# Agilent MassHunter VistaFlux for Qualitative Flux Analysis

## Technical Overview

### Introduction

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 may not result in altered abundance of metabolite intermediates. Stable isotope tracing has tremendous potential to help address these situations and drive deeper understanding of biological systems by providing dynamic information about the interconversion of metabolites. Qualitative flux analysis highlights the relative rate of these reactions.

In qualitative flux analysis, a stable isotope-labeled tracer (typically containing $^{13}C$, $^{15}N$, or $^{2}H$) is introduced into the biological system, resulting 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 mining the target metabolites, accounting for isotopologues, correcting for naturally occurring isotope abundance, and visualizing the results in a biological context. Agilent MassHunter VistaFlux is software designed to meet these challenges, and is designed as a qualitative flux analysis solution for MS-only data from Agilent TOF-based high resolution LC/MS systems (Figure 1). This technical overview describes the use of MassHunter VistaFlux for qualitative flux analysis.

Agilent Technologies

## Create Target Metabolite Lists: Pathways to PCDL and PCDL Manager

Agilent MassHunter Pathways to PCDL software (Figure 2) is designed to facilitate the creation of a custom Agilent Personal Compound Database and Library (PCDL) from metabolites present in pathway content contained within popular databases such as BioCyc, KEGG, and WikiPathways. Multiple pathways can be selected for investigating more complex biological networks. If a master database, such as Agilent METLIN PCDL, is referenced, all compound information (retention times, MS/MS spectra, compound identifiers, structures) from the reference database are included in the custom PCDL.

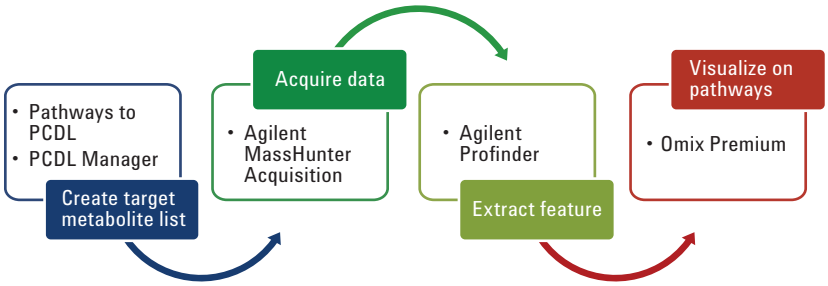



Figure 1. Agilent MassHunter VistaFlux Solution.

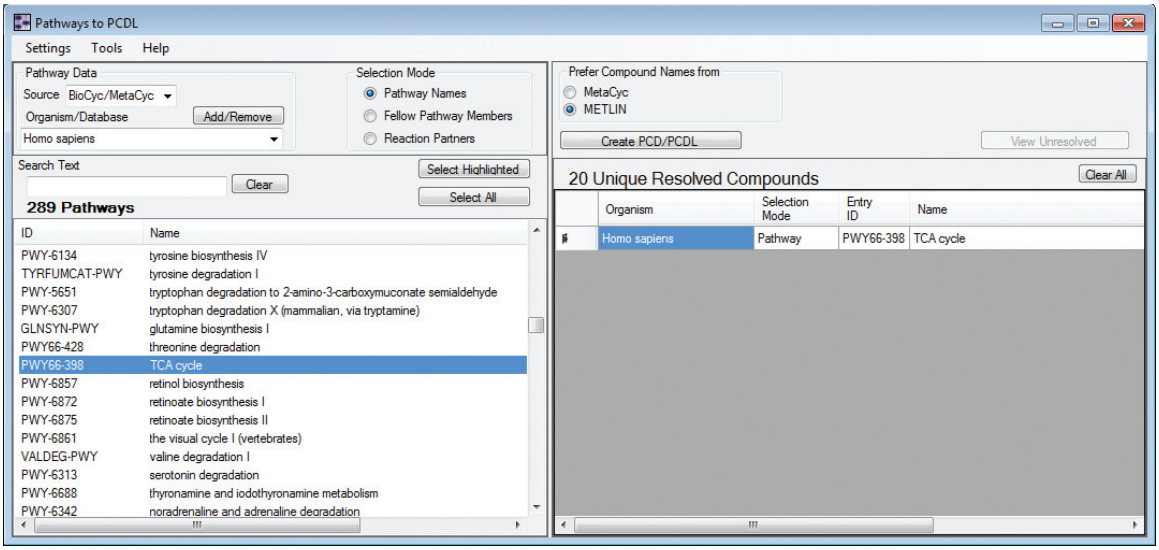



Figure 2. Creating a pathway-based target metabolite list in Agilent MassHunter Pathways to PCDL.

The resulting PCDL can be viewed and edited in Agilent MassHunter PCDL Manager (Figure 3). For example, metabolites that are not detected or not relevant to the experiment can be deleted, and additional compounds can be appended to the PCDL. For targeted isotopologue analysis, retention times must be imported from the master database or added for all metabolites of interest. This custom PCDL can then be used to direct the targeted mining of isotopologues in LC/MS data using Agilent MassHunter Profinder software.

## Acquire Data: Agilent MassHunter Acquisition

Target data analysis uses retention times to identify metabolites, thus a stable LC analytical method is critical for batch processing. In addition to separating isomeric metabolites, it is important to have adequate chromatographic separation for metabolites where the isotopologues may overlap. Interfering isotopes can impact isotopologue extraction and accuracy of the incorporation results. Improved chromatographic separation can be achieved using slower gradients, longer columns, and smaller particle stationary phases.

MassHunter Profinder can process centroid or profile data, however the latter gives better results as the advanced algorithmic features make use of the additional information content provided in profile spectra. The internal reference mass capability of the Agilent TOF systems is important in maintaining mass accuracy across large sample sets, thereby allowing Profinder to accurately distinguish metabolite isotopologues from coeluting compounds with similar masses. Because stable isotope incorporation occurs at different rates in the metabolites, it may be necessary to increase the amount of sample analyzed to adequately detect all isotopologues of interest.

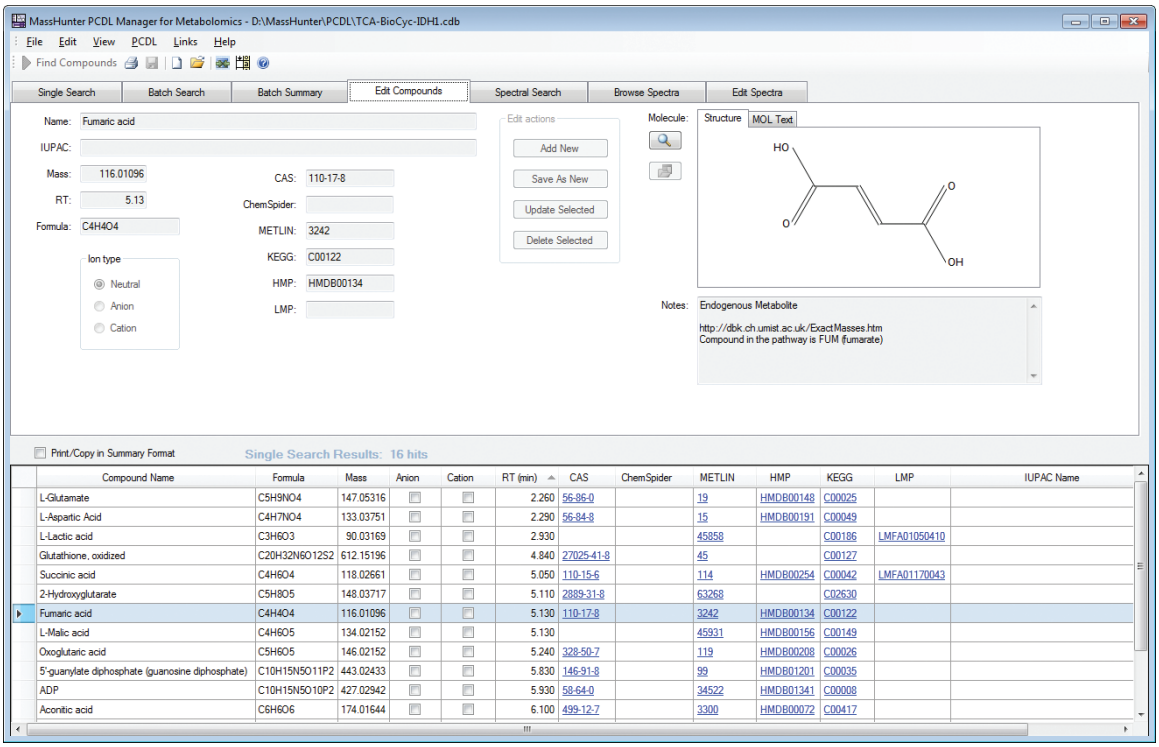



Figure 3. Target metabolite list displayed in PCDL Manager.

## Extract Isotopologues for Target Metabolites: MassHunter Profinder

MassHunter Profinder is a fast batch-processing software for mass spectrometric data that provides quality review and visualization of metabolite isotopologue incorporation. Because stable isotope incorporation occurs at different rates in different compounds of interest, and the distribution of isotopologues detected is expected to change over time, it is frequently necessary to conduct time-course studies. MassHunter Profinder has been designed to facilitate the analysis and review of such studies. It provides grouping of replicate data files, compound alignment, multiple chromatogram overlay, color by sample group, manual reintegration of peaks, and results export.

The batch isotopologue extraction algorithm[1] in MassHunter Profinder uses a target list of metabolites (.CDB) to extract all possible isotopologues based on metabolite chemical formulas. Key user settings for the extraction include choice of stable isotope label, label purity, mass tolerance, and retention time tolerance.

After extraction, for each metabolite MassHunter Profinder displays a compound summary table, the compound extracted ion chromatogram, the compound mass spectrum, and the isotopologue information for each LC/MS file in the batch (Figure 4). In MassHunter Profinder, isotopologue information can be corrected for natural isotope abundance, and can be shown as either absolute or relative abundance.

After batch processing, MassHunter Profinder easily exports batch results (.PFA format) to Omix Premium for pathway visualization and biological interpretation.

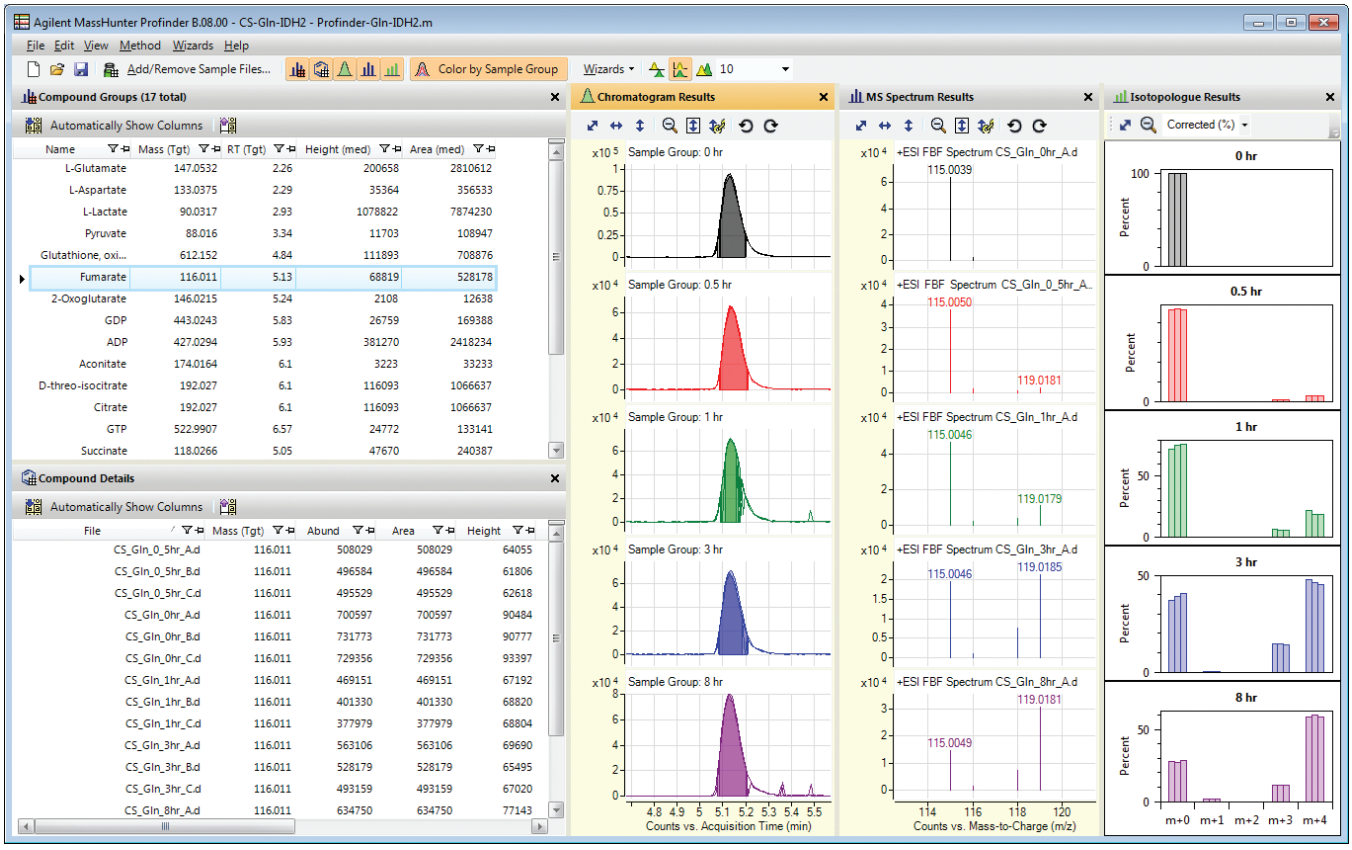



Figure 4. Agilent MassHunter Profinder results summary for a $^{13}C$ label time course study targeting the TCA cycle.

## Visualize Isotopologue Results on Pathways: Omix Premium

Omix Premium is designed to seamlessly import MassHunter Profinder results, and the .PFA batch export format contains all the information necessary for Omix Premium to easily visualize flux data on pathway maps. This includes sample group information, compound identifiers, and isotopologue abundances (Figure 5).

Omix Premium can import pathways from popular databases such as BioCyc and KEGG, or pathways can be manually created. Multiple pathways can be added and connected, or pathways can be user-modified. The pathway source in Omix Premium must match the identifier information used in the target metabolite PCDL to correctly map MassHunter Profinder results.

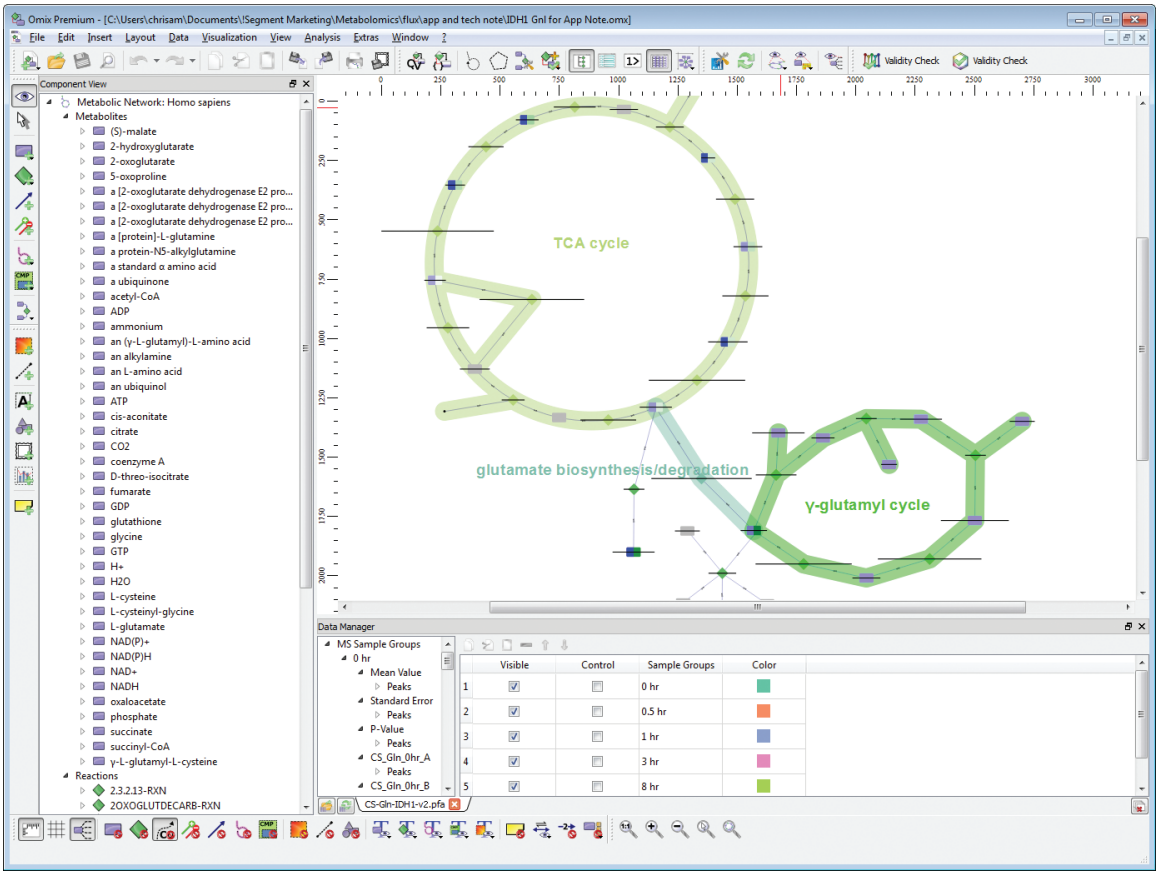


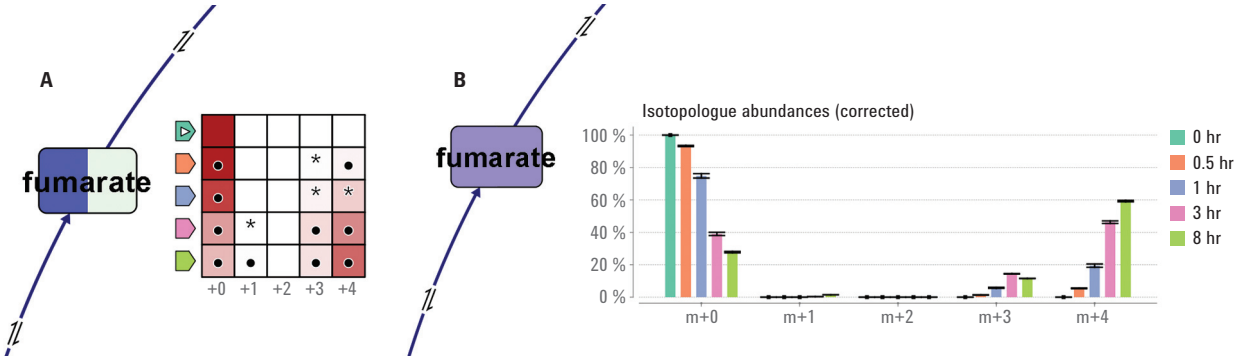

Figure 5. Omix Premium display of Agilent MassHunter Profinder results showing the sample group information is included in the .PFA batch result file import.

Omix Premium allows users to tailor the visualization by optionally displaying different information on pathway diagrams. For example, Figures 6A and 6B show several of the optional results visualizations of the fumarate node in the TCA cycle. In Figure 6A, the quilt plot shows time-point results on the y-axis, isotopologues on the x-axis, relative abundance by fill color intensity, and statistical significance by the symbols in the boxes (· and * as $p < 0.005$ and $p < 0.05$, respectively). The fumarate node is shaded to summarize overall metabolite abundance on the left side and the percent stable label incorporation as a color gradient on the right. Figure 6B shows the same results where the fumarate box is colored to indicate the abundance of the metabolite relative to other detected metabolites. An isotopologue plot is available for the different time-points collected in this particular experiment. Error bars indicate the standard deviation observed for the replicate measurements. This plot is similar to the isotopologue display in MassHunter Profinder, and provides a better view for estimating the percent of isotope incorporation. Both options clearly communicate that the M+0 isotopologue is declining as the M+4 isotopologue is increasingly labeled from the $^{13}C$ tracer with time.

Some metabolites such as ATP and ADP are ubiquitous and not labeled by the stable isotope tracer, but the measured abundance represents total cellular concentration, and still provides useful information for the interpretation of the overall experiment. Metabolite nodes for these compounds can be included, or shown separate from a specific pathway, with the same options for annotation and data visualization.

Time-course studies are extremely valuable for capturing the fluidity of metabolism. Omix Premium provides static and dynamic visualizations that make time-course results easier to interpret (Figure 7). These animations include options to show the isotope incorporation as node filling, and can easily be recorded to assemble a movie file.

Using both static and animated pathway visualizations, experimental results are easy to interpret and explain. Omix Premium visualization results can be exported as figures for publication, presentations, or reports.




Figures 6. A) A quilt plot view of the isotopologue abundances is shown for fumarate. B) shows a relative corrected isotopologue plot giving more details of the results.

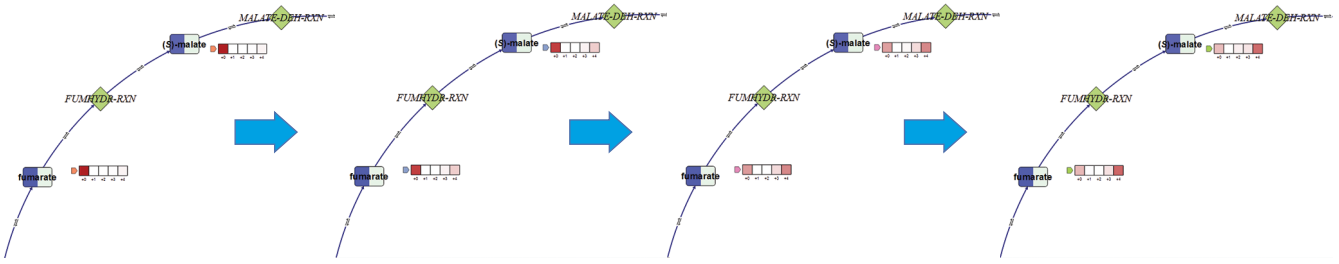



Figure 7. Stepwise visualization of flux for a time-course study representing 0.5, 1, 3, and 8-hour time points (from left to right) for fumarate and malate.

## Conclusions

Agilent MassHunter VistaFlux enables targeted qualitative flux analysis by reducing the difficulty of data processing. Experiments that previously involved the tedious manipulation of data and manual calculations in spreadsheets are now made possible with an efficient, routine workflow for batch processing of large sample sets. VistaFlux incorporates easy creation of a targeted metabolite list, a sophisticated isotopologue extraction algorithm, and flexible pathway visualization tools. VistaFlux extends metabolomics research by capturing the fluidity of metabolism, thus increasing biological understanding.

## Reference

1. MassHunter Profinder: Solving the Challenge of Isotopologue Extraction for Qualitative Flux Analysis, *Agilent Technologies*, publication number 5991-6817EN.

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