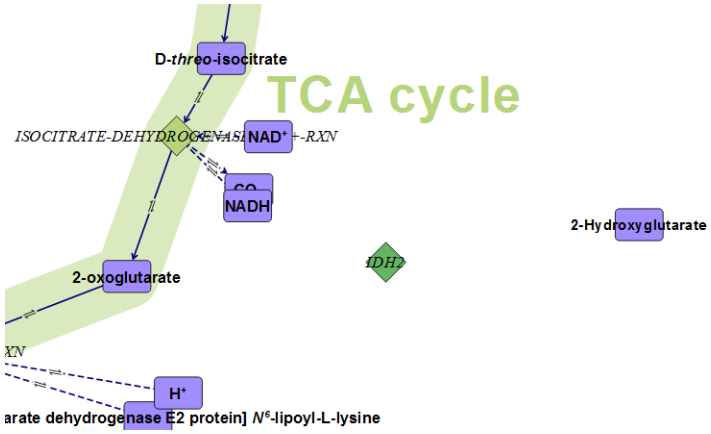


Figure 76 *Drawing Area showing the inserted IDH2 reaction*

Insert flux edges

e Click **Insert Flux Edge** on the toolbar to insert a flux edge from 2-hydroxyglutarate to the IDH2 reaction.

f Click on one of the nine control points around the 2-oxoglutarate metabolite. The center control point provides the smoothest network appearance.

g Drag the flux edge to one of the nine control points around the IDH2 reaction and release the flux edge. The center control point provides the smoothest network appearance.

h Click on one of the nine control points around the IDH2 reaction to insert a second flux edge from the IDH2 reaction to the 2-hydroxyglutarate metabolite. Since the Insert Flux Edge is still active you do not need to click **Insert Flux Edge** a second time. The center control point provides the smoothest network appearance.

i Drag the flux edge to one of the nine control points around the 2-hydroxyglutarate metabolite and release the flux edge. The center control point provides the smoothest network appearance.

j Click **Select** on the toolbar, or press Esc, to exit the process of inserting flux edges. Your network diagram should look similar to Figure 77 on page 82.

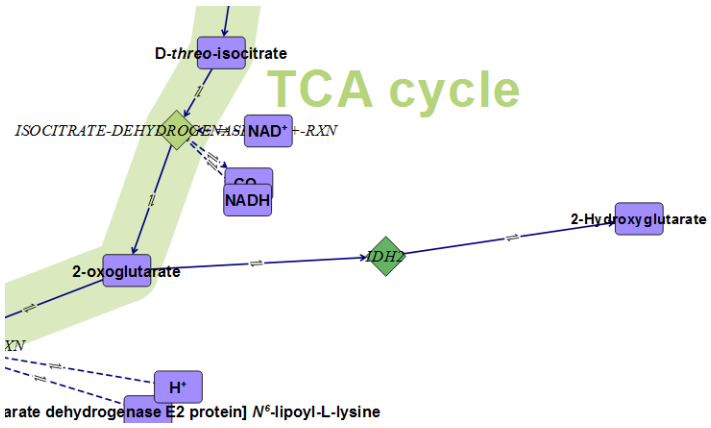


Figure 77 *Drawing Area showing the inserted IDH2 and flux edges*

2. Move the indirect metabolite glutathion near 2-hydroxyglutarate.

Since the glutathione metabolite is an indirect metabolite, position the glutathione metabolite near the 2-hydroxyglutarate metabolite.

- Drag the glutathione, oxidized metabolite to a position near the 2-hydroxyglutarate metabolite. Flux edges are not necessary to connect this metabolite to the network diagram (Figure 78).

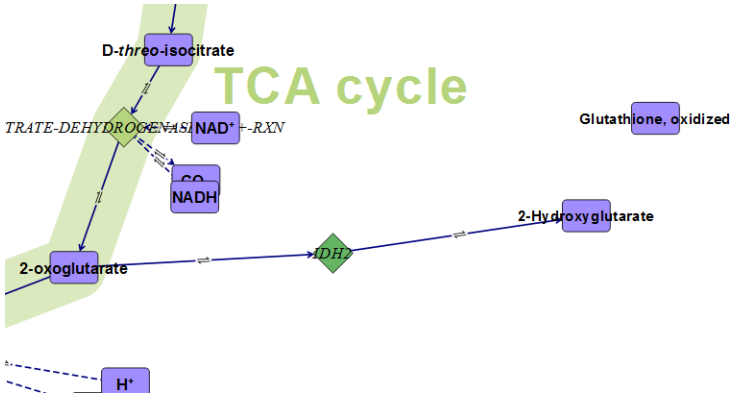


Figure 78 *Drawing Area showing the repositioned glutathion, oxidized metabolite*

3. Insert a new pathway containing L-aspartic acid.

Insert the aspartate biosynthesis pathway to show the relationship of the metabolite L-aspartic acid to the TCA cycle.

Delete metabolites

a Right-click the **L-aspartic acid** metabolite in the *Component View*, and then click **Delete** to delete the L-aspartic acid metabolite that was imported with the PFA file (Figure 79 on page 83).

b Right-click the **L-glutamate** metabolite in the *Component View*, and then click **Delete**.

Note: Because L-aspartic acid and L-glutamate are also part of the new pathway imported later in this step, deleting these metabolites *before* inserting the new pathway assures that the BioCyc database information is included in the network model (for example, chemical structure, database identifiers, and synonyms). The database information available in **Insert Pathway from BioCyc** in this case con-

tains more information, including the structural information, than the database used to create the PCDL used in Profinder.

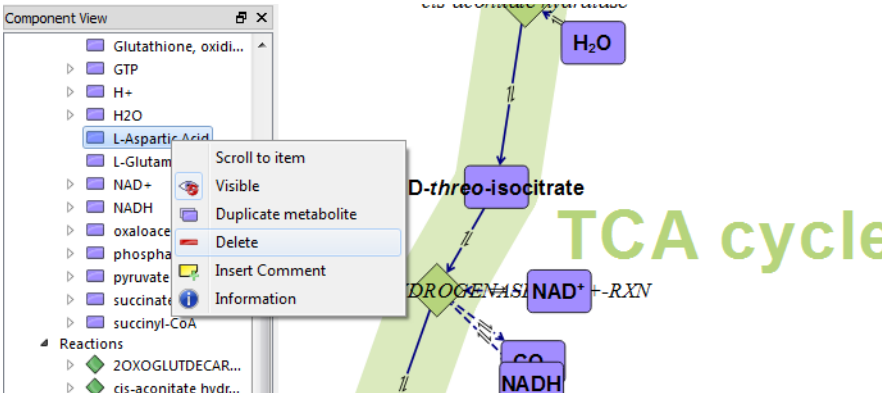


Figure 79 *Delete a metabolite from the Component View*

Insert a new pathway

c Click **Insert Pathway** > **Insert Pathway from BioCyc** on the toolbar, or click **Insert > Insert Pathway > Insert Pathway from BioCyc** on the menu bar, to insert the aspartate biosynthesis pathway.

d Type `aspartate` for **by name** search in the **Select Pathway** dialog box.

e Click the **Search** button (Figure 80).

f Click **aspartate biosynthesis** in the list of pathways that match your search text.

g Clear **import regulatory effects (inhibition and activation)**. Regulatory effects are only relevant for kinetic modeling and are not necessary for qualitative flux analysis.

h Click **OK**.

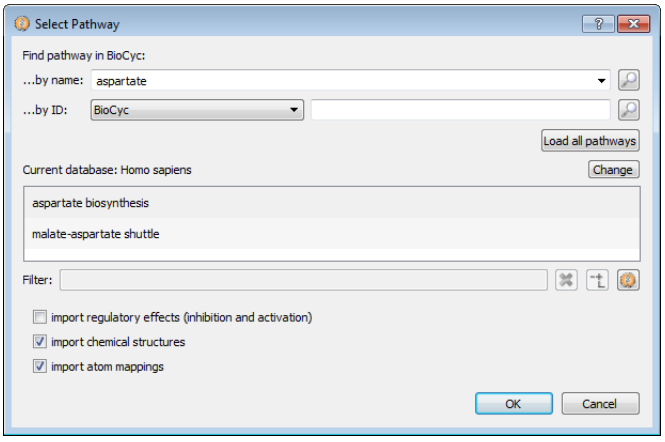


Figure 80 *Select Pathway dialog box*

i Drag the aspartate biosynthesis pathway near to and above the oxaloacetate metabolite on the TCA cycle, then click on the *Drawing Area*.

j Drag the pathway name aspartate biosynthesis to a position above the pathway so you can access the individual metabolites and reactions.

k Right-click on the ASPAMINOTRANS-RXN node, and then click **Automatically arrange single neighbors** to organize the nodes. Your network diagram should look similar to Figure 81.

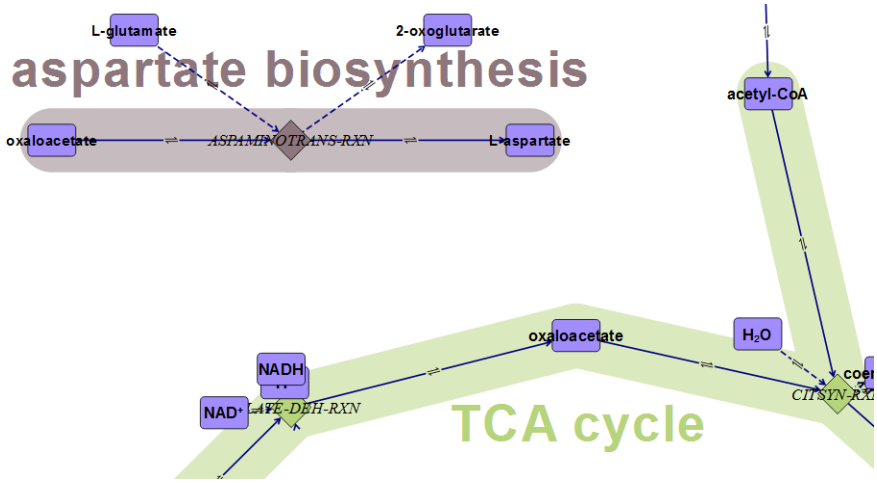


Figure 81 *Drawing Area showing the aspartate biosynthesis pathway next to the TCA cycle pathway*

Mirror a pathway

l Drag a selection box around the aspartate biosynthesis reaction (Figure 82).

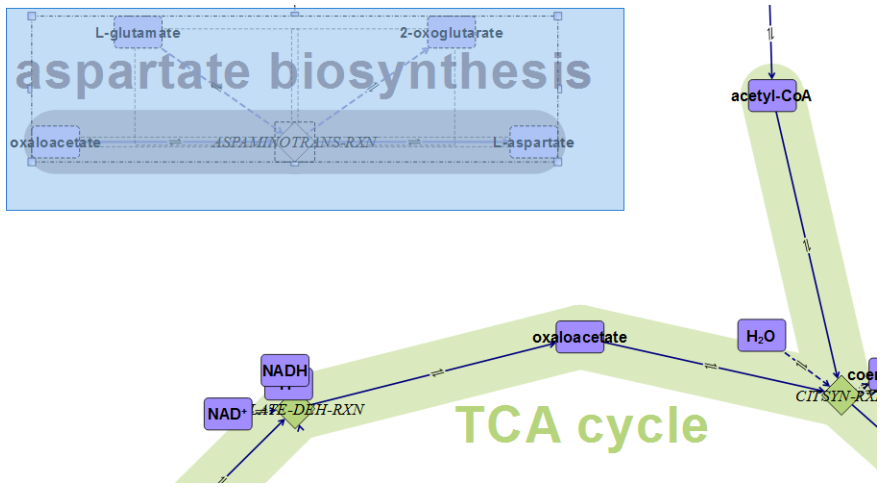


Figure 82 *Drawing Area showing the aspartate biosynthesis pathway next to the TCA cycle pathway*

m Right-click on an element of the selected the aspartate biosynthesis reaction.

n Click **Mirror horizontally**, or click the **Mirror horizontally** button, this places the oxaloacetate metabolites in each of the pathways near each other (Figure 83 on page 85).

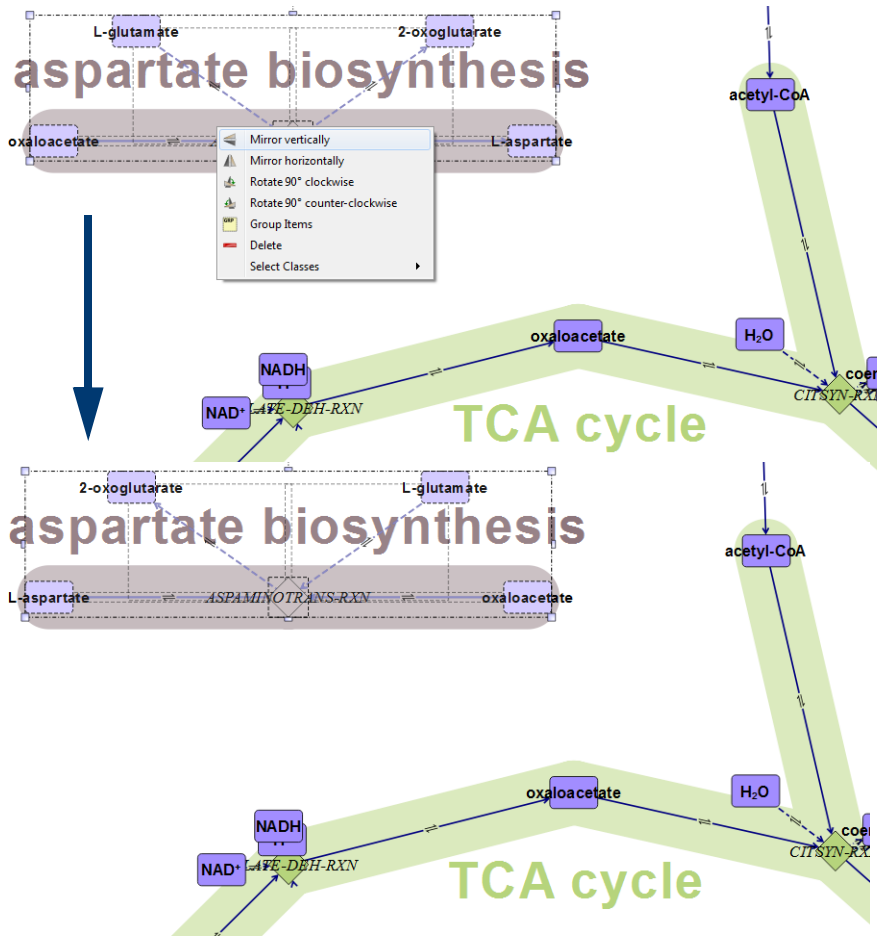


Figure 83 *Mirror a pathway to align the oxaloacetate metabolites near each other*

Unify a duplicate metabolite

o Click one oxaloacetate metabolite.

p Shift-click the other oxaloacetate metabolite to select both metabolites. A dashed rectangle encompassing both metabolites confirms that the metabolites are selected.

q Right-click the oxaloacetate metabolite that is part of the TCA cycle pathway.

Note: When you move, scale, or rotate a multi-node selection, any nodes that are assigned to an underlying pattern are ignored and remain unchanged on their position.

r Click **Unify selected duplicates** into this metabolite. The two pathways are now joined together (Figure 84 on page 86).

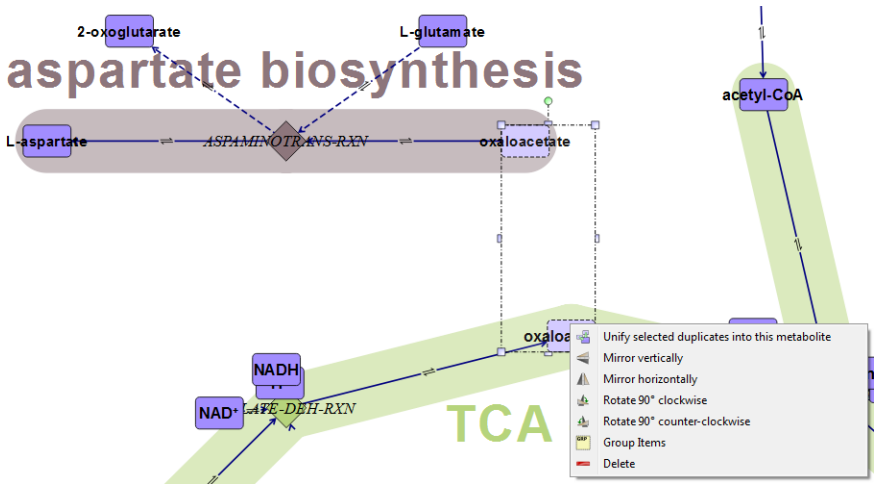


Figure 84 *Drawing Area showing the final arrangement of the aspartate biosynthesis pathway*

s Drag the metabolites, reactions, and descriptors to improve the pathway clarity similar to Figure 85.

Note: The aspartate biosynthesis reaction also includes the tracer metabolite, L-glutamate, that is the last metabolite from the target metabolite list imported from the PFA file. However, the tracer metabolite is part of another reaction pathway more directly associated with the mutant IDH2 reaction added in the next step.

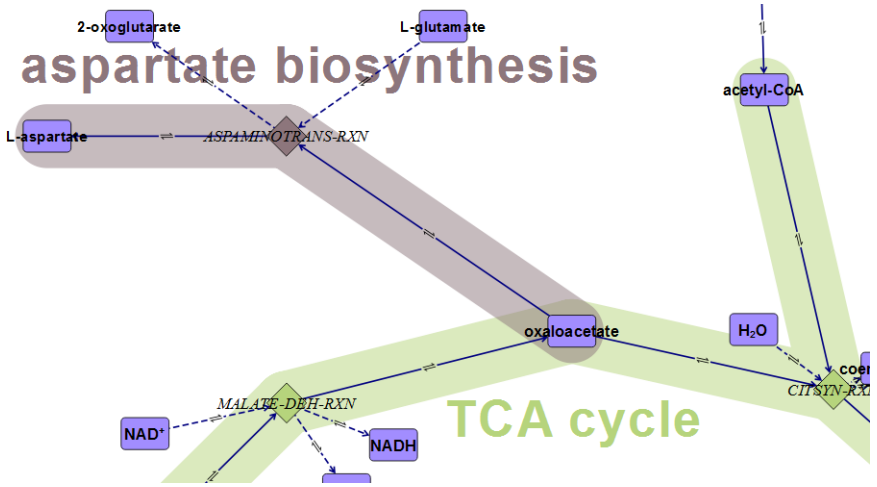


Figure 85 *Drawing Area showing the final arrangement of the aspartate biosynthesis pathway*

4. Insert a new pathway containing L-glutamate.

Insert the glutamate biosynthesis/degradation pathway to show the relationship of the metabolite L-glutamate to the TCA cycle near the IDH2 reaction.

Note: Because the L-glutamate metabolite imported from the PFA was previously deleted, the L-glutamate already present in the network model was created from the BioCyc database and therefore contains the most detailed information. L-Glutamate is not deleted before adding the new pathway in this step.

Insert a new pathway

a Click **Insert Pathway** > **Insert Pathway from BioCyc** on the toolbar, or click **Insert > Insert Pathway > Insert Pathway from BioCyc** on the menu bar to insert the glutamate biosynthesis/degradation pathway.

b Type `glutamate` for the **by name** search in the **Select Pathway** dialog box.

c Click the **Search** button (Figure 86 on page 87).

d Click **glutamate biosynthesis/degradation** in the list of pathways that match your search text.

e Clear **import regulatory effects (inhibition and activation)**. Regulatory effects are only relevant for kinetic modeling and are not necessary for qualitative flux analysis.

f Click **OK**.

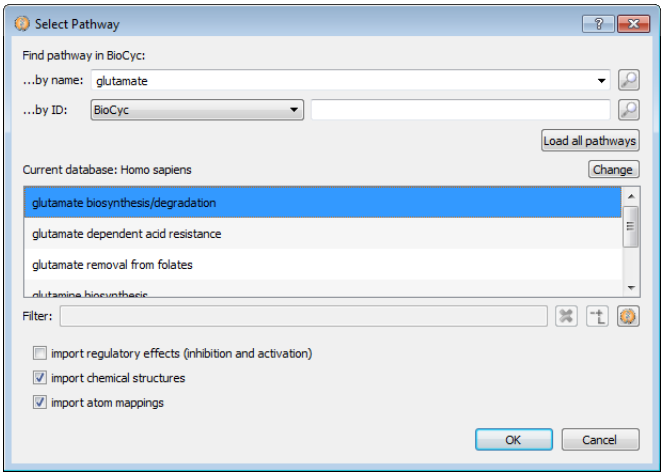


Figure 86 *Select Pathway dialog box*

g Drag the aspartate biosynthesis pathway near the 2-oxoglutarate metabolite on the TCA cycle, then click on the *Drawing Area*.

h Drag the pathway name glutamate biosynthesis/degradation to a position below the pathway so you can access the individual metabolites and reactions associated with the pathway (Figure 87).

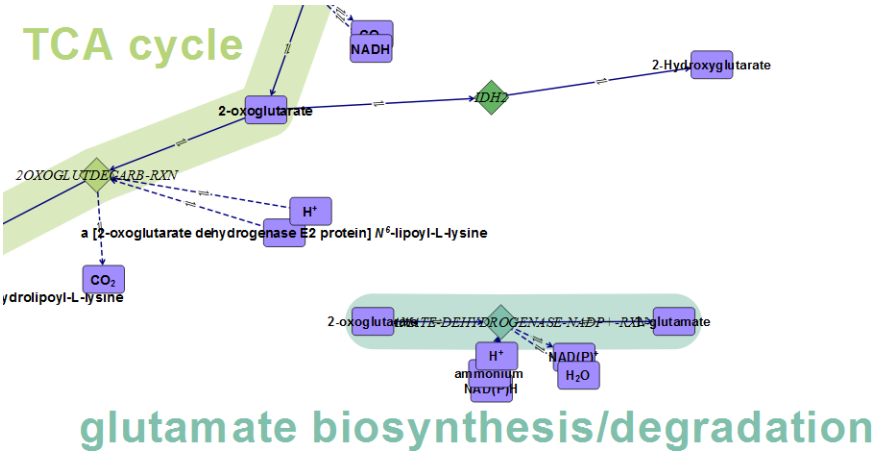


Figure 87 *Drawing Area with the glutamate biosynthesis/degradation pathway*

Unify a duplicate metabolite

i Click on one 2-oxoglutarate metabolite.

j Shift-click on the other 2-oxoglutarate metabolite to select both metabolites. A dashed rectangle encompassing both metabolites confirms that the metabolites are selected.

k Right-click on the 2-oxoglutarate metabolite that is part of the TCA cycle pathway.

l Click **Unify selected duplicates** into this metabolite. The two pathways are now joined together.

m Right-click on the GLUTAMATE-DEHYDROGENASE-NADP+-RXN reaction, and then click **Automatically arrange single neighbors** to improve the pathway clarity.

n Move the pathway nodes to create a layout that is similar to Figure 88.

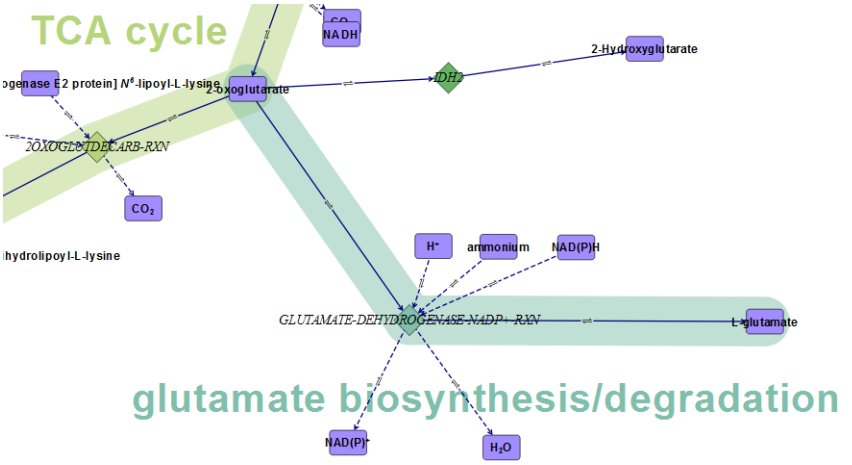


Figure 88 *Drawing Area showing the final arrangement of the glutamate biosynthesis/degradation pathway*

7. Adjust your network diagram using pattern and geometry tools

Adjust your pathways network diagram using pattern tools, geometry tools for edge segments, new pathways, and general visual placement in preparation for viewing your Profinder data within the *Drawing Area*. This is *Step 7* of "Review the workflow for creating a network diagram" on page 71.

Add a circle path

a Click the **Edit Layout Patterns** button on the toolbar. The *Drawing Area* changes; the network diagram is moved to the background and toolbars not related to the *Pattern Editor* are removed.

b Click the **Zoom Diagram** button on the toolbar so the entire pathway is visible in the *Drawing Area*.

c Click the **Insert Circle Pattern Path** button on the toolbar (Figure 89 **A** on page 89).

d Click in the *Drawing Area* over the CITCYN-RXN node to start the circle at the entry to the TCA cycle (Figure 89 **B** on page 89).

e Drag the dynamic circle view so that is aligned with and slightly larger than the general TCA cycle in the *Drawing Area* (Figure 89 **C**).

f Click on the *Drawing Area* to place the circle path. The beginning and end of the arc are indicated with red squares (Figure 89 **D**).

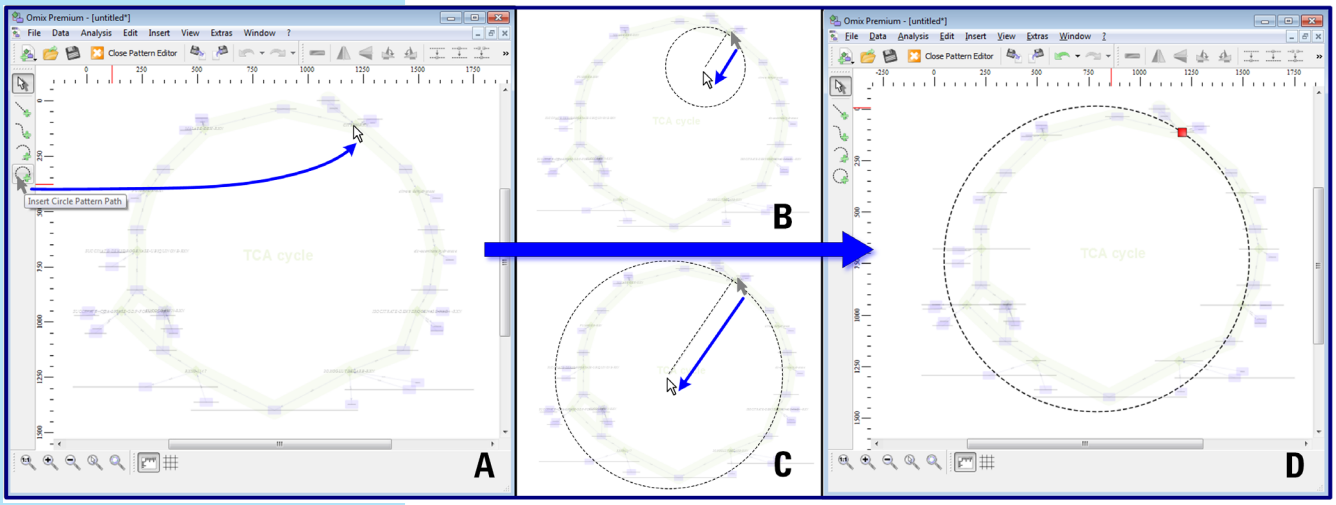


Figure 89 *Sequence of actions to draw a circle pattern in the Pattern Editor*

Note: When the circle is started and position properly over the TCA cycle, Omix Premium automatically guides you through alignment of the TCA cycle pathway to the pattern starting with the CITCYN-RXN node as shown in Figure 90 on page 90. Animated arrows on the circle shows that the alignment is proceeding in a clockwise direction. You direct the automatic layout using the **Add Nodes to Pattern** dialog box.

If the circle pattern is not properly inserted over the reaction the **Add Nodes to Pattern** dialog box does not appear. After you close the **Pattern Editor**, you can launch the **Add Nodes to Pattern** dialog box by dragging the CITCYN-RXN node onto the red square of the circle pattern that now appears in the background of the *Drawing Area*.

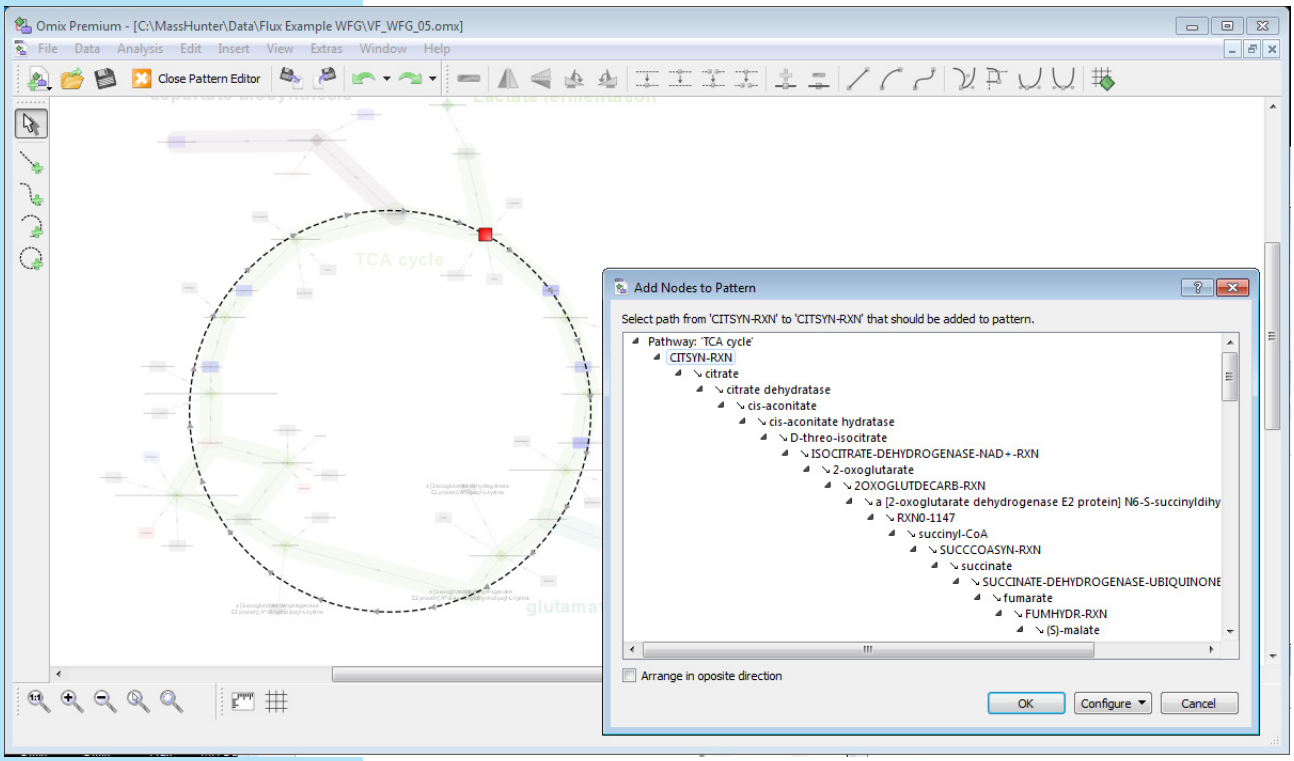


Figure 90 *Pattern editor and Add Nodes to Pattern dialog box*

Add nodes to the circle pattern

a Click on each successive metabolite and reaction to confirm the nodes you want to align to the circle pattern. When a node, a metabolite, or reaction is clicked the pathway is highlighted to provide a visual confirmation of the pattern alignment you selected (Figure 91 on page 91).

The beginning of the path is labeled *Pathway: 'TCA cycle'* and each successive node of the pathway is cascaded below and to the right. The starting node, CITCYN-RXN, is determined by where the circle was initially placed with respect to the pathway. When a pathway has a branch, an alternate cascaded sequence of nodes is presented further down the list of nodes. Move the **Add Nodes to Pattern** dialog box away from the *Drawing Area* so you can preview the layout path highlighted in yellow on the pathway diagram as shown in Figure 91 on page 91.

Mark **Arrange in opposite direction** if you want to arrange nodes in a counter-clockwise order.

b Click the second CITSYN-RXN node (highlighted in green) to select the nodes in a clockwise order as shown in Figure 92 on page 91. A green-highlighted node represents a complete and valid path through the network. You must select a green node to proceed with the layout process.

c Click **OK** to complete the node order. The network diagram is automatically fitted to the circle pattern and the edges are converted from lines to smooth curves that are fit onto the circle path (Figure 93 on page 92).

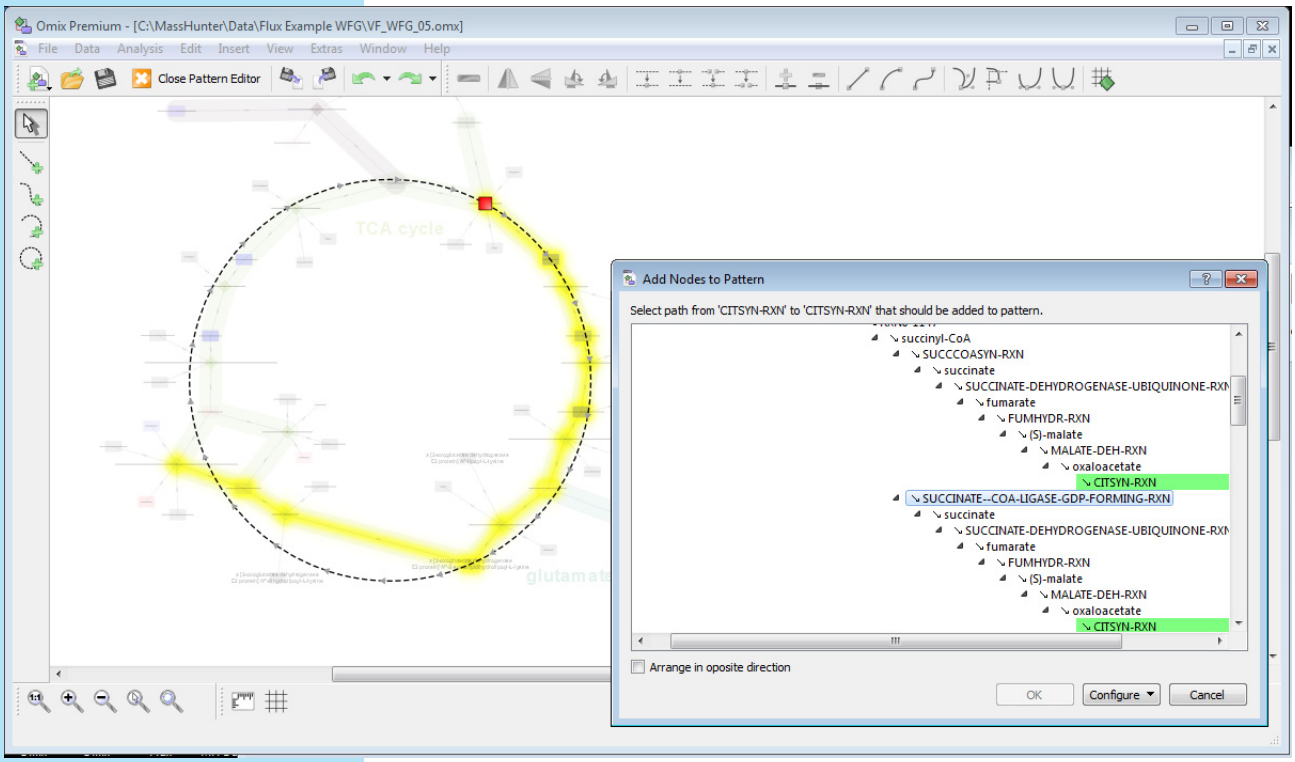


Figure 91 *Adding nodes to the circle path*

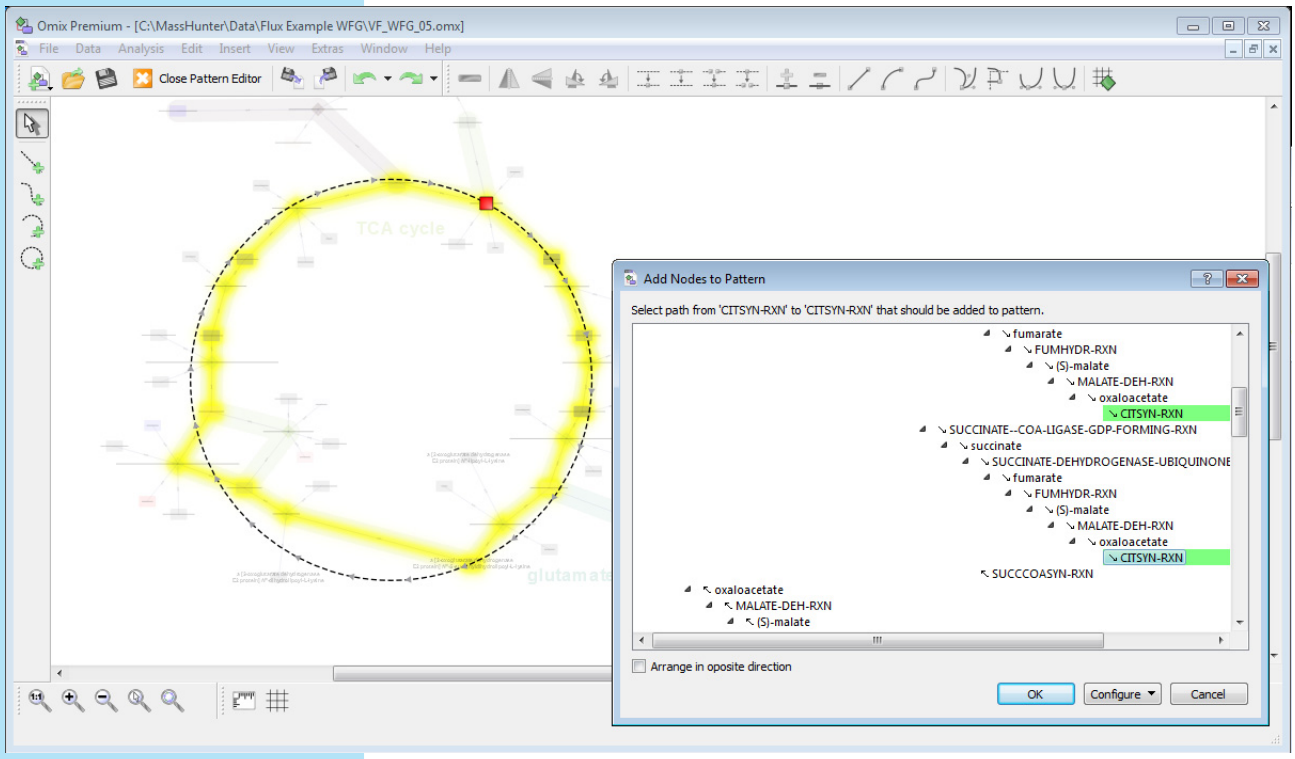


Figure 92 *Selecting the nodes to add to the path when there is a split in the pathway*

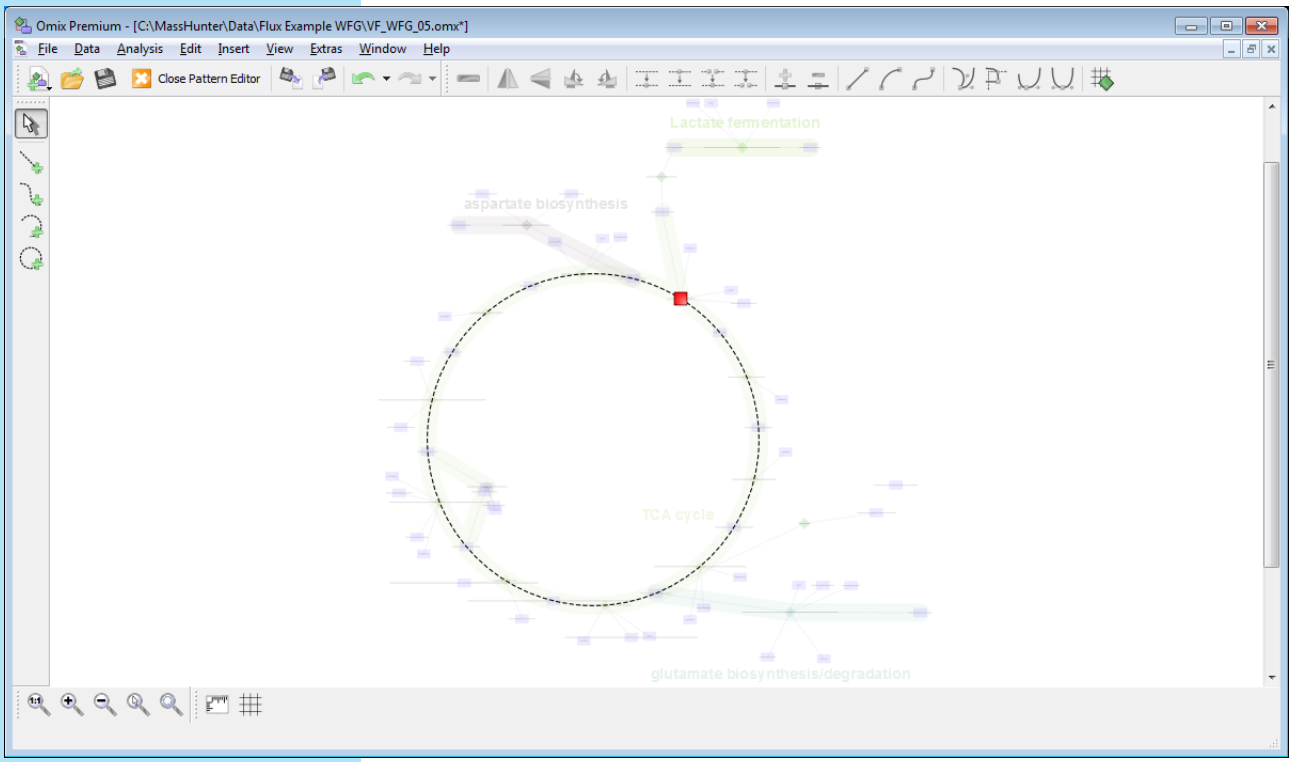


Figure 93 *The TCA cycle pathway fitted to a circle path*

Add a curve path

a Zoom the *Drawing Area* into the split path of the TCA cycle containing the SUCCOASYN-RXN node.

b Click the **Insert Curve Pattern Path** button on the toolbar.

c Click on the succinyl-CoA node, but not on the circle path, to start the curve path. It is not recommended to have the curve path connect to the circle path; this can lead to a confusion regarding which path a node and its edges follow. This is the first control point for the curve.

d Click on the *Drawing Area* near, but to the lower-right of the SUCCINATE-COA-LISAGE-GDP-FORMING-RXN node. This is the second control point for the curve.

e Click again on the *Drawing Area* near, but to the upper left of the SUCCINATE-COA-LISAGE-GDP-FORMING-RXN node. This is the third control point for the curve.

f Click on SUCCINATE-COA-LISAGE-GDP-FORMING-RXN node, but not on the circle path, to complete the line path. This is the fourth, and final, control point for the curve. A curve path is completed when you place four control points (Figure 94 on page 93).

g Click **Close Pattern Editor** Close Pattern Editor on the toolbar. The circle and curve paths are visible behind your pathways network diagram.

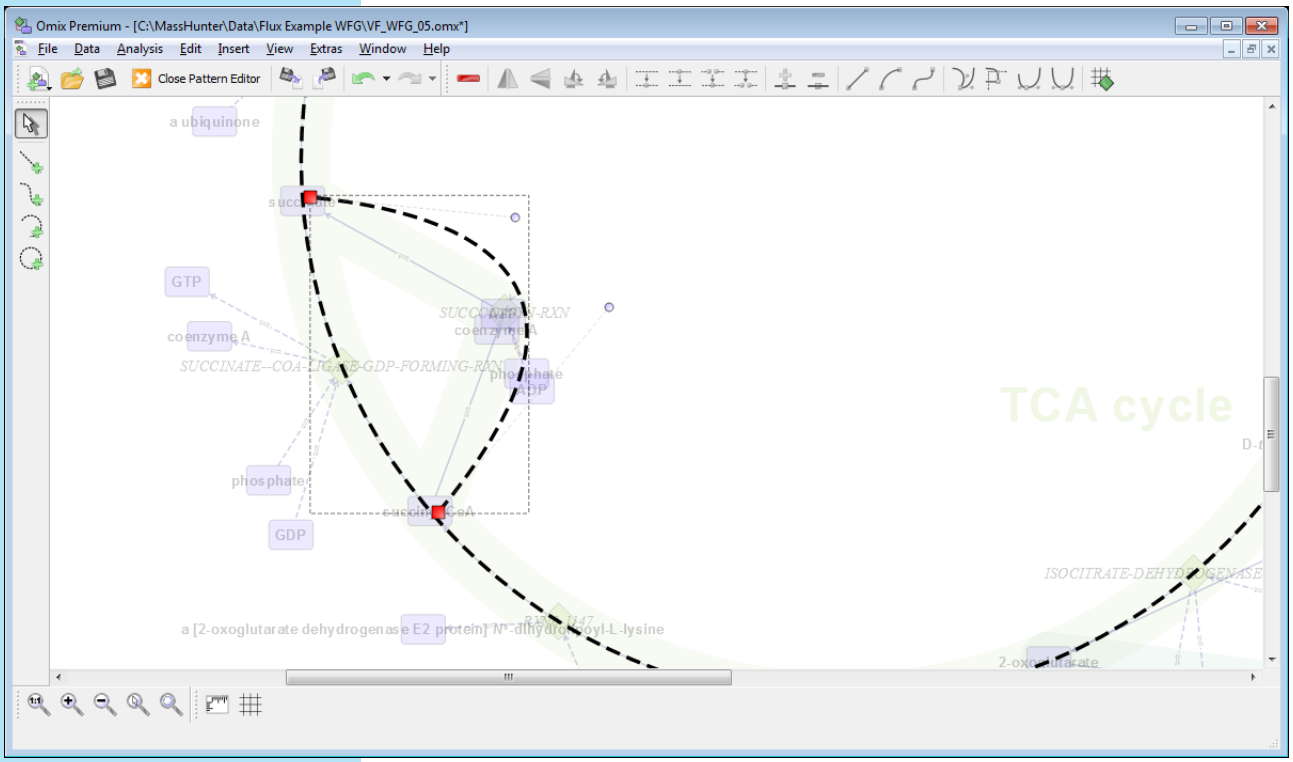


Figure 94 *Curve path in the pattern editor*

Add a node to a path

a Drag the SUCCCOASYN-RXN node to the curve path. By moving the reaction node to a position close to the underlying path, Omix Premium automatically snaps the node to the path.

b Drag the metabolites connected to the SUCCCOASYN-RXN node to view the reaction edges.

Align an edge to a path

a Click the edge between the succinyl-CoA and SUCCCOASYN-RXN nodes. The edge is surrounded by a dashed rectangle.

b Click the edge between the succinyl-CoA and SUCCCOASYN-RXN nodes a second time to enable changing the edge geometry. A red circle appears on the edge (Figure 95).

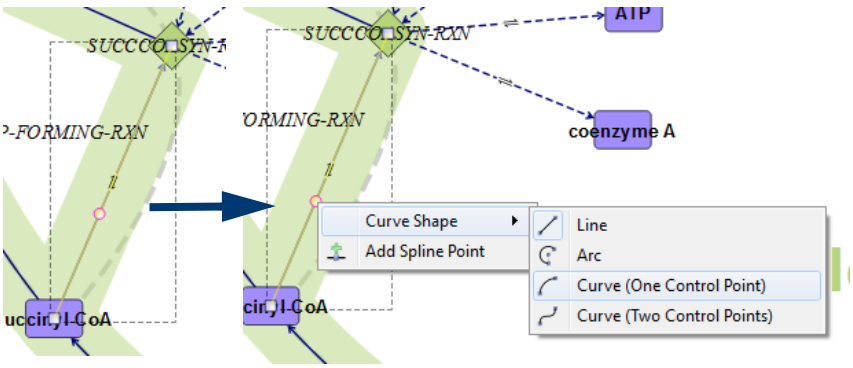


Figure 95 *Select a line edge to convert to curve edge*

c Right-click the red circle (control point) on the edge, then click **Curve Shape > Curve (One Control Point)** to change the edge line into a curve (Figure 95 on page 94).

d Drag the edge control point, now a black circle, to match the edge to the arc path (Figure 96).

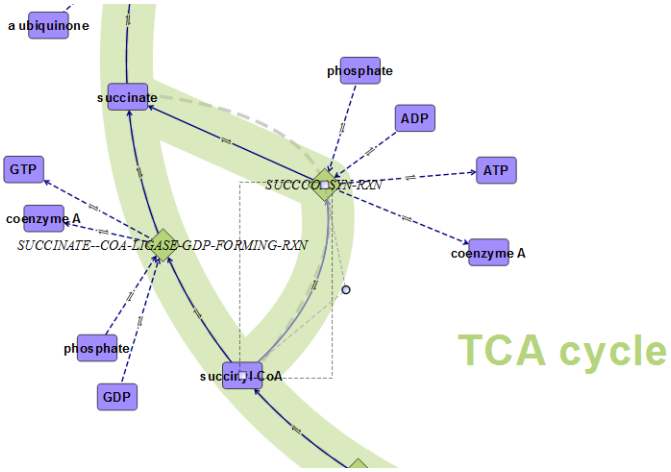


Figure 96 *Adjust a curve edge to follow the background curve pattern*

e Repeat the edge conversion to a curve and alignment for the edge between the SUCCCOASYN-RXN and succinate nodes (Figure 97).

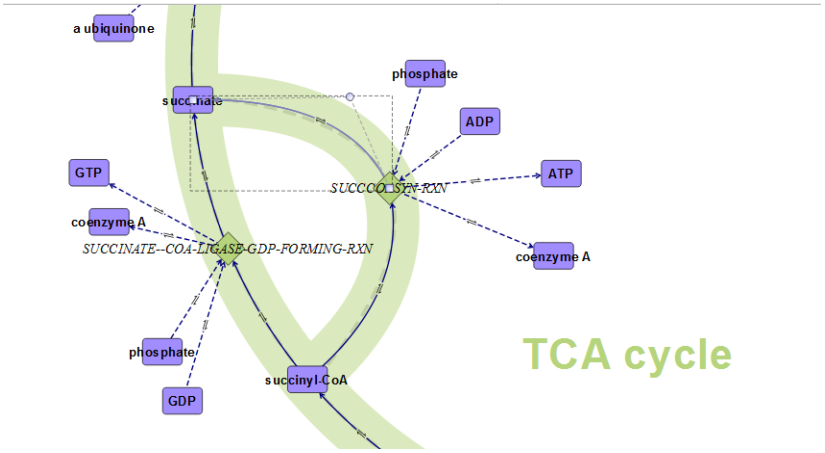


Figure 97 *Split in the TCA cycle pathway aligned to a background curve path*

f Click the **Zoom Diagram** button on the toolbar so the entire pathway is visible in the *Drawing Area* (Figure 98 on page 95).

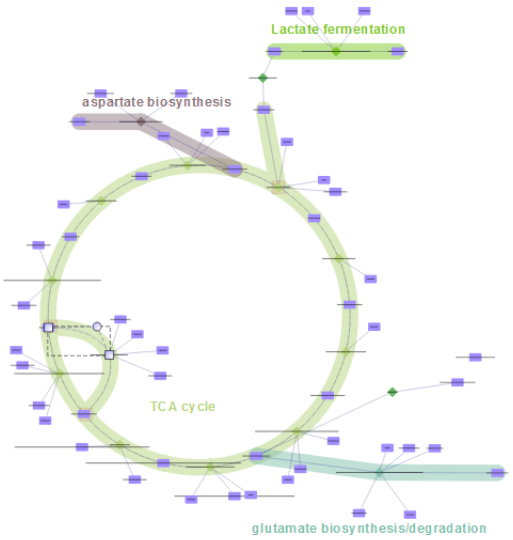


Figure 98 *Network diagram after repositioning nodes and aligning to patterns using the Pattern Editor*

Create a new pathway

You can define a new pathway and add reaction, edge, and metabolite nodes to the pathway using the *Component View* window. The new pathway can be formatted and adjusted in a manner identical to imported pathways.

a Click the **Insert Pathway** button > **Insert Pathway** on the toolbar.

b Type `2-Hydroxyglutarate formation` for the **Name** in the **New Pathway** dialog box (Figure 99).

c Mark the **IDH2** reaction on the list of Available Reactions. The list of available reactions contains reactions that are not already associated with a pathway.

d Click **OK**. The new pathway appears in the Pathways tree in the *Component View* window.

Note: Since the IDH2 reaction node already has reaction edges that connect it with the metabolites 2-hydroxyglutarate and 2-oxoglutarate, the entire pathway is created from this operation (Figure 102 on page 96).

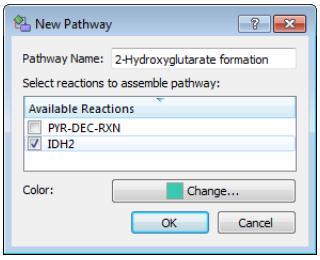


Figure 99 *New Pathway dialog box*

Create and fill an empty pathway

If you click **OK** in the **New Pathway** dialog box (Figure 99 on page 95), but did not select a reaction to assign to the new pathway, a notice is displayed. You can click **Yes** to create an empty pathway or Click **No** to assign a reaction to the new pathway (Figure 100).

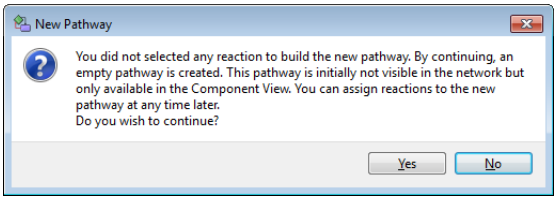


Figure 100 *New Pathway created without a reaction notice*

a Click the **IDH2** reaction node in the *Component Viewer* window.

b Drag the **IDH2** reaction node to the **2-Hydroxyglutarate formation** pathway in the *Component Viewer* window (Figure 101).

Note: Since the IDH2 reaction node already has reaction edges that connect it with the metabolites 2-hydroxyglutarate and 2-oxoglutarate, the entire pathway is created with a single drag and drop operation (Figure 102).

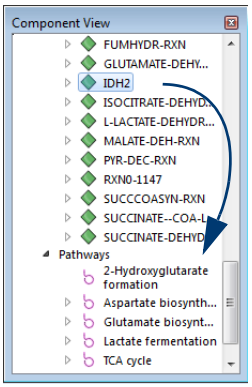


Figure 101 *Drag a reaction to a pathway in the Component View dialog box*

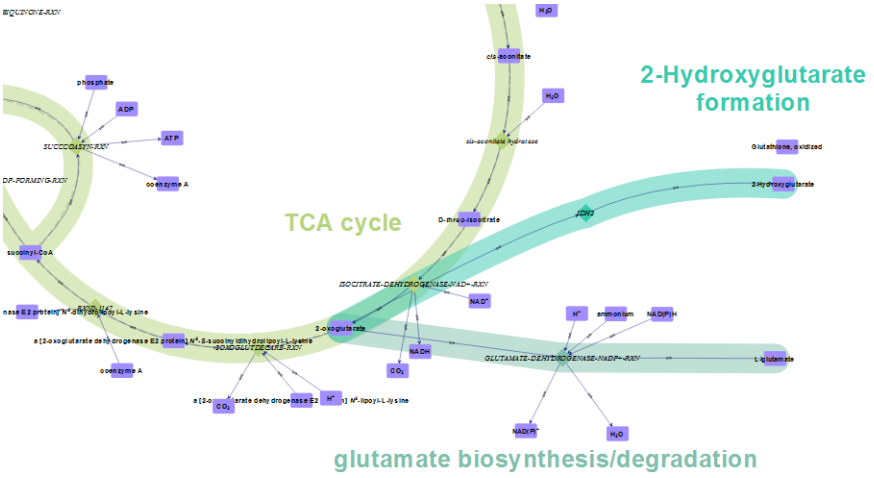


Figure 102 *2-Hydroxyglutarate formation pathway after smoothing the edges*

Add a new pathway

a Click the **Insert Pathway** button > **Insert Pathway** on the toolbar.

b Type `Pyruvate to TCA cycle` for the **Name** in the **New Pathway** dialog box (Figure 103).

c Mark the **PYR-DEC-RXN** reaction on the list of Available Reactions. The list of available reactions contains reactions that are not already associated with a pathway.

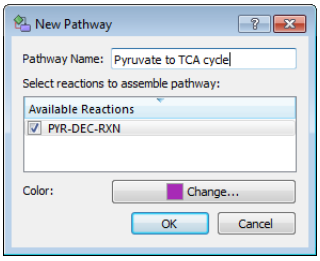


Figure 103 *New Pathway dialog box*

d Click **OK**. The new pathway appears in the Pathways tree in the *Component View* window.

Note: Since the PYR-DEC-RXN reaction node already has reaction edges that connect it with the Lactate fermentation pathway, the entire pathway is created from this operation (Figure 104).

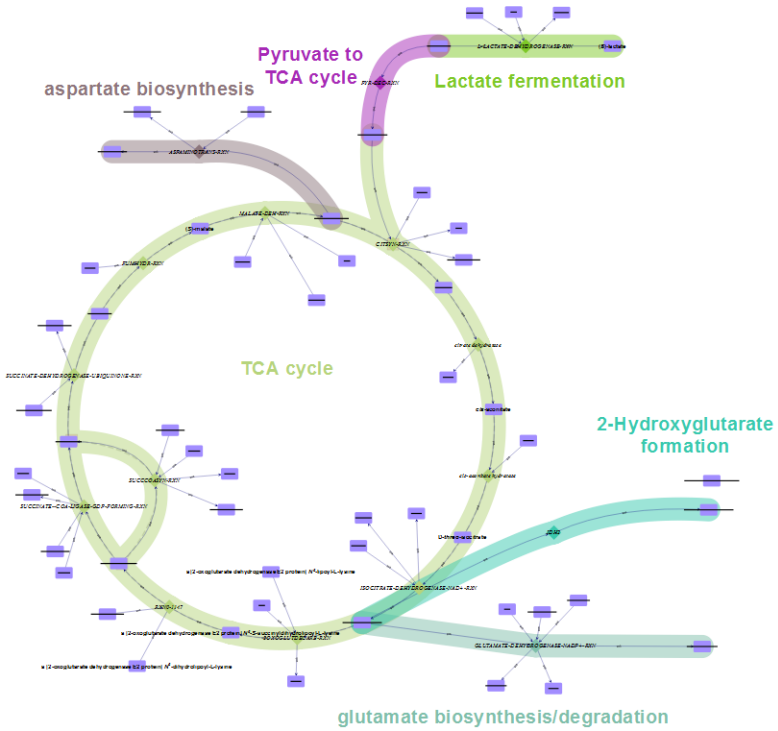


Figure 104 *Complete network diagram with line smoothing*

8. Save your network diagram

Save your pathways network diagram as an Omix Premium (OMX) document. This is *Step 8* of "Review the workflow for creating a network diagram" on page 71.

Note: When your Omix Premium document has unsaved changes an asterisk appears at the end of the file name in the title bar (Figure 105).

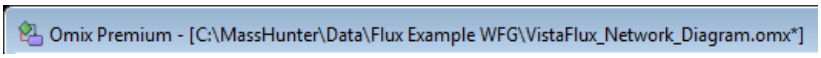


Figure 105 *Unsaved changes in your document are indicated by an asterisk in the title bar*

a Click **File > Save** or click the **Save** button on the toolbar.

If this is the first time you are saving your project, you are prompted for the folder and file name as described in the following steps. The first time you save your project is the same as when you click **File > Save As**.

b Navigate to the folder to save your Omix Premium network in the **Save Omix Network Model** dialog box.

c Enter `VistaFlux_Network_Diagram` for the **File name** (Figure 106).

d Click **Save**.

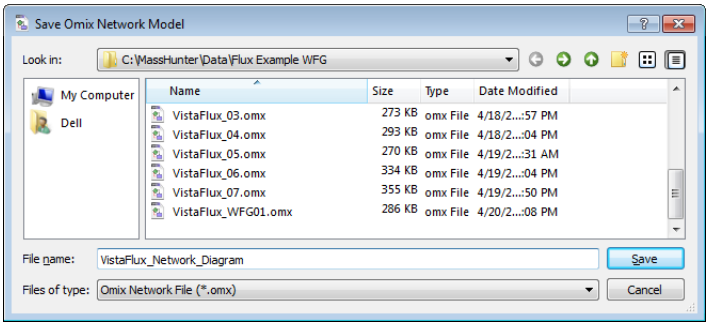


Figure 106 *Save Omix Network Model dialog box*

Import your Profinder Project

Import compounds and isotopologue extraction results from your Profinder project using the PFA file you saved in "Export your results as a Profinder Archive" on page 65. See "4. Add compounds from your Profinder Archive file" on page 78 for steps to use the PFA file to add the target compounds to your network diagram.

a Click **Data > Open Data Table**. If the *Data Manager* window is already open you can click the **Open Data Table** button, a small button located at the bottom-left of the *Data Manager* window.

b Navigate to the folder and select file VistaFlux_WFG.pfa (Figure 107).

c Click **Open**. Profinder compounds and isotopologue results are imported into the *Data Manager* window. The *Data Manager* window is automatically opened and your Profinder data is added to your document.

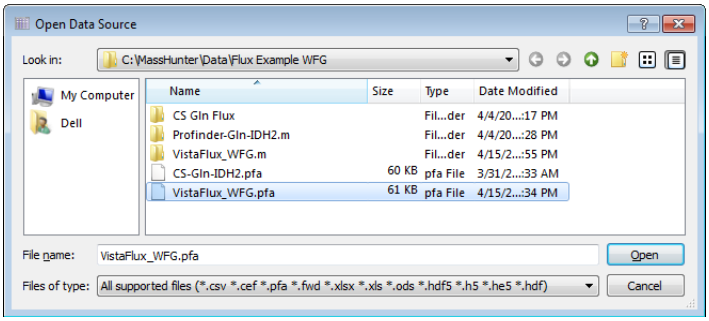
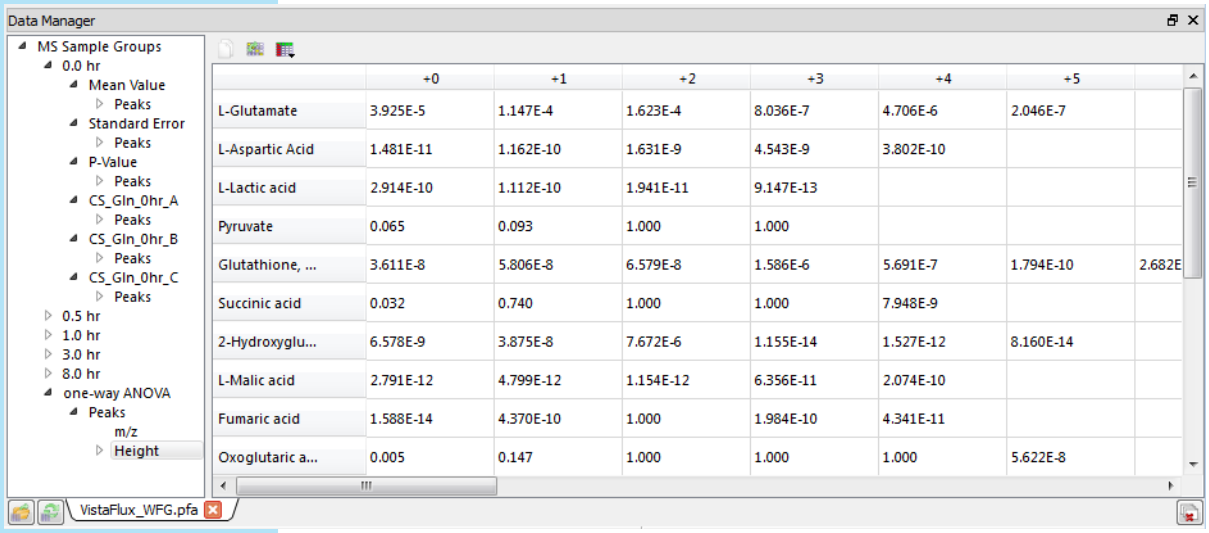


Figure 107 *Open Data Source dialog box*

d Review the information contained in the Data Manager window (Figure 108).



	+0	+1	+2	+3	+4	+5	
L-Glutamate	3.925E-5	1.147E-4	1.623E-4	8.036E-7	4.706E-6	2.046E-7	
L-Aspartic Acid	1.481E-11	1.162E-10	1.631E-9	4.543E-9	3.802E-10		
L-Lactic acid	2.914E-10	1.112E-10	1.941E-11	9.147E-13			
Pyruvate	0.065	0.093	1.000	1.000			
Glutathione, ...	3.611E-8	5.806E-8	6.579E-8	1.586E-6	5.691E-7	1.794E-10	2.682E
Succinic acid	0.032	0.740	1.000	1.000	7.948E-9		
2-Hydroxyglu...	6.578E-9	3.875E-8	7.672E-6	1.155E-14	1.527E-12	8.160E-14	
L-Malic acid	2.791E-12	4.799E-12	1.154E-12	6.356E-11	2.074E-10		
Fumaric acid	1.588E-14	4.370E-10	1.000	1.984E-10	4.341E-11		
Oxoglutaric a...	0.005	0.147	1.000	1.000	1.000	5.622E-8	

Figure 108 *Example view of the Data Manager window*

e Click **Data > Show Data Manager**, or click the **Data Manager** button on the toolbar, to close the *Data Manager* window. The Profinder data is retained by the Omix Premium document when the *Data Manager* window is closed.

Click **Data > Show Data Manager**, or click the **Data Manager** button, again to re-open the *Data Manager* window.

Import Profinder results without a pathway

Import your target metabolites and isotopologue results into an Omix Premium document without a pathway and visualize the data as a collection of compounds.

When you import a PFA file from Profinder, you are notified when one or more compounds in your PFA file do not match any compounds in your Omix Premium document; when you choose to insert the unmatched compounds from your PFA you can use all of the MassVisualizer tools to review your Profinder results without first creating a pathways network diagram.

1. Launch Omix Premium.

Double-click the Omix Premium icon located on your desktop, or click **Start > All Programs > Omix Premium > Omix Premium**. When Omix Premium opens the document area is arranged to facilitate a quick means to review the software features, open a new document, and open a recent document.

2. Create a new document.

a Click **New** in the *Document Area* (Figure 109).

b Click **New Omix Network**.

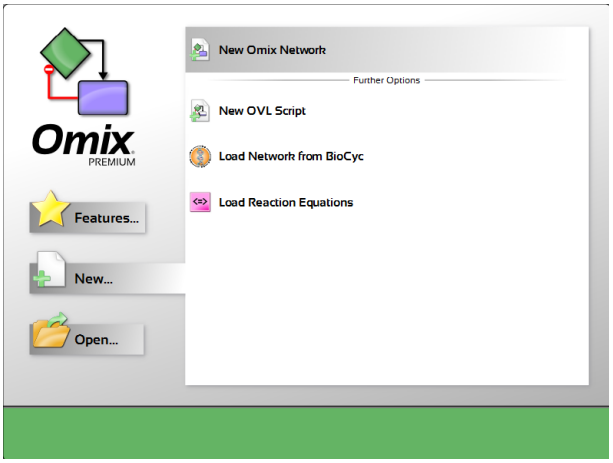


Figure 109 *Create a new document in Omix Premium*

3. Import your Profinder results

a Click **Data > Open Data Source**.

b Navigate to the folder and select your PFA file (Figure 110).

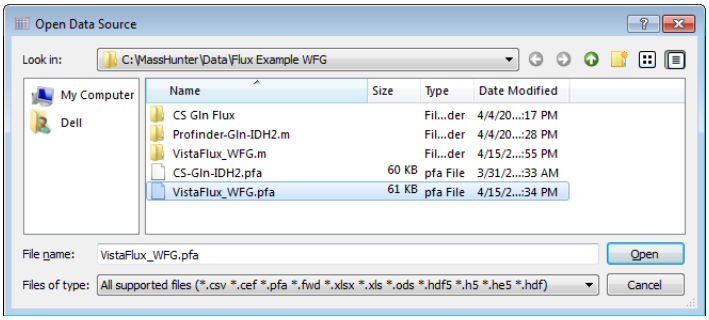


Figure 110 *Open Data Source dialog box*

c Click **Open**.

Activate Agilent MassVisualizer plug-in

d Click **Yes** in the new **Open Data Source** dialog box to activate Agilent MassVisualizer (Figure 111). You can chose to activate the Agilent MassVisualizer plug-in later; however, MassVisualizer must be activated at this time in this workflow.

Note: If you do not activate the MassVisualizer plug-in you cannot add missing compounds in the next step. Click **Extras > Document Extensions > Agilent MassVisualizer** to manually enable MassVisualizer and continue to the next step.

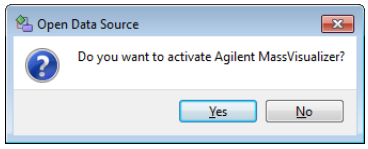


Figure 111 *Open Data Source dialog box*

e Click **Data > Show Data Manager** to close the *Data Manager* window. The purpose for opening your Profinder project is to obtain the compounds that are not present in the TCA cycle and lactate fermentation pathways; the *Data Manager* window is not necessary to import the missing compounds.

---

4. Insert the compounds from your Profinder results into the new document.

a Click **Visualization > Agilent MassVisualizer > Check for missing compounds**. If you have not previously checked for missing compounds, when you next click the **Show Abundance Changes** button Omix Premium will automatically check for missing compounds.

Note: When there are compounds in the PFA file that do not match compounds in the open document, the **Compound Availability** dialog box is opened after you click the **Show Abundance Changes** button. This gives you the opportunity to import metabolites that have mass spectral data to a pathway diagram that does not yet contain nodes for them. Otherwise the **Abundance Change Visualization** dialog box is opened.

b Review the list of metabolites in the Profinder Archive file that are not matched to a compound in the open document in the **Compound Availability** dialog box. In this case all of the target compounds in the PFA file are not available in the network diagram (Figure 112).

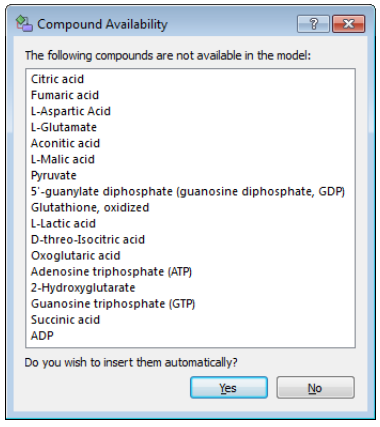


Figure 112 *Compound Availability dialog box*

c Click **Yes** to add the compounds from the PFA file.

d Click any where on the *Drawing Area* to visualize the compounds in Omix Premium. The compounds are added to your new document as separate, unconnected nodes arranged in a vertical column (Figure 113).

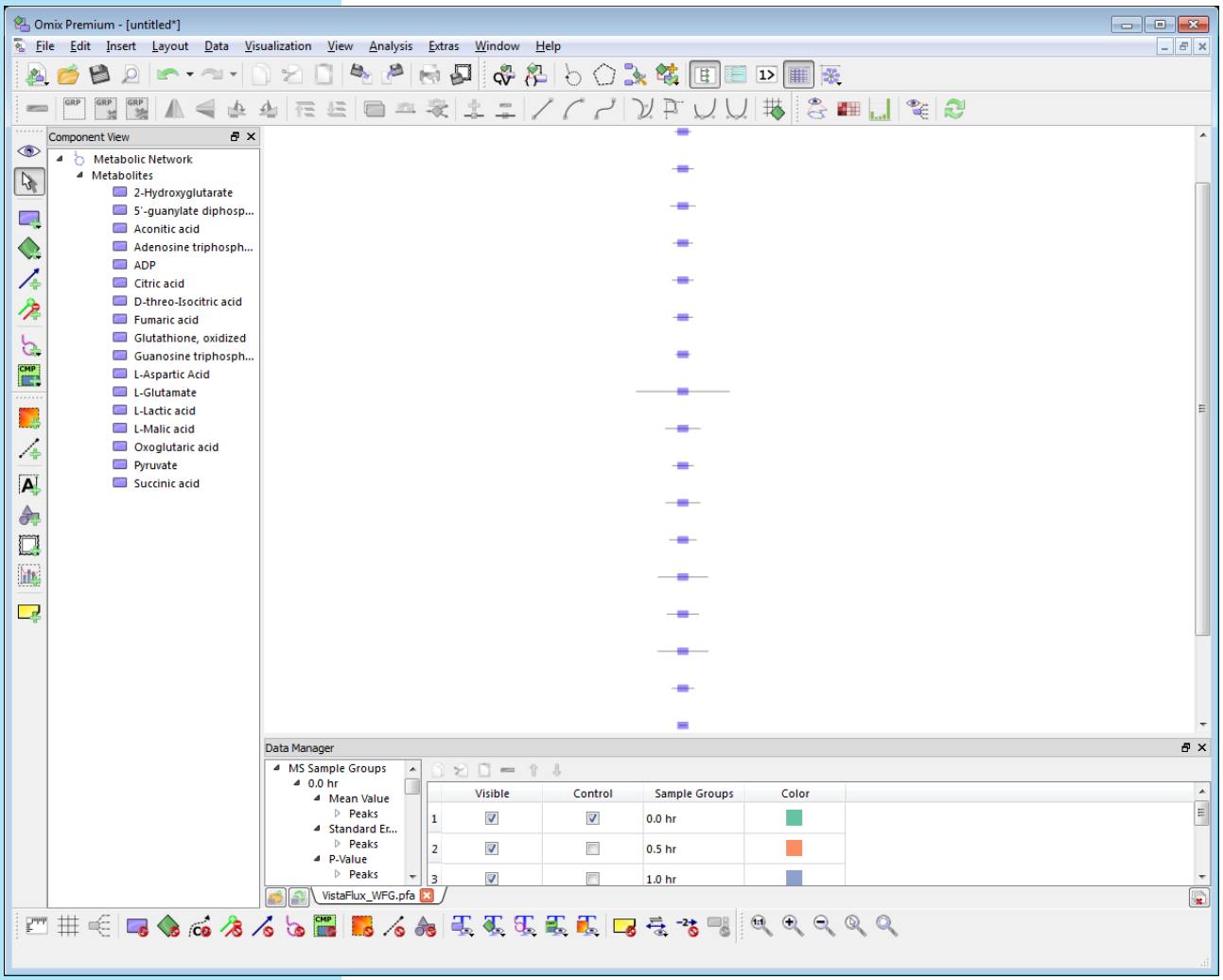


Figure 113 *Target metabolites added to a new Omix Premium document*

Display isotopologue metabolite data and highlight pathways

The MassVisualizer plug-in for Omix Premium provides you with the ability to visualize isotopologue data from Profinder; you can view absolute and relative abundances, labeling incorporation, and statistics within the context of your network diagram.

MassVisualizer overview

After you import your isotopologue results into Omix Premium, you can use the MassVisualizer toolbar and menu (Figure 114) to add new views to your network diagram. MassVisualizer provides you with four classes of visualization tools: Abundance Changes, Quilt Plots, Bar Charts, and Background Information with Visualization on Demand.

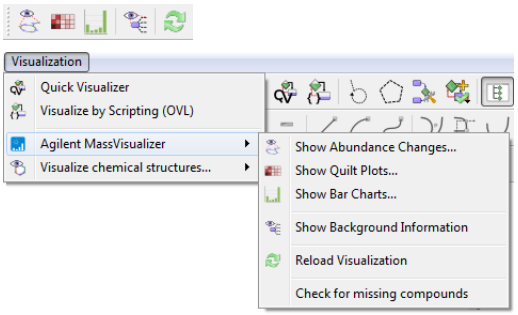


Figure 114 *Views of the MassVisualizer toolbar (top) and menu (bottom) when you visualize your results by "all sample groups." When you visualize your results by "individual sample group," sample group control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to use to view your results by each individual group.*

Abundance Change

Visualize up to three different summary statistics within the metabolite node: (1) metabolite change per group versus the control group, (2) labeling incorporation per group, and (3) fractional labeling percent per group. This visualization mode cannot be used with *Draw structures on the metabolites*. Abundance change parameters are viewed in the **Abundance Change Visualization** dialog box (Figure 115 on page 104) and are represented on the metabolite node as shown in Figure 116 on page 104. See "Edit Color Coding and Color" on page 106 for editing the colors.

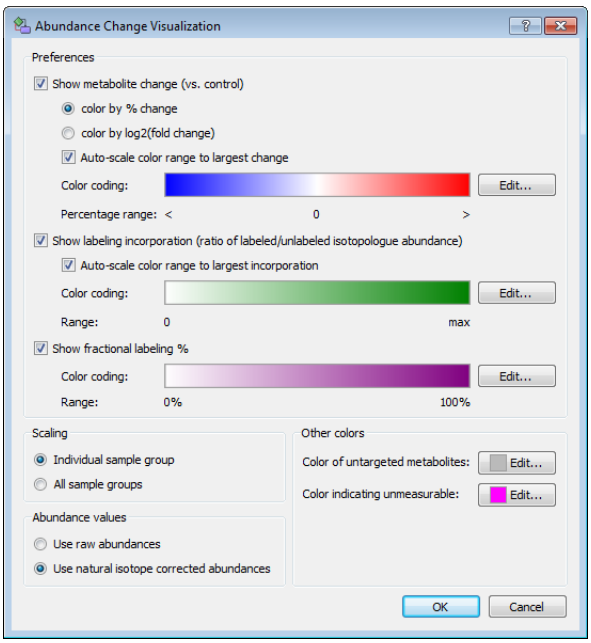


Figure 115 *Abundance Change Visualization dialog box*

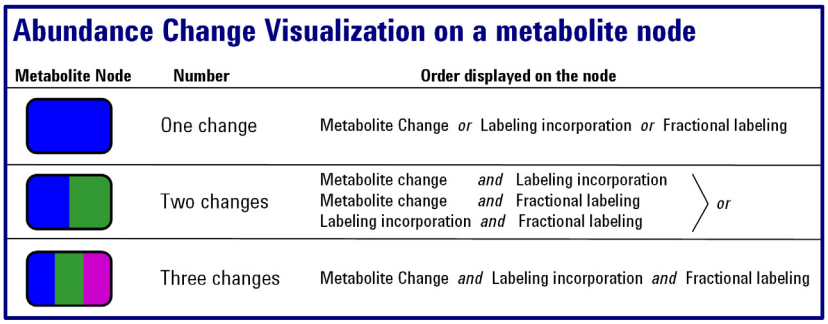


Figure 116 *Metabolite fold change visualization*

Quilt Plots

Visualize the isotopologue abundances, by individual or all sample groups, in quilt plots next to the metabolite nodes. Indications of statistical significance of the isotopologue abundances among the groups can be enabled and adjusted by either a pairwise Welch's t-test versus control, or a one-way ANOVA of each group against every other group. Quilt plot parameters are viewed in the **Quilt Plots** dialog box (Figure 117 on page 105). See "Edit Color Coding and Color" on page 106 for editing the colors.

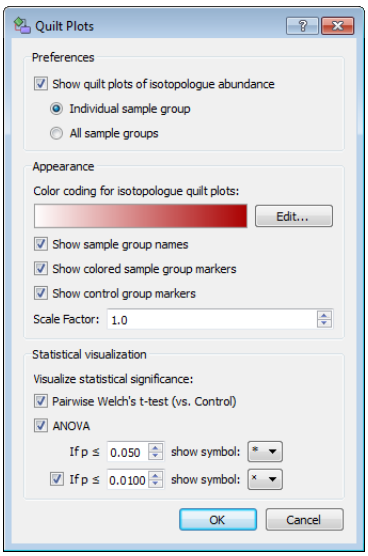


Figure 117 *Quilt Plots dialog box*

Bar Charts

Visualize various isotopologue summaries in bar charts displayed next to the metabolite nodes. You can view bar charts containing metabolite abundance, label incorporation, fractional labeling, and isotopologue histograms. Bar chart parameters are viewed in the **Bar Charts** dialog box (Figure 118). The parameters in the **Bar Charts** dialog box change based on the *Preferences* selection. See "Edit Color Coding and Color" on page 106 for editing the colors.

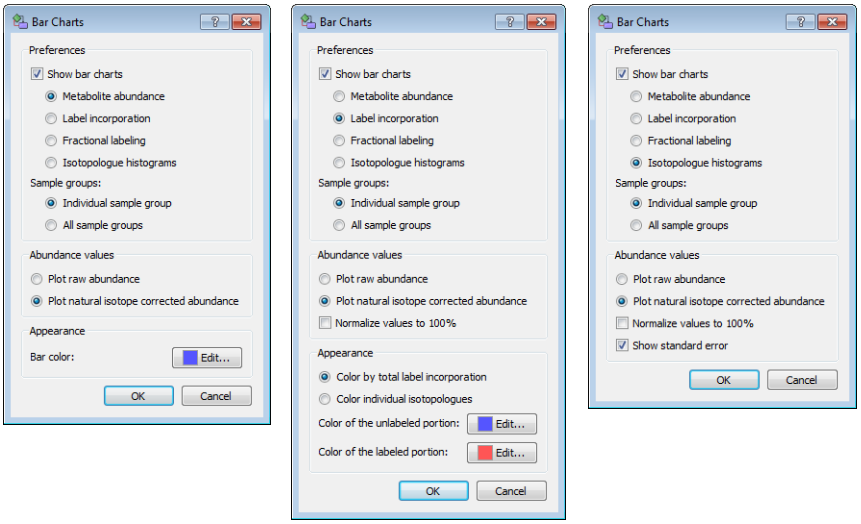


Figure 118 *Bar Charts dialog box variations based on the preferences selection (the dialog box when Fractional labeling is selected is identical to when Metabolite abundance is selected)*

Edit Color Coding and Color

Each of the MassVisualizer tools (Abundance Changes, Quilt Plots, and Bar Charts) offers you the ability to edit the colors used to display your data. Omix Premium provides you with parameters to edit sequential color coding, diverging color coding, and single colors.

Sequential color coding uses a value range of 0 to 1 (or 100%) to compute the corresponding color. Percentage values are not directly related to fold change values; the colors for the percentages are used to represent relative changes. For example, label incorporation is represented by a value between zero (no incorporation) and a maximum incorporation; therefore, sequential color coding uses 0% to encode zero label incorporation and 100% to encode the maximum amount of label incorporation.

Diverging color coding uses a value range of minimum-zero-maximum to compute the corresponding color. For fold change visualization, a diverging color coding is used because fold change can be positive (increasing change) or negative (decreasing change). The color coding value of 50% encodes no fold change. 0% encodes the negative fold change (minimum) and 100% encodes the positive fold change (maximum).

A **single color** is used to color a metabolite node when the metabolite is untargeted or unmeasurable, as well as the color of the bars in a bar chart.

a Click the Edit... button next to any **Color coding** gradient scale to launch the **Edit Color Coding** dialog box. This allows you to create a custom gradient (Figure 119).

b Click the Set Color button in the **Edit Color Coding** dialog box, or click the Edit... button in the **Abundance Change Visualization** and **Bar Charts** dialog boxes, to launch the **Choose Color** dialog box. This allows you to select your preferred color (Figure 119).

c Select your preferred color using any of the *Color Triangle*, *Color Circle*, *Color Area*, *Color Palette* tabs in the **Choose Color** dialog box.

d Click **OK** in the respective dialog boxes to save your color selection.

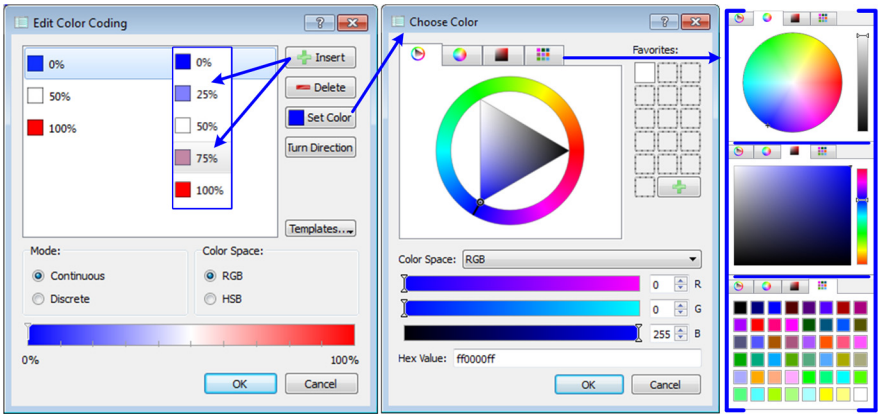


Figure 119 *Edit Color Coding and Choose Color dialog boxes*

Select a control group

Various statistical views in the MassVisualizer toolbar are "versus the control." You can change the control group at any time in the *Data Manager* window.

a Click **Data > Show Data Manager**, or click the **Data Manager** button on the toolbar.

b Mark the *Control* column for *Group 1* (Figure 120). To mark, or clear a mark, in the data table; double-click on your selection under the *Control* column.

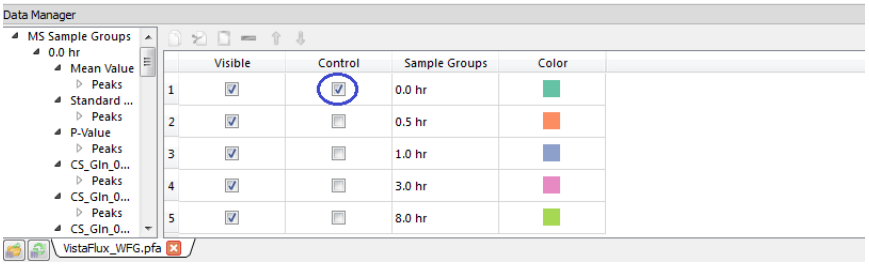


Figure 120 *Select a control group in the Data Manger window*

Enable visualization of abundance changes on your network diagram

Visualization of abundance changes is illustrated using the lower-right section of the example network diagram, where the *2-hydroxyglutarate formation* and *glutamate biosynthesis/degradation* pathways join the *TCA cycle* pathway.

Zoom the *Drawing Area* so you can see the section of the network diagram similar to Figure 121.

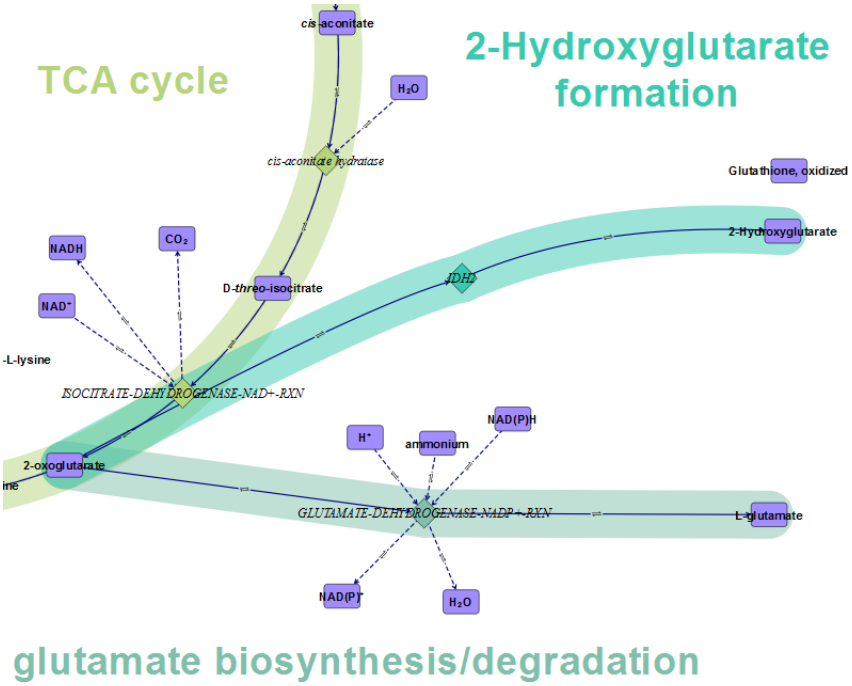


Figure 121 *Zoom of the Drawing Area before adding abundance change visualization*

Metabolite change

When you view metabolite change, the metabolite abundance change can be viewed as a percent change or log2 scaled, referred to as a fold change. When you clear **Auto-scale color range to largest change** custom values can be entered for the lower and upper bounds of the metabolite abundance change.

a Click the **Show Abundance Changes** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Abundance Changes**.

b Mark **Show metabolite change (vs. control)** in the **Abundance Change Visualization** dialog box (Figure 115 on page 104).

c Click **Individual sample group**.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. The color of the *target metabolite nodes* change to reflect the maximum percent or fold change of the metabolite abundance for the current group (selected using the slider in the MassVisualizer toolbar) relative to the control group: the color gradient varies from up, increasing abundance (red), to down, decreasing abundance (blue).

d Click **All sample groups**.

When your results are visualized by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. The color of the *target metabolite nodes* change to reflect the maximum percent, or fold change, across all of the groups in your experiment relative to the control group: the color gradient varies from up, increasing abundance (red), to down, decreasing abundance (blue) (Figure 122).

e Click **OK**.

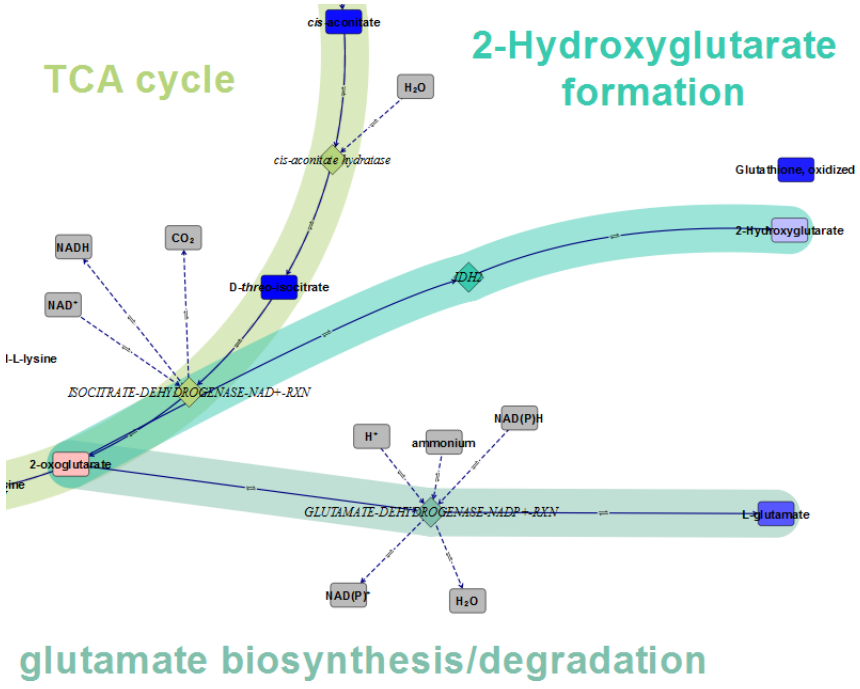


Figure 122 *Zoom Drawing Area with all sample groups abundance change visualization*

Labeling incorporation change

When you view labeling incorporation you can select automatic scaling of the range (0 to the maximum value) or you can specify an upper bound of the incorporation. When you clear **Auto-scale color range to largest incorporation** you can enter a custom value for the upper bound of the labeling incorporation.

a Click the **Show Abundance Changes** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Abundance Changes**.

b Mark **Show labeling incorporation (ratio of labeled/unlabeled isotopologue abundances)** in the **Abundance Change Visualization** dialog box (Figure 115 on page 104).

c Click **Individual sample group**.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. The color of the *target metabolite nodes* change to reflect the largest change in label incorporation for the current group (selected using the slider in the MassVisualizer toolbar): the color gradient varies from low label incorporation (white) to high label incorporation (green).

d Click **All sample groups**.

When you visualize your results by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. The color of the *target metabolite nodes* change to reflect the largest change in label incorporation across all of the groups in your experiment: the color gradient varies from low label incorporation (white) to high label incorporation (green), see Figure 123.

e Click **OK**.

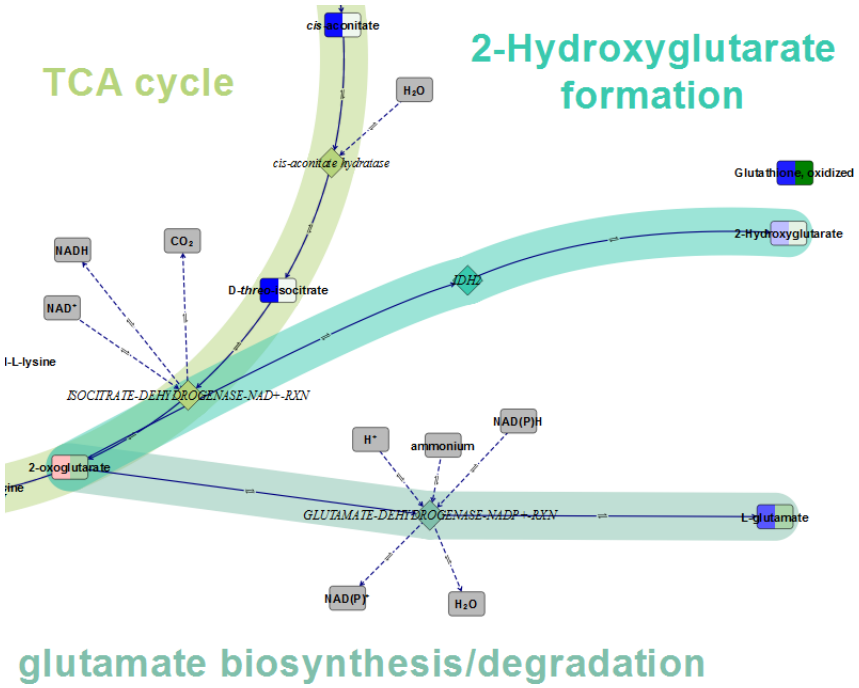


Figure 123 *Zoom Drawing Area with two abundance change visualizations*

Fractional labeling %

a Click the **Show Abundance Changes** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Abundance Changes**.

b Mark **Show fractional labeling %** in the **Abundance Change Visualization** dialog box (Figure 115 on page 104).

c Click **Individual sample group**.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. The color of the *target metabolite nodes* change to reflect the largest change in fractional labeling for the current group (selected using the slider in the MassVisualizer toolbar) relative to the control group: the color gradient varies from low fractional labeling (white) Click **All sample groups**.

When your results are visualized by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. The color of the *target metabolite nodes* change to reflect the largest change in fractional labeling across all of the groups in your experiment relative to the control group: the color gradient varies from low fractional labeling (white) to high fractional labeling (violet), see Figure 124.

d Click **OK**.

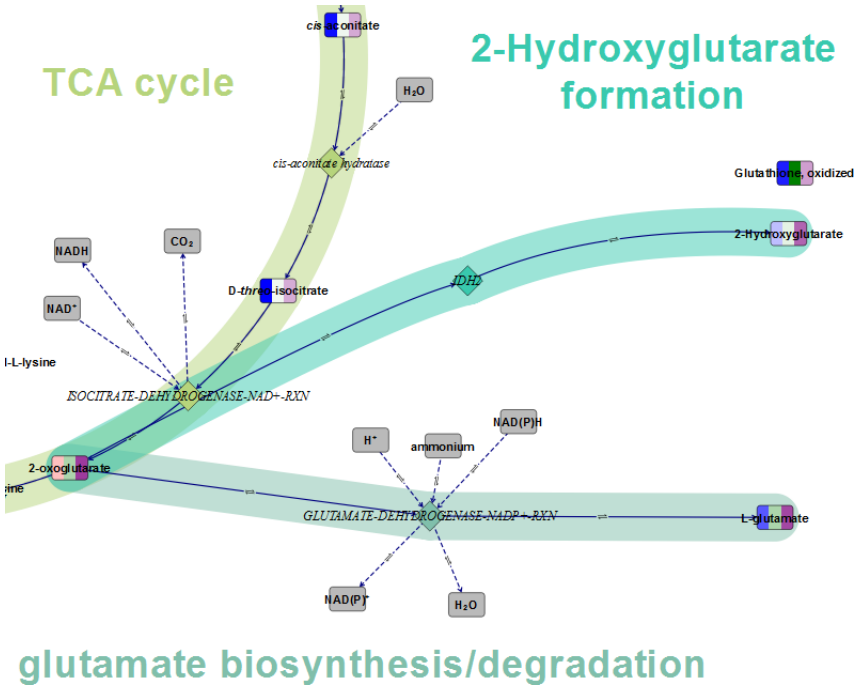


Figure 124 *Zoom Drawing Area with three abundance change visualizations*

Enable visualization of quilt plots on your network diagram

Visualization of quilt plots is illustrated using the lower-right section of the example network diagram, where the 2-hydroxyglutarate and glutamate biosynthesis/degradation pathways join the TCA cycle.

Zoom the *Drawing Area* so you can see the section of the network diagram similar to Figure 121 on page 107.

Quilt plots

a Click the **Show Quilt Plots** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Quilt Plots**.

b Mark **Show quilt plots of isotopologue abundances** in the **Quilt Plots** dialog box (Figure 117 on page 105).

c Click **Individual sample group**.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. Quilt plots are shown next to the metabolite node. The color of the columns in the *single isotopologue data row* change to reflect the maximum isotopologue abundance for the current group (selected using the slider in the MassVisualizer toolbar): the color gradient varies from low isotopologue abundance (white) to high isotopologue abundance (red).

d Click **All sample groups**.

When your results are visualized by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. Quilt plots are shown next to the metabolite node and there is a row of data for each group. The color of the columns in the *isotopologue data rows* change to reflect the maximum isotopologue abundance for each group: the color gradient varies from low isotopologue abundance (white) to high isotopologue abundance (red), see Figure 123 on page 109.

Statistical visualization

e Mark **Pairwise Welch's t-test (vs. Control)**.

The Welch's t-test is a version of the Student's t-test. Unlike the Student's t-test that assumes a normal distribution, the Welch's t-test does not, and is more reliable when the test is performed on sample groups with unequal sample sizes and variance. Welch's t-test results are indicated symbolically, the symbols are defined by a threshold p-value and a symbol, within the quilt chart. The Welch's t-test is applied in a pairwise manner, where each experimental group is tested against the control group (specified in the Data Manager - see Figure 120 on page 107).

p-value

A p-value of 0.05 is similar to stating that, if the mean values for each group are identical, then a 5% chance or less exists of observing a difference in the mean of the group values as large as you observed. In other words, statistical treatment of random sampling from identical populations with a p-value set at 0.05 leads to a difference smaller than you observed in 95% of the experiments, and larger than you observed in 5% of the experiments. Another way to express a p-value of 0.05 is that there is a 5% chance or less of the observed difference being due to mere random chance.

Note: At times a really large change may have low significance (small p-value). This can occur when there are small absolute differences in the abundances of the isotopomers (see the quilt plot for 2-oxoglutarate with two thresholds).

f Mark **ANOVA**.

Analysis of variance (in the case of Omix Premium, a one-way ANOVA) compares the means and variance of *each group* versus *all other groups* in a "round-robin" fashion. This test is similar to a Student's t-test. A one-way ANOVA produces an F statistic, which in turn is expressed as a p-value from the F distribution. The results are shown symbolically below the quilt plots.

Note: Multiple testing correction is not applied to ANOVA results in Omix Premium. This may increase the false positive error rate slightly, depending on the experimental design.

g Mark the second threshold so that the quilt plots can show up to two thresholds of statistical significance for each test.

h Click **OK**.

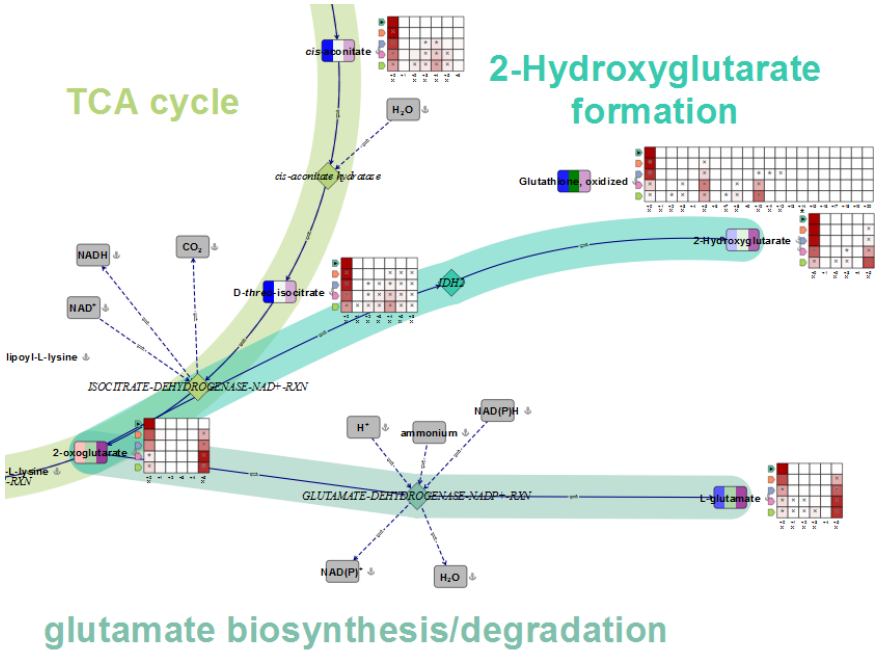


Figure 125 *Zoom Drawing Area with three abundance changes and quilt plots visualization*

Enable visualization of bar charts on your network diagram

Visualization of bar charts is illustrated using the lower-right section of the example network diagram, where the 2-hydroxyglutarate and glutamate biosynthesis/degradation pathways join the TCA cycle. The bar charts visualization options are similar to the **Isotopologue Results** in MassHunter Profinder, with the difference being that in Omix Premium you can view the results for all of the metabolites at the same time, and in the context of your biochemical network.

Zoom the *Drawing Area* so you can see the section of the network diagram similar to Figure 121 on page 107.

Bar charts, label incorporation

a Click the **Show Bar Charts** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Bar Charts**.

b Mark **Show bar charts** in the **Bar Charts** dialog box.

c Click **label incorporation** under the *Preferences* group heading. The parameters in the **Bar Charts** dialog box change based on the *Preferences* selection as illustrated in Figure 118 on page 105.

d Click **Individual sample group** in the **Bar Charts** dialog box.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. Bar charts are shown next to the metabolite node. The color of the bars on the chart in the *individual sample group* reflect the labeled and unlabeled isotopologue abundance or percentage for the current group (selected using the slider in the MassVisualizer toolbar): the color for the unlabeled isotopologue is blue and the labeled isotopologue is red.

e Click **All sample groups** in the **Bar Charts** dialog box.

When your results are visualized by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. Bar charts are shown next to the metabolite node. The color of bars on the chart rows for the *all sample groups* reflect the labeled and unlabeled isotopologue abundance or percentage for each group: the color for the unlabeled isotopologue is blue and the labeled isotopologue is red, see Figure 126 on page 114.

f Click **OK**.

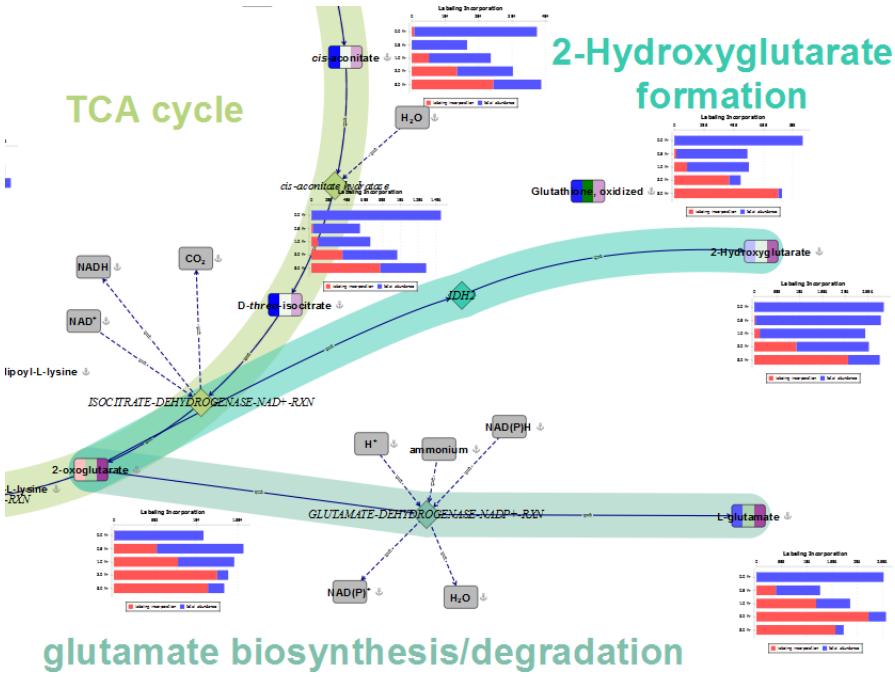


Figure 126 *Zoom Drawing Area with three abundance changes and bar charts visualization with label incorporation*

Bar charts, isotopologue histograms

a Click the **Show Bar Charts** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Bar Charts**.

b Click **isotopologue histograms** under the *Preferences* group heading. The parameters in the **Bar Charts** dialog box change based on the *Preferences* selection as illustrated in Figure 118 on page 105.

c Click **Individual sample group** in the **Bar Charts** dialog box.

When your results are visualized by **individual sample group**, control buttons are visible in the toolbar and the menu (Figure 141 on page 125) for you to view your results by stepping through each individual group. Bar charts are shown next to the metabolite node. The bars on the chart in the *individual sample group* reflect the isotopologue abundance or percentage for the current group (selected using the slider in the MassVisualizer toolbar).

d Click **All sample groups** in the **Bar Charts** dialog box.

When your results are visualized by **all sample groups**, the control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103. Bar charts are shown next to the metabolite node. The color of the bars on the chart rows for the *all sample groups* reflect the isotopologue abundance or percentage for each group, see Figure 127 on page 115.

e Click **OK**.

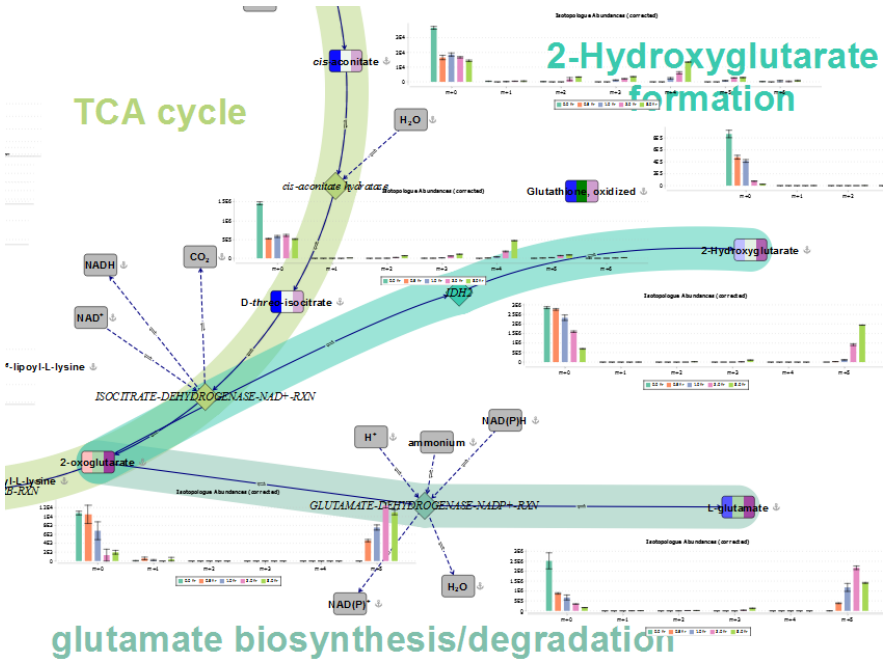


Figure 127 *Zoom Drawing Area with three abundance changes and bar charts visualization with isotopologue histograms*

Visualize chemical structures your network diagram

Metabolites created from curated databases include structural information. You can display the chemical structures of the metabolites next to the metabolite nodes or replace the metabolite nodes with the structures.

Note: You must have the Chemical Structures plug-in enabled to visualize chemical structures. When pathways are loading from BioCyc you must mark **import chemical structures** in the **Select Pathway** dialog box, or when you click **Options** in the **Load from BioCyc** dialog box when you are creating a new document from BioCyc.

Zoom the *Drawing Area* so you can see the section of the network diagram similar to Figure 121 on page 107.

a Click the **Visualize chemical structures** button > **Draw structures next to metabolites** from the toolbar, or click **Visualization > Visualize chemical structures > Draw structures next to metabolites**.

b Review and adjust the positions of the elements of your network diagram in the *Drawing Area* (Figure 128 on page 116).

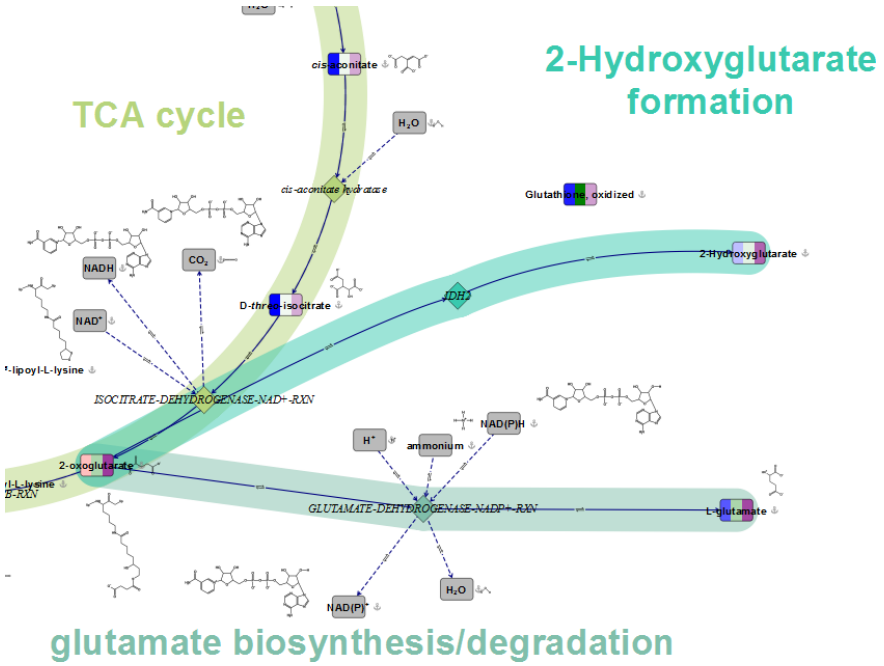


Figure 128 *Zoom Drawing Area with three abundance changes and chemical structures visualization*

Node anchor

When charts and chemical structures are added to your network diagram, these *accessories* are positioned relative to the small anchor that is shown next to each node. The anchor (Figure 129) is movable, and you can be place the anchor anywhere relative to the node (top, bottom left, and right).

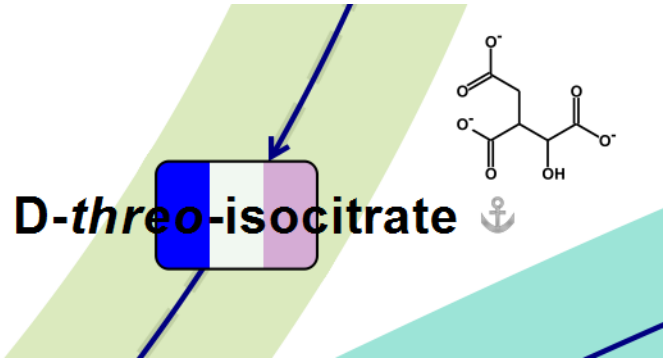


Figure 129 *Anchor used to position accessories in your network diagram*

The anchor has a layout property for aligning the accessories as shown in Figure 130 on page 117. The accessories can be aligned freehand, horizontally, vertically, or according to the diagram standard. *Freehand* is shown in the *Property Editor* window, when the accessories are placed manually (relative to the anchor).

You can also edit many other parameters associated with your metabolite, and other features of your network diagram, using the *Property Editor* window.

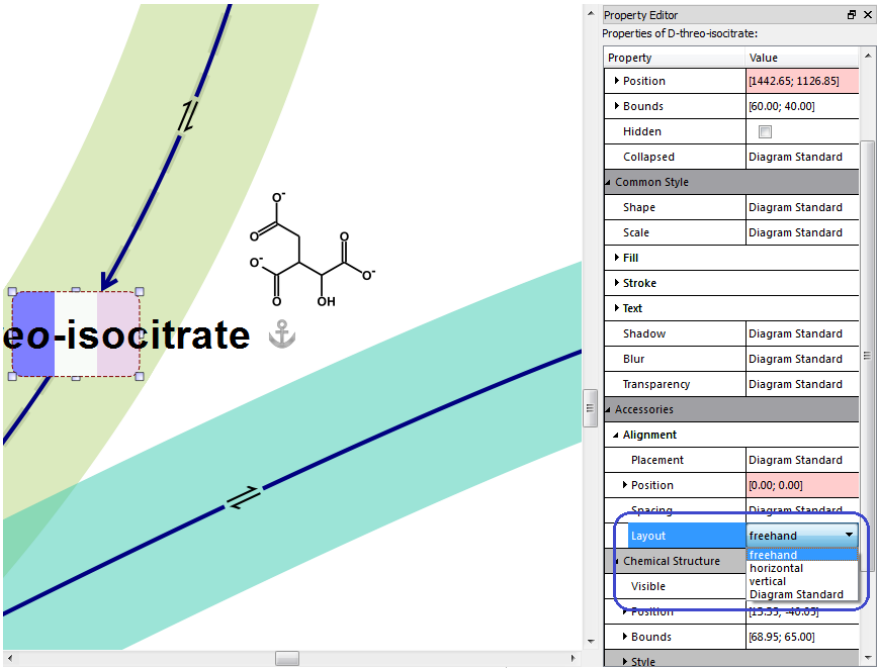


Figure 130 *Layout properties for the Anchor*

View all of your Profinder results using Visualization on Demand

Visualization on Demand allows you view all of the background information on your target metabolites.

a Click the **Show Background Information** button from the toolbar, or click **Visualization > Agilent MassVisualizer > Show Background Information**. This loads your Profinder results into an active state in your Omix Premium session.

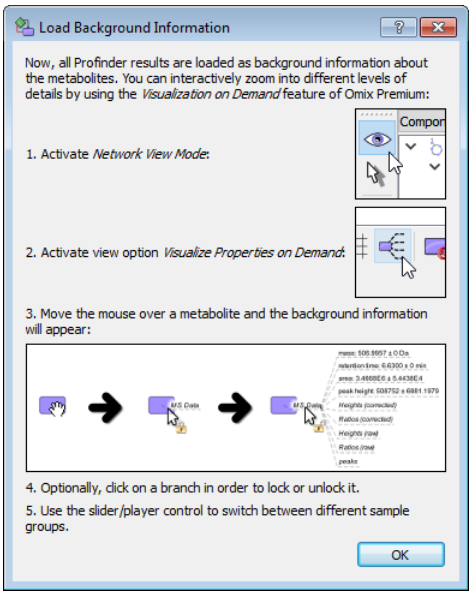


Figure 131 *Load Background Information dialog box*

The **Load Background Information** dialog box shows you the steps to enable visualization on demand.

b Click the **Network View Mode** button from the toolbar.

c Click the **Visualize Properties on Demand** button from the toolbar, or click **View > Visualize Properties on Demand**.

d Move your mouse slowly over a metabolite and then click on branches of the information tree to lock the information in view on your *Drawing Area*. Click a branch again to unlock (hide) the information (

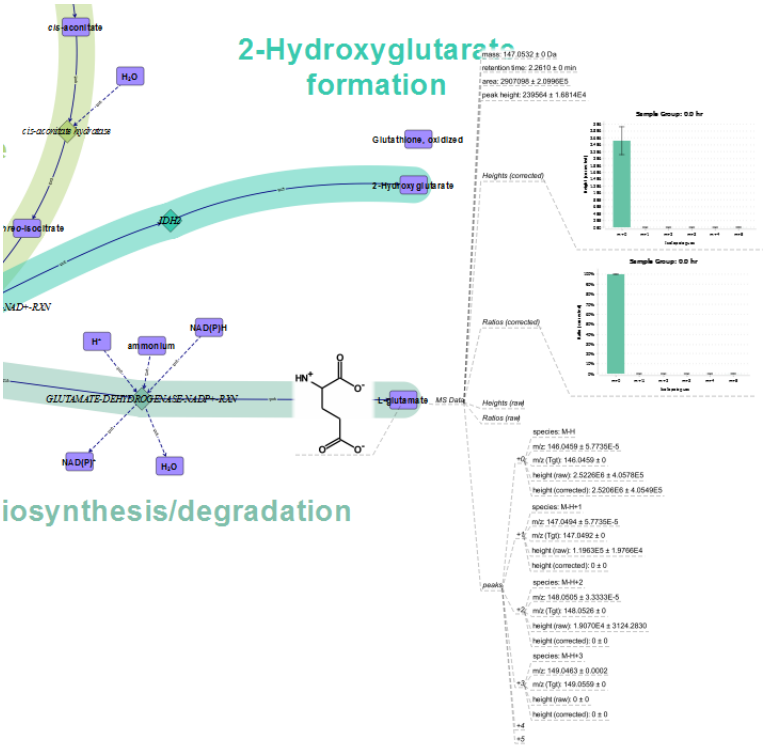


Figure 132 *Sample visualization on demand for L-glutamate*

Generate static images of your pathways network

When you have created your network diagram, imported your isotopologue results from Profinder, and developed an understanding of your experiment in the context of the biological network, you can generate static images of your qualitative flux analysis. With Omix Premium you can arrange, scale, highlight, and enable/disable nearly every feature of the network diagram and results visualization. Omix Premium provides you with powerful tools to help you present your qualitative flux analysis in reports, publications, and presentations.

Click the **Network View Mode** button from the toolbar to disable editing mode and enable pan, zoom, and many other view controls of your network diagram.

Show only the pathway metabolites

a Click the **Hide Cofactors** button from the toolbar to hide the cofactors to the reaction nodes.

Note: If you are in editing mode, the cofactors in the *Drawing Area* remain visible but take on a lighter shade (ghosted) to indicate they do not appear in the view mode and during print, export, and image save operations.

b Click the **Hide Reactions** button from the toolbar to hide the reaction nodes. The diamond shapes are removed from the pathways.

c Click the **Appearance of Reaction Labels** **button > Hidden** from the toolbar to hide the text description of the reaction nodes. The reaction node descriptions are removed from the pathways (Figure 133).

d Click **File > Save Image** to save a sample image of the network diagram. The saved image is identical to the *Drawing Area* in view mode.

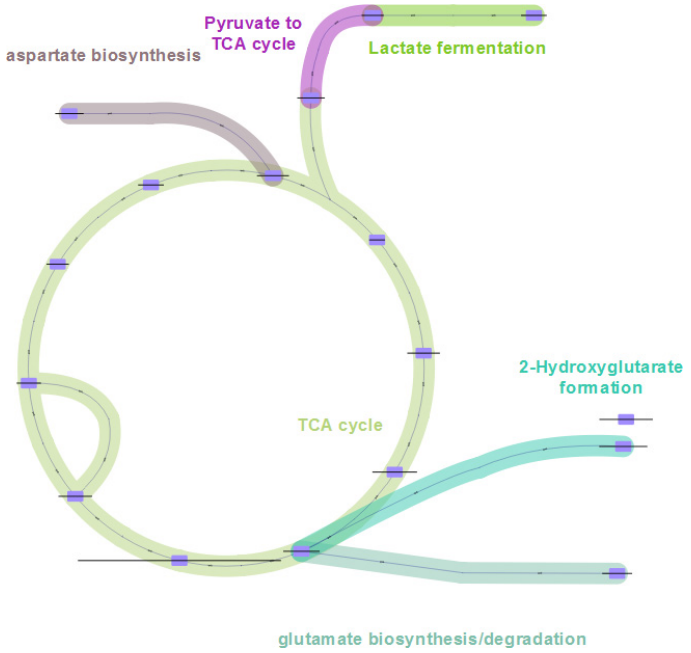


Figure 133 *View of the network diagram in view mode*

Show chemical structures on metabolite nodes

The chemical structures tend to be initially drawn small with respect to the network diagram. This example makes the chemical structures more prominent.

a Click the **Visualize chemical structures** button **> Draw structure on metabolites** from the toolbar to replace the metabolite rectangles with the chemical structures.

b Click the **Select** button from the toolbar to enter the editing mode

c Zoom the *Drawing Area* to view a chemical structure of *(S)-lactate* in detail.

d Click on a chemical structure to enable the control nodes.

e Drag a corner control node on the chemical structure to make the chemical structure larger (Figure 134).

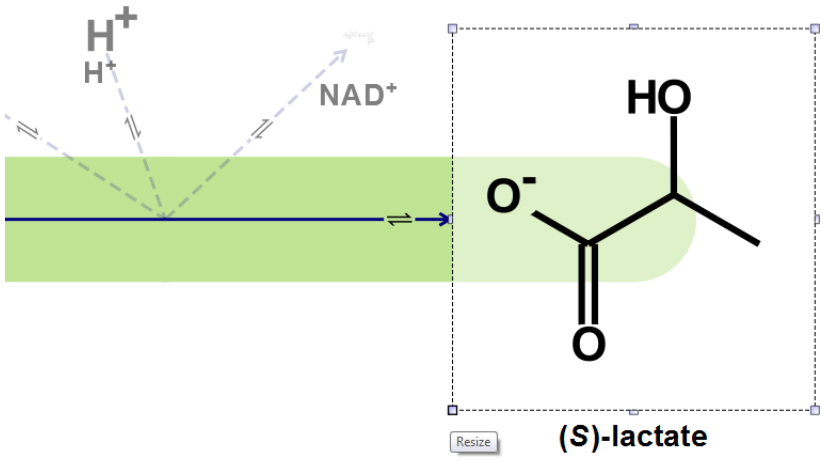


Figure 134 *Change the size of a chemical structure on the network diagram, note that the cofactors are lighter indicating they do not appear in the view mode*

f Click the **Network View Mode** button from the toolbar to disable editing mode (Figure 135).

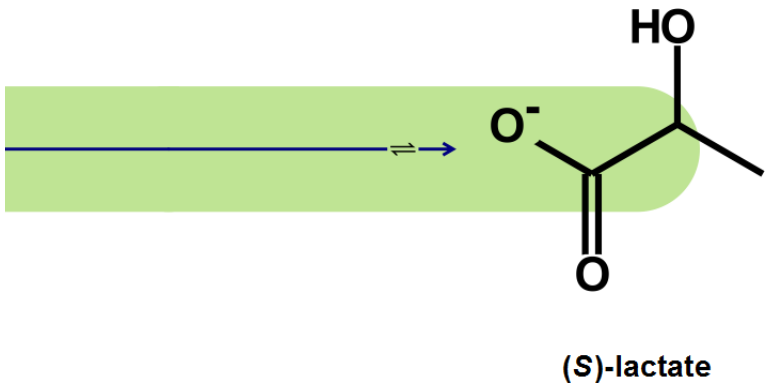


Figure 135 *Resized chemical structure*

Emphasize the target metabolites

a Click the **Visualize chemical structures** button **> Draw structure on metabolites** from the toolbar to remove the chemical structures and return the names of the metabolites to the nodes.

b Click the **Select** button from the toolbar to enter the editing mode

c Click the **Show Property Editor** button from the toolbar.

Change a metabolite font

d Click the *(S)-lactate* metabolite.

e Click the cell for the **Font value** under *Common Style, Text* in the *Property Editor* window to edit properties of the font for this metabolite (Figure 136).

f Select **72** for **Size** in the **Select Font** dialog box.

g Click **OK**.

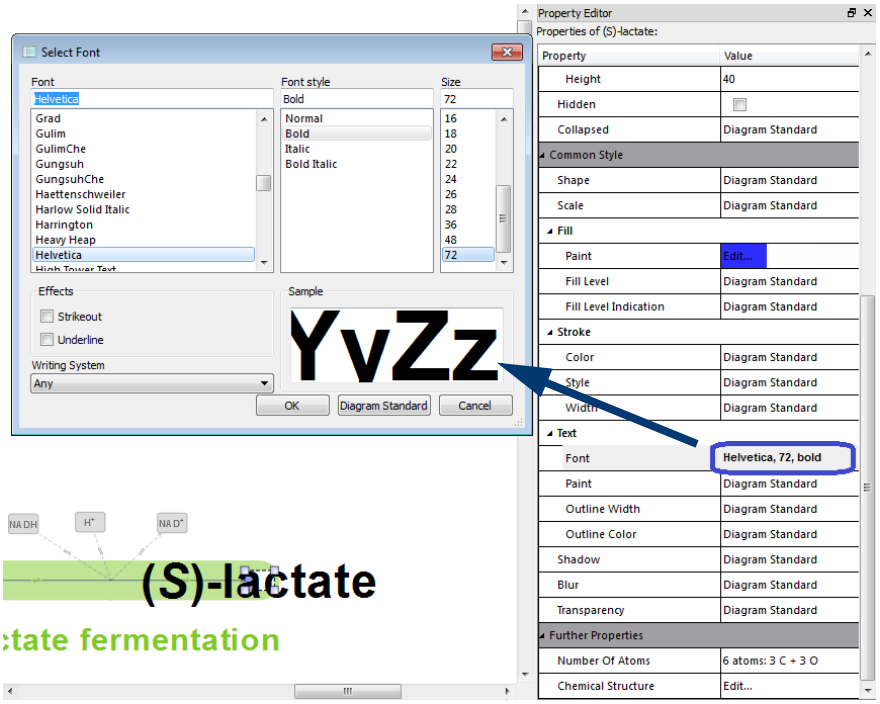


Figure 136 *Edit the font properties of a metabolite in the property editor*

Hide a metabolite label

h Click the *acetyl-CoA* metabolite.

i Click the cell for the **Placement value** under *Label, Base* in the *Property Editor* window to change the value of the label for this metabolite (Figure 137 on page 122).

j Click **Hidden**.

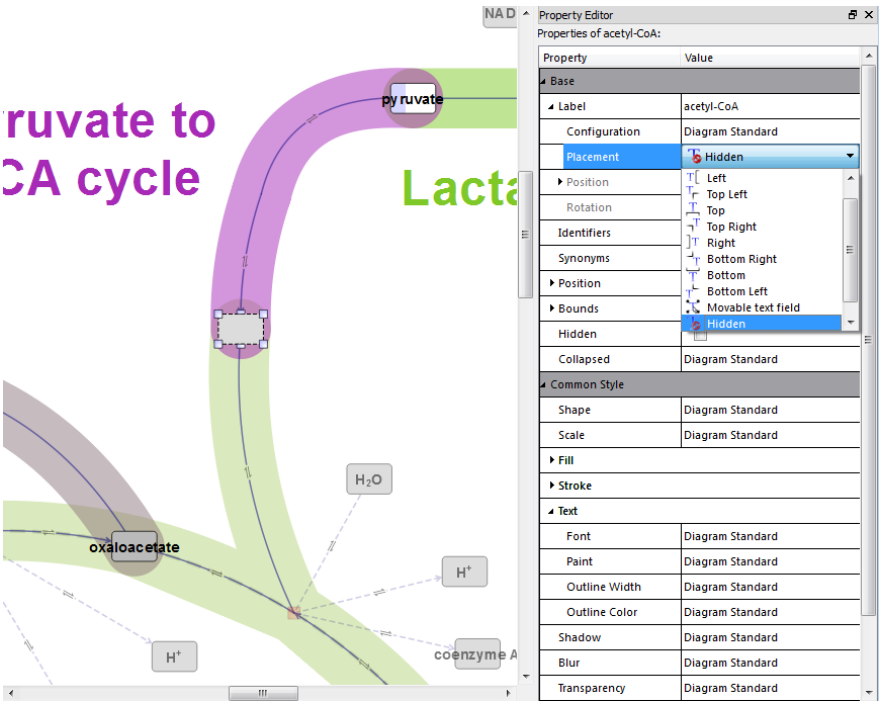


Figure 137 *Edit the font properties of a metabolite in the property editor*

k Repeat "Change a metabolite font" and "Hide a metabolite label" to increase the font size for the target metabolites and hide the labels of the non-target metabolites. You can perform the font adjustments on more than one metabolite at a time.

l Click the **Hide Metabolites** button from the toolbar to hide non-target metabolite nodes.

m Click the **Hide Reactions** button from the toolbar to hide reactions nodes.

n Click the **Hide Cofactors** button from the toolbar to hide cofactors.

o Click the **Hide Effector edges** button from the toolbar to hide effector edges.

p Click the **Hide Flux edges** button from the toolbar to hide the flux edges.

q Click the **Network View Mode** button from the toolbar to disable editing mode and view your network (Figure 138 on page 123).

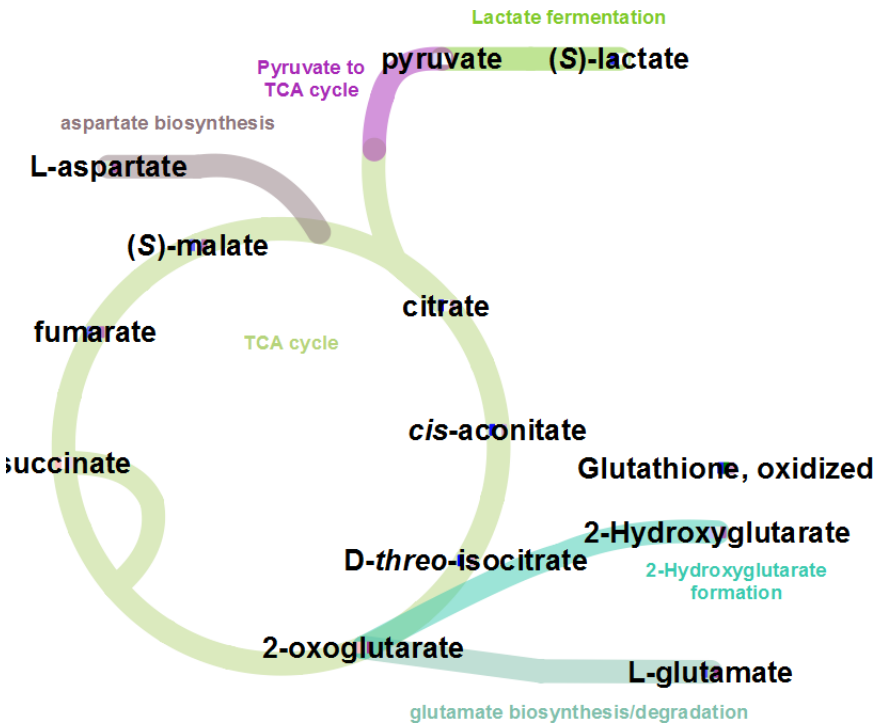


Figure 138 *Network diagram after adjusting the metabolite labels*

r Click the **Visualize chemical structures** button > **Draw structure next to metabolites** to add chemical structures.

s Click the **Select** button from the toolbar to enter the editing mode

t Resize the chemical structures of the target metabolites.

u Click the **Network View Mode** button from the toolbar to disable editing mode and view your network diagram (Figure 139).

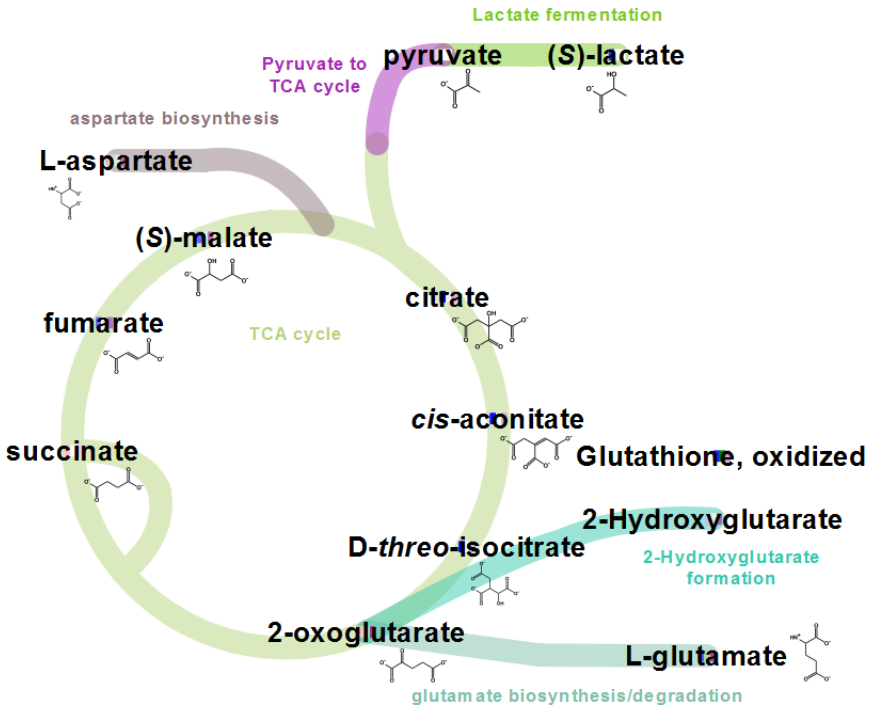


Figure 139 *Network diagram after adjusting the metabolite labels and adding chemical structures*

Add plots and charts to your network diagram

With the network diagram cleaned up to the essential elements from your flux analysis, you can add quilt plots and/or bar charts to your network diagram.

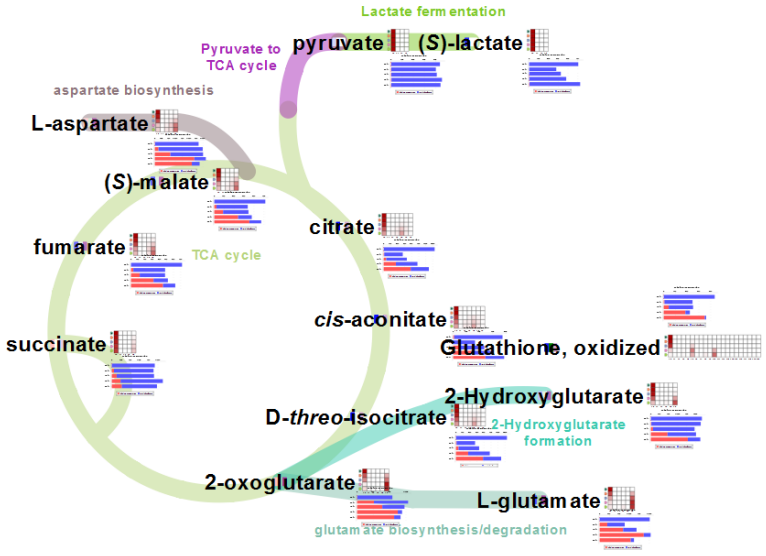


Figure 140 *Network diagram with charts*

Step through time points and network activity

In "Display isotopologue metabolite data and highlight pathways" on page 103 each of the fold change, quilt plot, and bar chart results can be displayed with a visualization preference of **Individual sample group** in the respective dialog boxes. When **Individual sample group** is selected, the fold change, quilt plot, and bar chart data is presented for the current group as selected using the slider in the MassVisualizer toolbar.

Click any of the control buttons available on the MassVisualizer toolbar or **Visualization** menu to step through the time points (sample groups). Figure 141 shows the control buttons available with MassVisualizer; Stop, Back, Play, Next, Slider, and Record.

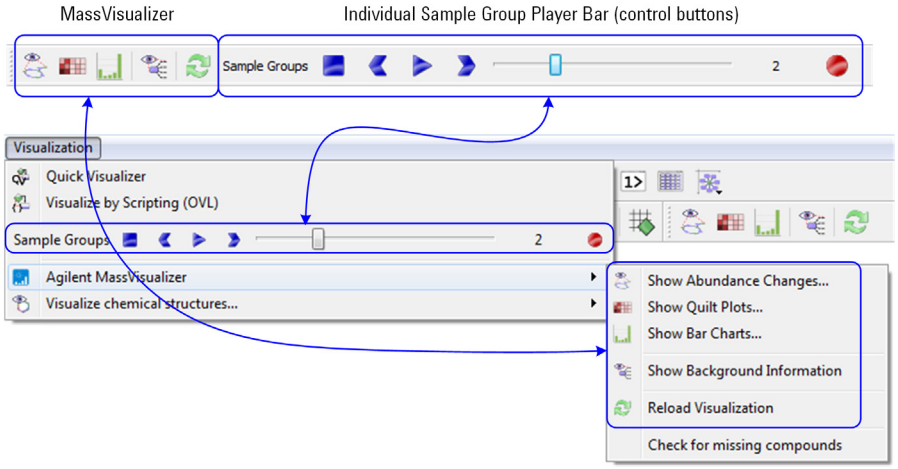


Figure 141 *MassVisualizer toolbar (top) and menu (bottom) when you visualize your results by "individual sample group." You use the control buttons to step through the time points (sample groups). When you visualize your results by "all sample groups," the sample group control buttons are not displayed, and the toolbar and the menu appear as shown in Figure 114 on page 103.*

Save your Omix Premium project

At any time during your session with Omix Premium, you can save your pathways network diagram as an Omix Premium (OMX) document. Saving your session with a descriptive name and a sequential number for the file name saves you time if you want to return to prior network diagrams after you have adjusted and customized various elements.

Note: When your Omix Premium document has unsaved changes an asterisk appears at the end of the file name in the title bar (Figure 142).

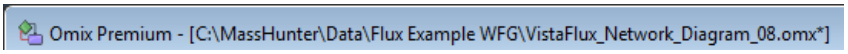


Figure 142 *Unsaved changes in your document are indicated by an asterisk in the title bar. This example uses a descriptive name and a sequential number for the file name.*

a Click **File > Save** or click the **Save the network document to file** button on the toolbar.

b Navigate to the folder to save your Omix Premium network in the **Save Omix Network Model** dialog box.

c Enter a descriptive name and a sequential number for the **File name** (Figure 143).

d Click **Save**.

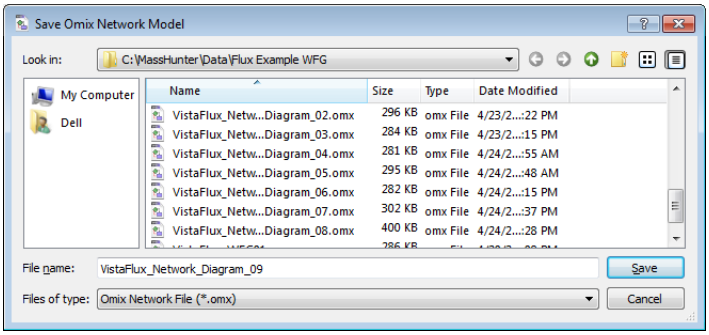


Figure 143 *Save Omix Network Model dialog box*

Next step...

You have now completed the fourth step of the VistaFlux Software workflow. In the next workflow step you export your results.

Export results

Your network model and qualitative flux analysis results can be exported for reports and presentations.

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Export tables, charts and images

When you have created your network diagram, imported your isotopologue results from Profinder, developed an understanding of your experiment in the context of the biological network, and generated static images, you can export your network diagram for presentations and publications.

Your options to export images of your qualitative flux analysis include:

- Print to system printers, see "Print to system printer using clippings"
- Export to biochemical formats, see "Export to biochemical formats" on page 130
- Save to graphics file formats, see "Save to graphics file formats" on page 130
- Copy PFA data file tables from *Data Manager* to other programs, such as presentation and spreadsheet software.

Print to system printer using clippings

a Click the **Network View Mode** button from the toolbar to disable editing mode and view your network diagram.

b Click **File > Edit Diagram Clippings** to enter the *Diagram Clipping Editor* mode.

c Click the **Insert Diagram Clipping** button from the toolbar.

d Drag the clipping selection frame to encompass the area you want to assign to a clipping.

e Type in a name for the clipping region you have defined in the **New Diagram Clipping** dialog box (Figure 144).

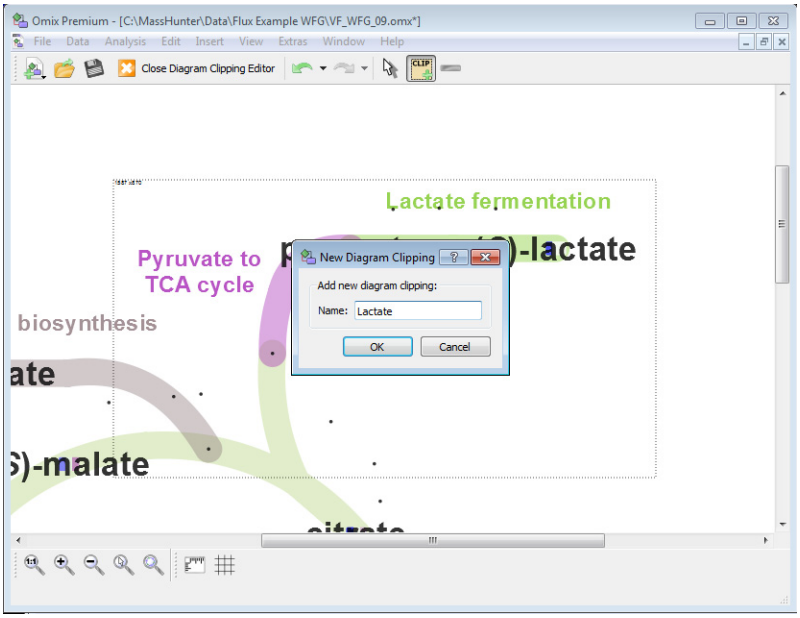


Figure 144 *Diagram Clipping Editor*

f Click **OK**.

g Click **Close Diagram Clipping Editor**.

h Click **File > Print**, or type Ctrl-P, to print your network diagram to any system printer installed on your personal computer.

i Select the system printer to use in the **Print** dialog box.

j Click **Print**. This action does not start a printing; the next dialog box allows you to set your clipping, print options, and generate a preview.

k Select your *clipping*, or <Print All>, in the **Print Metabolic Network** dialog box.

l Select **Fit to Page Width** and other parameters to use in the **Print Metabolic Network** dialog box (Figure 145).

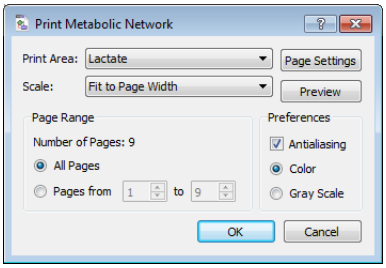


Figure 145 *Print Metabolic Network dialog box*

m Click **Preview** to review the output before sending it to your system printer (Figure 146). The clipping is fit to the page, and if there is room on the page additional elements from your network diagram are also added to the clipping.

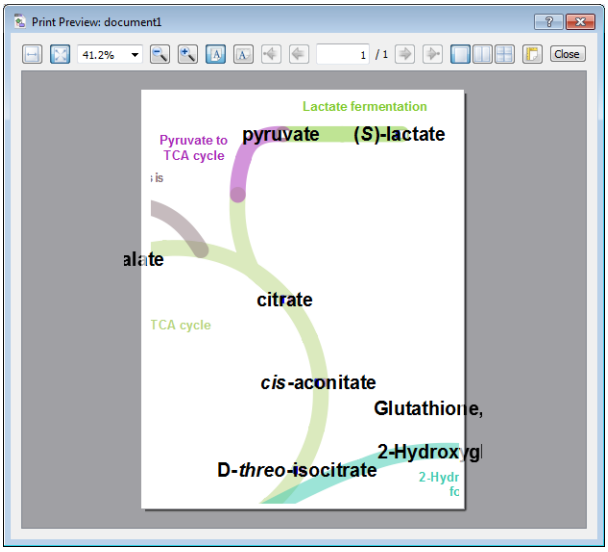


Figure 146 *Preview view of your network diagram*

n Click **Close** to return to the **Print Metabolic Network** dialog box.

o Click **OK** to print your network diagram (Figure 145).

Export to biochemical formats

a Click **File > Export > Export Network File**, or type Ctrl-E, to export your network diagram to standard flux, systems biology, and stoichiometry file formats.

b Navigate to the folder to save your network model in the **Export Omix Network Model** dialog box.

c Type the **File name**.

d Select the file type to export your file (Figure 147).

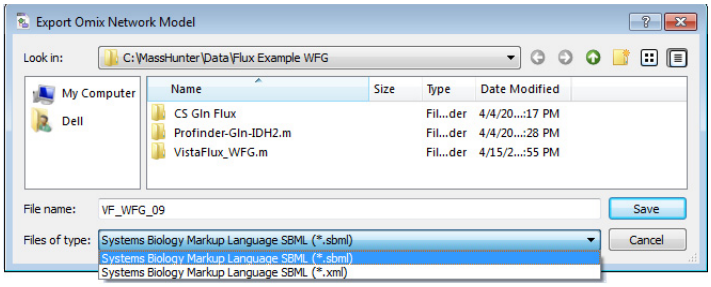


Figure 147 *File types in the Export Omix Network Model dialog box*

e Click **Save** to export your network diagram.

Save to graphics file formats

a Click **File > Save Image** to save your entire network diagram, not just the current view in the *Drawing Area*, to a graphics or presentation file format.

b Navigate to the folder to save your network diagram in the **Export Omix Network Diagram** dialog box.

c Type the **File name**.

d Select the file type to export your file (Figure 148).

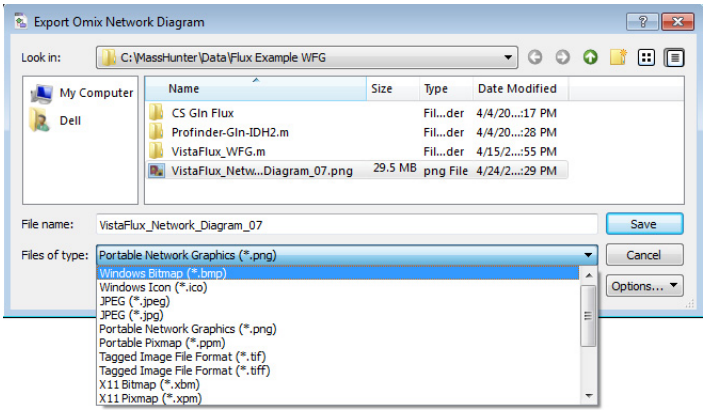


Figure 148 *File types in the Print Metabolic Diagram dialog box*

e Click **Save** to export your network diagram image.

Copy tables

a Click the top left header cell of any table in the **Data Manager** window to select the table.

b Click the **Copy** icon in the top of the **Data Manager** window (Figure 149).

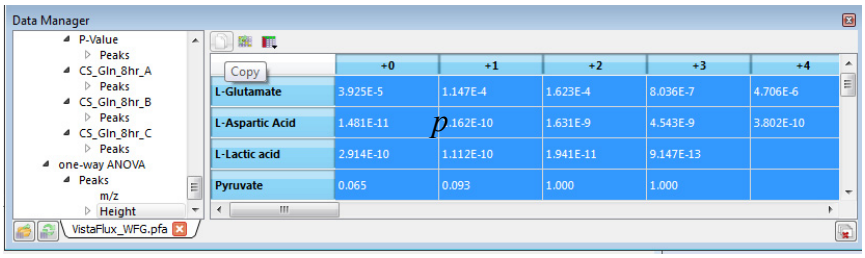


Figure 149 *Copy a table in the Data Manager window*

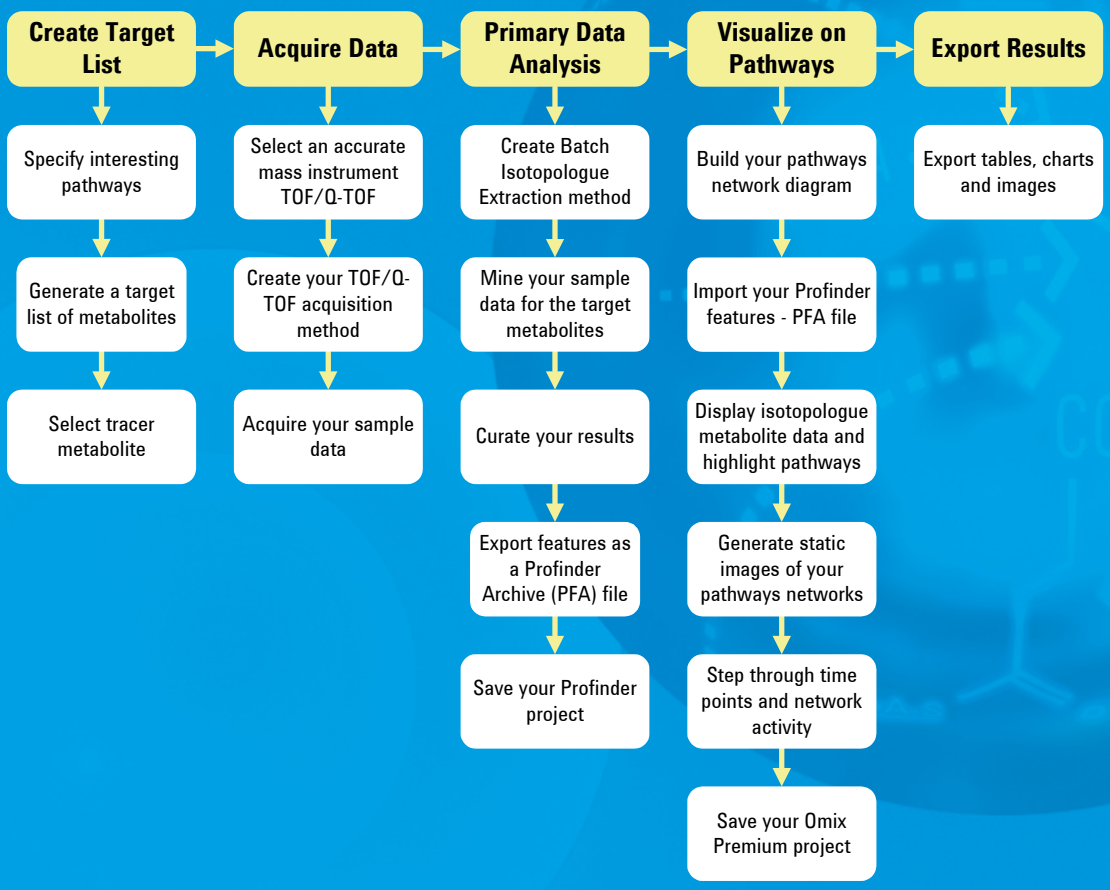
c Paste the table into a spreadsheet or other office software product.

Conclusion

You have now completed the VistaFlux Software workflow.

Reference information

This chapter consists of definitions and references. The definitions section includes a list of terms and their definitions as used in this workflow. The references section includes citations to Agilent publications that help you use Agilent products.



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Agilent Technologies

Definitions

This section contains a list of terms and their definitions as used in this workflow. Review of the terms and definitions presented in this section helps you understand the software wizards and the class prediction workflow.

Abundance profile

Relative or absolute signal intensities of the extracted compounds identified in your sample derived from the chromatographic/mass spectral data.

Algorithm

Mathematical calculations and related parameters that are applied to sample data to produce a result that may be used to represent or classify a sample.

Alignment

Adjustment of the chromatographic retention time of eluting components to improve the correlation among data sets, based on the elution of specific component(s) that are (1) naturally present in each sample or (2) deliberately added to the sample through spiking the sample with a known compound or set of compounds that does not interfere with the sample.

ANOVA

Abbreviation for analysis of variance which is a statistical method that simultaneously compares the means between two or more attributes, parameters, or groups of a sample data set. ANOVA is used to determine if a statistical difference exists between the means of two or more data sets (groups) and thereby prove or disprove the hypothesis. See also Student's t-test and Welch's t-test.

Bar chart

A two-dimensional representation of data that uses rectangles that originate from one axis, an axis that represents a non-numerical value, to represent a numerical value defined by the second axis.

Baselining

A technique used to view and compare data, that involves converting the original data values to values that are expressed as changes relative to a calculated statistical value, derived from the data. The calculated statistical value is referred to as the baseline.

Bioinformatics

The use of computers, statistics, and informational techniques to increase the understanding of biological processes.

Biomarker

An organic molecule whose presence and concentration in a biological sample indicates a normal or altered function of higher level biological activity.

Carbohydrate

An organic molecule consisting entirely of carbon, hydrogen, and oxygen that is important to living organisms.

CEF file

A file format called a compound exchange file (CEF) that is used to exchange data between Agilent software. CEF files are used to share molecular features between MassHunter software.

Cell	The fundamental unit of an organism consisting of several sets of biochemical functions within an enclosing membrane. Animals and plants are made of one or more cells that combine to form tissues and perform living functions.
Class	A grouping of samples organized for a study based on having a similar or identical likeness such as origin, condition (age, disease, treatment, extraction procedure), or another trait that is relevant to an experiment.
Co-elution	When ion signals elute from a chromatographic column at nominally the same time. This can be due to the ion signals belonging to adducts of the same compound, alternative charge states of the same compound, or two or more compounds sharing similar chemical properties, causing them to be unresolvable by the chosen chromatography.
Complex	Class of compounds consisting of two or more proteins that physically bind each other. Their combined form is biologically active and stable.
Composite spectrum	A compound spectrum generated to represent the molecular feature that includes more than one ion, isotope, or adduct (not just M + H) and is used by Mass Profiler Professional for recursive analysis and ID Browser.
Compound	A metabolite that may be individually referred to as a compound, descriptor, element, entity, feature, or metabolite during the various steps presented during various workflows.
Compound group	A single compound that is targeted, or found, in any of the sample data files in a project. For example, if 20 compounds are found in the first data file in the project, then there are at least 20 compound groups in the project. If additional unique compounds are found in the remaining data files for your project, then additional compound groups are created.
Control	During analyses of your sample data, replicate samples are averaged together in groups representing the independent variable in your experiment. When one group is used as a reference against which statistical variation of the other groups is compared, the one group is referred to as a control.
Data	Information that represents, in a form suitable for storing and processing by a computer, the qualitative or quantitative attributes of a subject. Examples include LC/MS data consisting fundamentally of time, ion m/z, and ion abundance from a chemical sample.
Data processing	Conversion of data into meaningful information. Computers are employed to enable rapid recording and handling of large amounts of data, i.e., MassHunter Workstation and Mass Profiler Professional.

Data reduction	See reduction.
Deconvolution	An algorithmic feature extraction of compound ion signals by coelution and in some cases, grouping of isotope clusters by *m/z*, charge state, and adduct/deduct ion species.
Dependent variable	An element in a data set that can only be observed as a result of the influence from the variation of an independent variable. For example, a pharmaceutical compound structure and quantity may be controlled as two independent variables while the metabolite profile presents a host of small-molecule products that make up the dependent variables of a study.
Document	A network diagram created in Omix Premium. Also referred to as a network model.
Edge	A visual representation of the connection between a reaction and a metabolite when creating a network diagram.
Effector	A small molecule that regulates biological activity in a pathway.
Element	A metabolite that may be individually referred to as a compound, molecular feature, element, or entity during the various steps of data analysis. An element can also refer to one of many pieces of graphical information used to create a network diagram.
Environment	The natural and/or controlled conditions that surround an organism as it lives and operates.
Enzyme	Proteins acting as biocatalysts in a metabolomic reaction. These entities are particularly important in depicting a biochemical network.
Experiment	Data acquired in an attempt to understand causality where tests or analyses are defined and performed on an organism to discover something that is not yet known, to demonstrate as proof of something that is known, or to find out whether something is effective.
Extraction	The process of retrieving a deliberate subset of data from a larger data set whereby the subset of the data preserves the meaningful information, not the redundant and less meaningful information. Also known as data extraction or feature extraction.
F statistic	A value used with a one-way ANOVA test to determine if the difference between the mean values of two sample groups are significant.
Feature	Independent, distinct characteristic of a phenomena and data under observation. Features are an important part of the identification of patterns - pattern recognition -

	within data whether processed by a human or by artificial intelligence, such as MassHunter Profinder. During the various workflows a feature may be individually referred to as a compound, descriptor, element, entity, or metabolite.
Feature extraction	The extraction of compound signals from complex, multidimensional data and its background noise. This results in a reduction of data size and complexity through the removal of redundant and non-specific data. Careful feature extraction yields a smaller data set that is more easily processed without any compromise in the information quality.
Feature selection	The identification of important, or non-important, variables and the variable relationships in a data set using both analytical and a priori knowledge about the data.
Filter	The process of establishing criteria by which entities are removed (filtered) from further analysis during data analysis.
Flux	A representation of the magnitude and direction of chemical flow through a pathway.
Filter by flag	A flag is a term used to denote a quality of an entity within a sample. A flag indicates if the entity was detected in each sample as follows: Present means the entity was detected, Absent means the entity was not detected, and Marginal means the signal for the entity was saturated.
Fold change	A measure of the amount of change expressed in the ratio of the amount of change from the original value versus the original value. A fold change can be positive (increasing) or negative (decreasing)
Hypothesis	A proposition made to explain certain facts and tentatively accepted to provide a basis for further investigation. A proposed explanation for observable phenomena may or may not be supported by the analytical data. Statistical data analysis is performed to quantify the probability that the hypothesis is true. Also known as the scientific hypothesis.
Hypothetical	A statement based on, involving, or having the nature of a hypothesis for the purposes of serving as an example and not necessarily based on an actuality.
Identified compound	Chromatographic components that have an assigned, exact identity, such as compound name and molecular formula, based on prior assessment or comparison with a database. See also Unidentified Compound.
Independent variable	An essential element, constituent, attribute, or quality in a data set that is deliberately controlled in an experiment. An independent variable may be referred to as a parameter and is assigned a parameter name during the various steps of data analysis.

Isotope

An element that has a different number of neutrons from another element with the same number of protons. Examples include ^{12}C versus ^{13}C,^{14}N versus ^{15}N, and ^{1}H versus ^{2}H, each of these elements represent different isotopes of carbon, nitrogen, and hydrogen, respectively.

Isotopologue

Molecules that contain the same molecular formula and structure but differ in their isotopic composition through the substitution of one or more atoms with a different isotope. The exact location of the isotope in the molecule, while important chemically, is not important in flux analysis, just the number of isotopes in the molecule.

Isotopomer

Molecules that contain the same molecular formula, structure, and number of isotopes but differ in the specific atomic location of the isotopes in the molecular structure.

Label

The isotope that is used to enrich a compound used for flux analyses.

Labeling

When an isotope of an atom is substituted for the naturally occurring atom, the resulting compound is referred to as being labeled. Metabolites in a cell can become labeled when a isotopically enriched compound is introduced to the cellular metabolism. An experiment that studies the rate that metabolites become labeled through metabolism are referred to as metabolic flux analysis or qualitative flux analysis.

Lipidomics

Identification and quantification of cellular lipids from an organism in a specified biological situation. The study of lipids is a subset of metabolomics.

Mass variation

Using the mass to charge (*m/z*) resolution to improve compound identification. Compounds with nearly identical and identical chromatographic behavior are deconvoluted by adjusting the m/z range for extracting ion chromatograms.

Mean

The numerical result of dividing the sum of the data values by the number of individual data observations.

Metabolism

The chemical reactions and physical processes whereby living organisms convert ingested compounds into other compounds, structures, energy and waste.

Metabolite

Small organic molecules that are intermediate compounds and products produced as part of metabolism. Metabolites are important modulators, substrates, byproducts, and building blocks of many different biological processes. A metabolite may be individually referred to as a compound, molecular feature, element, or entity during the various steps of data analysis.

Metabolome

The complete set of small-molecule metabolites that may be found within a biological sample. Small molecules are typically in the range of 50 to 600 Da.

Metabolomics	The process of identification and quantification of all metabolites of an organism in a specified biological situation. See Metabonomics for the study of the change in the metabolites in response to externalities.
Metabonomics	The metabolic response to externalities such as drugs, environmental factors, and disease. See Metabolomics for the identification and quantitation of metabolites.
Model	Another name to refer to a network diagram.
Network	A set of metabolite and reaction nodes that can be assembled with additional information to represent the operation biochemical system.
Network diagram	A graphical visualization of metabolite and reaction nodes, effectors, and flux edges that together represent the operation biochemical system.
Node	A representation of a metabolite or reaction when you create a network diagram.
Normalization	A technique used to adjust the ion intensity of mass spectral data from an absolute value based on the signal measured at the detector to a relative intensity of 0 to 100 percent based on the signal of either (1) the ion of the greatest intensity or (2) a specific ion in the mass spectrum.
Null hypothesis	The default position taken by the hypothesis that no effect or correlation of the independent variables exists with respect to the measurements taken from the samples. An example null hypothesis: "No effect or correlation exits between a change in the independent variables (e.g., treatment) and a change in the dependent variables (e.g., metabolic profile)."
Observation	Data acquired in an attempt to understand causality where no ability exists to (1) control how subjects are sampled and/or (2) control the exposure each sample group receives.
Organism	A group of biochemical systems that function together as a whole thereby creating an individual living entity, such as an animal, plant, or microorganism. Individual living entities may be multicellular or unicellular. See also specimen.
Pathway	A sequence of reactions and metabolites that represent the chemical reactions that occur in a cell.
p-value	The probability of obtaining a statistical result that is comparable to or greater in magnitude than the result that was actually observed, assuming that the null hypothesis is true. The null hypothesis is stated that no correlation exists between the independent variables and the measurements taken from the samples. Rejection of the null hypothesis is typically made when the p-value is less than 0.05 or 0.01. A

	p-value of 0.05 or 0.01 may be restated as a 5% or 1% chance of rejecting the null hypothesis when it is true. When the null hypothesis is rejected, the result is said to be statistically significant meaning that a correlation exists between the independent variables and the measurements as specified in the hypothesis.
Peptide	Linear chain of amino acids that is shorter than a protein. The length of a peptide is sufficiently short that it is easily made synthetically from the constituent amino acids.
Peptide bond	The covalent bond formed by the reaction of a carboxyl group with an amine group between two molecules, e.g., between amino acids.
PFA file	A file format called a Profinder Archive (CEF) that is used to exchange data between Profinder and Omix premium.
Quilt plot	A graphical representation of data using a two-dimensional grid and where each grid employs a color used to indicate magnitude instead of numerical text.
Proteomics	The study of the structure and function of proteins occurring in living organisms.
Recursive	Reapplying the same algorithm to a subset of a previous result in order to generate an improved result.
Recursive feature finding	A multi-step process that improves the accuracy of finding statistically significant features in sample data files. This is employed by MassHunter Profinder.
Reduction	The process whereby the number of variables in a data set is decreased to improve computation time and information quality, such as an extracted ion chromatogram obtained from LC/MS data files. Reduction provides smaller, viewable and interpretable data sets by employing feature selection and feature extraction. Also known as dimension reduction and data reduction. This is part of most of the advanced processes employed by Mass Profiler Professional.
Regression analysis	Mathematical techniques for analyzing data to identify the relationship between dependent and independent variables present in the data. Information is gained from the estimation, regression, or the sign and proportionality of the effects of the independent variables on the dependent variables.
Replicate	Multiple identical samples collected from a population so that the sample evaluation results in a value that more closely approximates the true value.
Sample	A part, piece, or item that is taken from a specimen and understood as being representative of the larger specimen (e.g., blood sample, cell culture, body fluid, aliquot) or population. An analysis may be derived from samples taken at a particular geographical location, taken at a specific period of time during an experiment, or taken

	before or after a specific treatment. A small number of specimens used to represent a whole class or group.
Sample group	An experimental condition, such as the time a sample was acquired after an experiment was started, assigned to replicate samples. Larger number of samples in a sample group improve the statistical significance of your qualitative flux analysis.
Sampling	The process of taking samples that have a statistical representation of the population under evaluation.
Spline curve	A smooth curve representation to connect nodes in a network diagram. The degree of curve applied to a spline is controlled by spline points.
Spline point	The degree of curve applied to a spline curve is controlled by moving points that are referred to as spline points.
Standard	A chemical or mixture of chemicals used to compare the quality of analytical results or to measure and compensate the precise offset or drift incurred over a set of analyses.
Statistics	The mathematical process employed in manipulating numerical data from scientific experiments to derive meaningful information. This is part of the principal component analysis, t-test, and ANOVA processes employed by Mass Profiler Professional.
Student's t-Test	A statistical test to determine whether the mean of the data differs significantly from that expected if the samples followed a normal distribution in the population. The test may also be used to assess statistical significance between the means of two normally distributed data sets (groups). See also ANOVA.
Subject	A chemical or biological sample taken from a specimen, or a whole specimen, that undergoes a treatment, experiment, or an analysis for the purposes of further understanding.
Tracer	A stable isotope labeled compound, referred to as a tracer, is introduced into the biological system for flux analysis. The tracer typically contains multiple atoms of ^{13}C, ^{15}N, or ^{2}H.
Unidentified compound	Chromatographic components that are only uniquely denoted by their mass and retention times and which have not been assigned an exact identity, such as compound name and molecular formula. Unidentified compounds are typically produced by feature finding and deconvolution algorithms. See also Identified Compound.

Variable

An element in a data set that assumes changing values, e.g., values that are not constant over the entire data set. The two types of variables are independent and dependent.

Volume

The area of the extracted compound chromatogram (ECC). The ECC is formed from the sum of the individual ion abundances within the compound spectrum at each retention time in the specified time window. The compound volume generated by MFE is used by Mass Profiler Professional to make quantitative comparisons.

Welch's t-test

A version of the Student's t-test. Unlike the Student's t-test that assumes a normal distribution, the Welch's t-test does not assume normal variance and is more reliable when the test is performed on sample groups with unequal sample sizes and unequal variance. See also Student's t-test.

Wizard

A sequence of dialog boxes presented by Mass Profiler Professional that guides you through well-defined steps to enter information, organize data, and perform analyses.

References

This section consists of citations to Agilent manuals, primers, application notes, presentations, product brochures, technical overviews, videos, and software that help you use Agilent products and perform your qualitative flux analyses.

Manuals

Check www.agilent.com for the most current version of these manuals. Check the installation media or the online Help of your installed software for the manuals that apply to your version software.

- *MassHunter Pathways to PCDL Software Quick Start Guide* (G6825-90008, Revision A, November 2012)
- *MassHunter Personal Compound Database and Library Manager Quick Start Guide* (G3336-90022, Revision A, September 2014)
- *MassHunter PCDL for Qualitative Analysis Familiarization Guide* (G5883-90002, Revision B, February 2016)
- *MassHunter METLIN Metabolite PCD/PCDL Quick Start Guide* (G6825-90009, Revision A, October 2014)
- *MassHunter Profinder Software Quick Start Guide* (G3835-90027, Revision A, April 2016)

Application Notes

- ^{13}C-Glutamine Qualitative Flux Analysis of a Chondrosarcoma Cell Line Using Agilent VistaFlux (5991-6810EN, May 2016)

Technical Overviews

- Agilent MassHunter Profinder: Solving the Challenge of Isotopologue Extraction for Qualitative Flux Analysis (5991-6817EN, April 2016)
- Agilent MassHunter VistaFlux for Qualitative Flux Analysis (5991-6756EN, April 2016)

Presentations

- Introducing VistaFlux Software for High Performance Qualitative Flux Analysis (http://www.agilent.com/en-us/promotions/flux, April 2016)
- Advances in Instrumentation and Software for Metabolomics Research (Advances in Instrumentation and Software for Metabolomics.pdf, September 18, 2012)
- Workflows to Support Automated Class Prediction with Complex Samples (WP20_405__Workflows_Support_Automated_Class_Prediction.pdf, June 25, 2012)
- Multi-omics Analysis Software for Targeted Identification of Key Biological Pathways (May 3, 2012)
- Predictive Classification of Contaminants Encountered During the Distillation of Shochu, a Distilled Beverage Native to Japan (ASMS_2011_ThP_316.pdf, June 23, 2011)
- Metabolomics LCMS Approach to: Identifying Red Wines according to their variety and Investigating Malaria infected red blood cells (November 3, 2010)

- Small Molecule Metabolomics (November 3, 2010)
- Presentation: Metabolome Analysis from Sample Prep through Data Analysis (November 3, 2010)

Acknowledgments and Citations

This section contains acknowledgments and citations for the BioCyc and KEGG pathways databases.

BioCyc Pathway/Genome Databases

Includes BioCyc Pathway/Genome databases from the Bioinformatics Research Group at SRI International®, used under license.

http://www.biocyc.org/

Citation based on use of BioCyc databases or the Pathway Tools software

If you use BioCyc databases or the Pathway Tools software in your research, cite relevant publications as described on the BioCyc website:

http://biocyc.org/publications.shtml

For example, users who publish research results in scientific journals based on use of data from the EcoCyc Pathway/Genome database should cite:

Keseler et al., *Nucleic Acids Research* **39**:D583-90, 2011.

Users who publish research results in scientific journals based on use of data from most other BioCyc Pathway/Genome databases should cite:

Caspi et al., *Nucleic Acids Research* **40**:D742-53, 2012.

KEGG Database

Includes KEGG (Kyoto Encyclopedia of Genes and Genomes) databases developed by Kanehisa Laboratories.

http://www.genome.jp/kegg/

Citation based on use of KEGG Database

If you use the KEGG database in your research, cite relevant publications as described on the KEGG website:

http://www.genome.jp/kegg/kegg1.html

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Revision A, May 2016

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