Enabling Stable Isotope Label Tracing with MassHunter VistaFlux

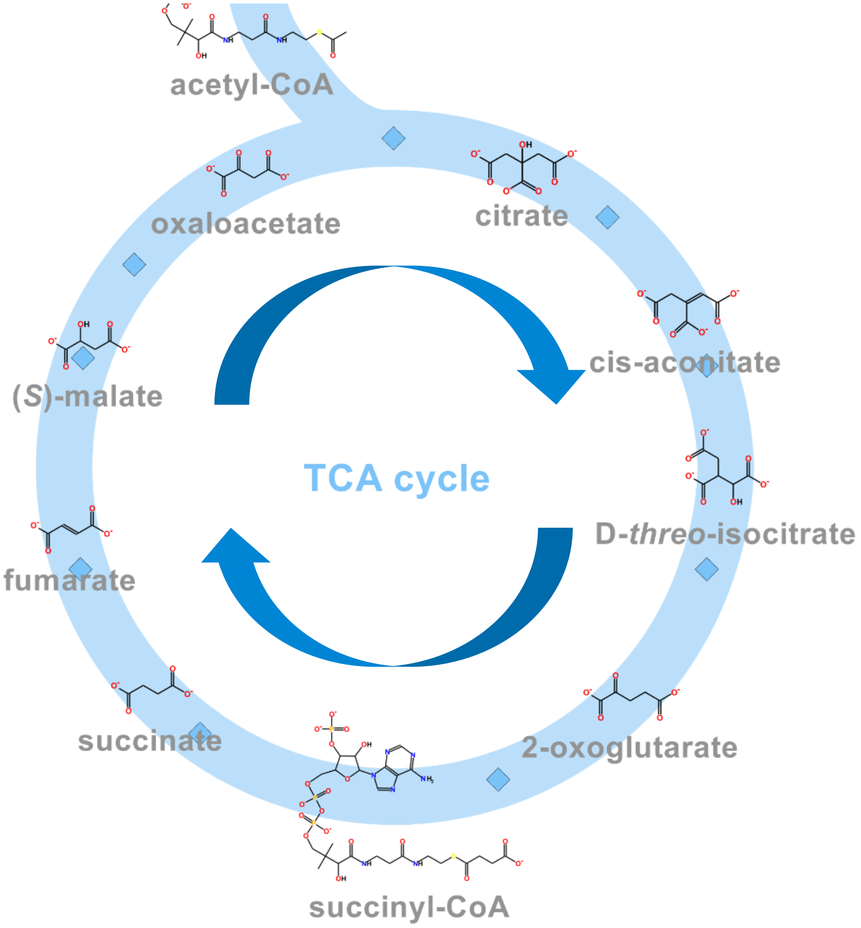
May 2016

The Measure of Confidence

Stable Isotope Tracing Using MassHunter VistaFlux

Qualitative Flux Analysis

- Metabolomics provides static information on cellular molecular composition
- Qualitative flux analysis reveals *in vivo* pathway activity
- Qualitative flux analysis tracks the flow of metabolites through a pathway

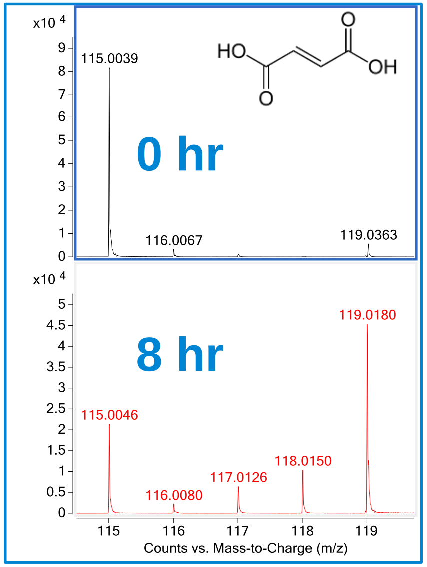


VistaFlux Stable Isotope Tracing

Isotopologue Tracking

Use of stable isotope labels (^{13}C, ^{15}N, and ^{2}H)

Monitor stable isotope incorporation



Fumarate $C_4H_4O_4$

Isotopologues

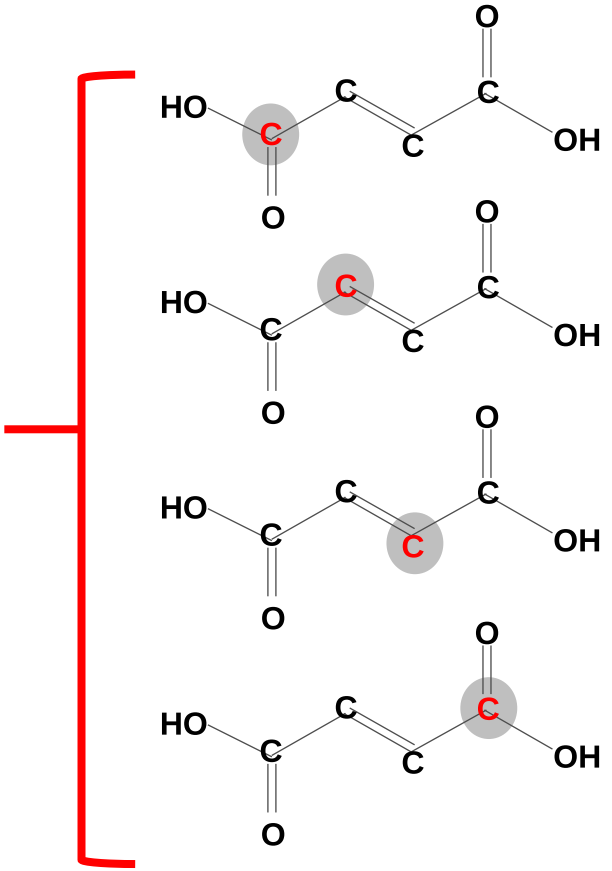
M+0: $^{13}C_0C_4H_4O_4$

M+1: $^{13}C_1C_3H_4O_4$

M+2: $^{13}C_2C_2H_4O_4$

M+3: $^{13}C_3C_1H_4O_4$

M+4: $^{13}C_4C_0H_4O_4$



4 M+1 Isotopomers

Manual Qualitative Flux Analysis Workflow

Current Flux Data Analysis

Create target metabolite list

Determine *m/z* for all possible isotopologues

Extract isotopologues for targets

Integrate peaks and copy results to Excel

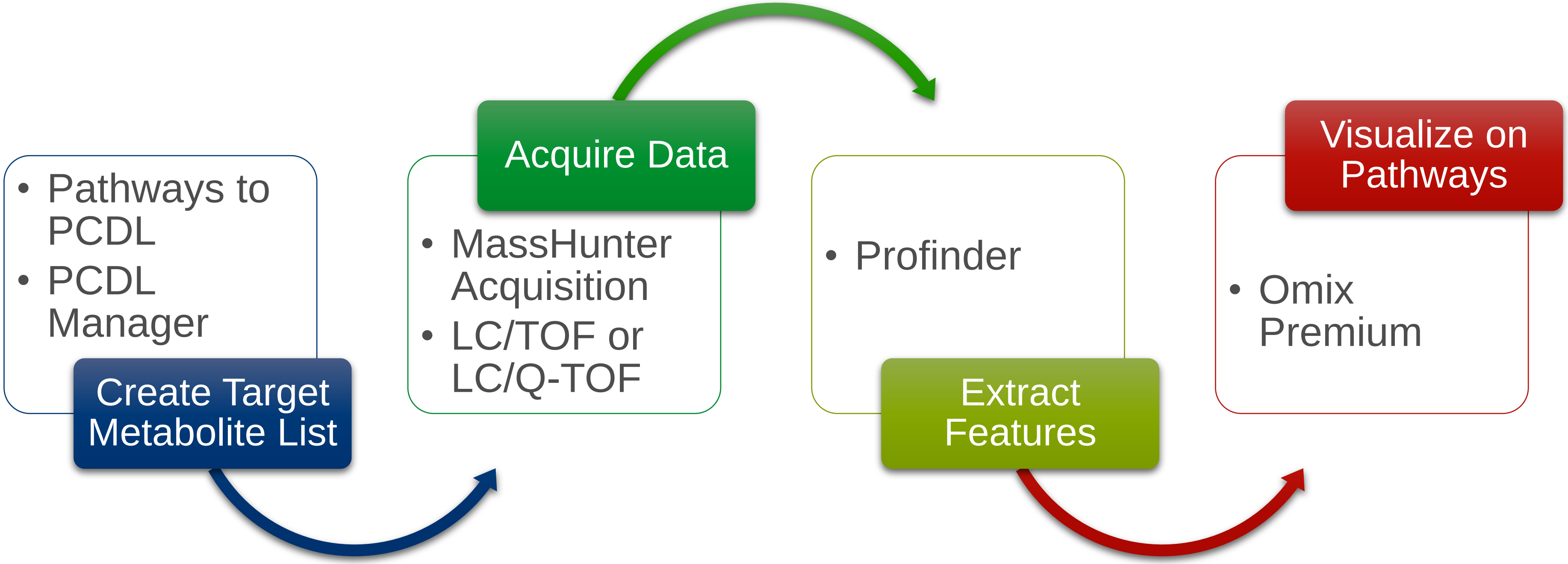
Correct for natural isotope abundance

Manually visualize on pathways

- Tedious, error-prone and time-consuming
- Limits the number of compounds analyzed

Batch Qualitative Flux Analysis Workflow

Agilent's VistaFlux Workflow



Create Target Metabolite List

- Pathways to PCDL
- PCDL Manager

Acquire Data

- MassHunter Acquisition
- LC/TOF or LC/Q-TOF

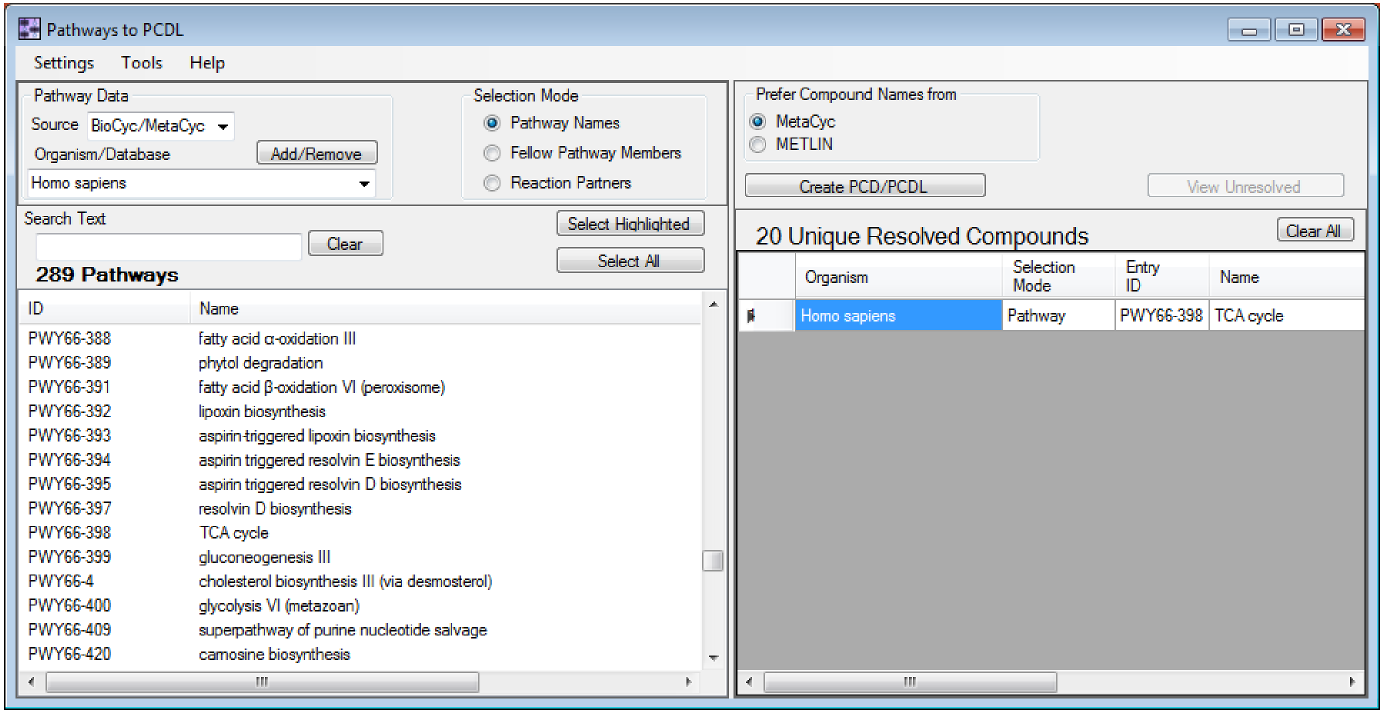
Extract Features

- Profinder

Visualize on Pathways

- Omix Premium

Target List Creation Based on Pathways of Interest



Pathway Data

Source: BioCyc/MetaCyc

Organism/Database: Homo sapiens

Selection Mode

● Pathway Names
○ Fellow Pathway Members
○ Reaction Partners

Prefer Compound Names from

● MetaCyc
○ METLIN

Search Text

289 Pathways

ID	Name
PWY66-388	fatty acid α-oxidation III
PWY66-389	phytol degradation
PWY66-391	fatty acid β-oxidation VI (peroxisome)
PWY66-392	lipoxin biosynthesis
PWY66-393	aspirin-triggered lipoxin biosynthesis
PWY66-394	aspirin triggered resolvin E biosynthesis
PWY66-395	aspirin triggered resolvin D biosynthesis
PWY66-397	resolvin D biosynthesis
PWY66-398	TCA cycle
PWY66-399	gluconeogenesis III
PWY66-4	cholesterol biosynthesis III (via desmosterol)
PWY66-400	glycolysis VI (metazoan)
PWY66-409	superpathway of purine nucleotide salvage
PWY66-420	carnosine biosynthesis

20 Unique Resolved Compounds

Organism	Selection Mode	Entry ID	Name
Homo sapiens	Pathway	PWY66-398	TCA cycle

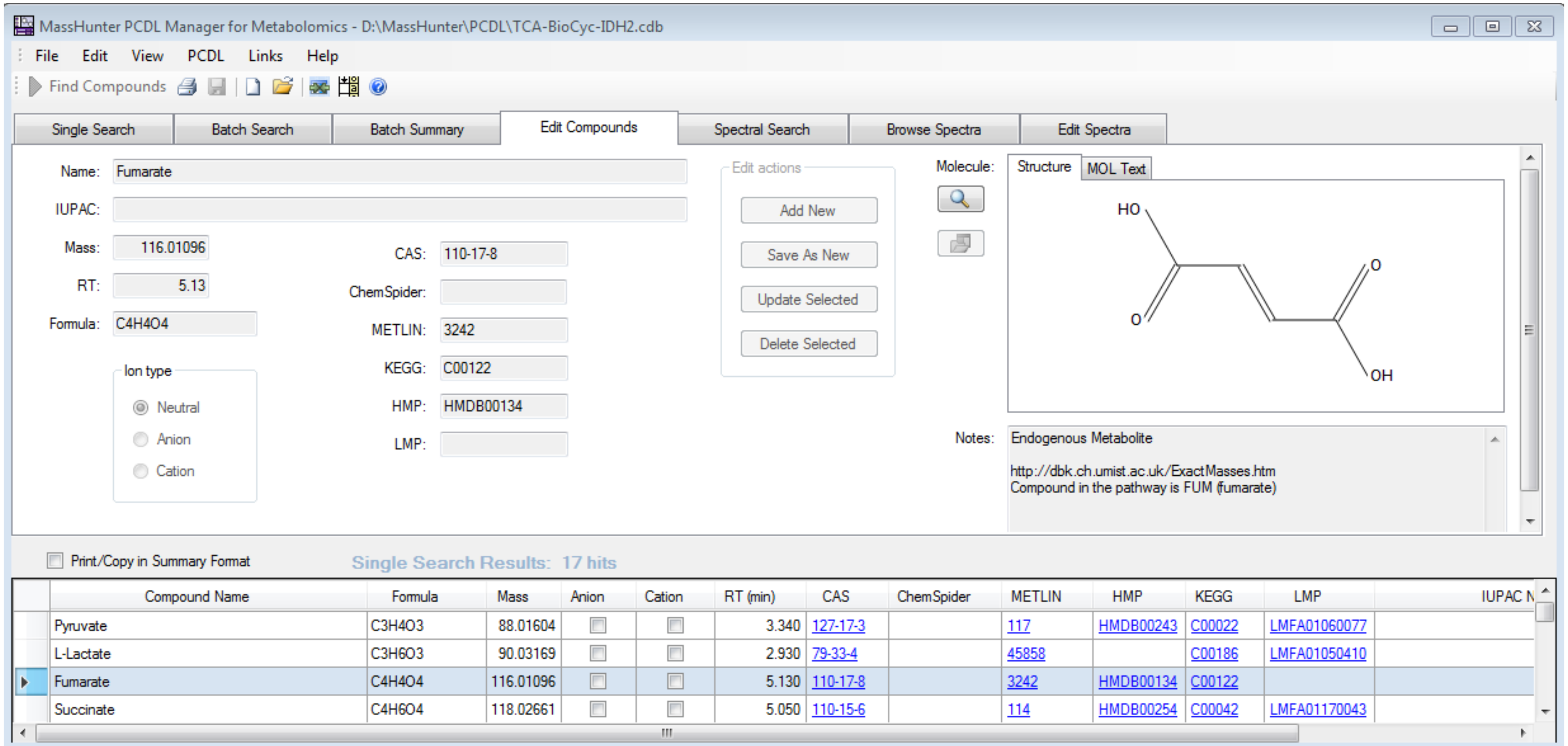
Select pathway(s) of interest in Agilent’s Pathways to PCDL software

- Supports BioCyc, KEGG and WikiPathways

Generate a metabolite list as a database (PCDL)

- Includes all metabolites (eliminates duplicates)
- Includes name, structure, empirical formula and identifiers

Edit Database to Create Target Metabolite List



MassHunter PCDL Manager for Metabolomics - D:\MassHunter\PCDL\TCA-BioCyc-IDH2.cdb

Name: Fumarate
IUPAC:
Mass: 116.01096
RT: 5.13
Formula: C4H4O4

Ion type
● Neutral
○ Anion
○ Cation

CAS: 110-17-8
ChemSpider:
METLIN: 3242
KEGG: C00122
HMP: HMDB00134
LMP:

Notes: Endogenous Metabolite

http://dbk.ch.umist.ac.uk/ExactMasses.htm
Compound in the pathway is FUM (fumarate)

☐ Print/Copy in Summary Format

Single Search Results: 17 hits

Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	METLIN	HMP	KEGG	LMP	IUPAC N
Pyruvate	C3H4O3	88.01604	☐	☐	3.340	127-17-3		117	HMDB00243	C00022	LMFA01060077	
L-Lactate	C3H6O3	90.03169	☐	☐	2.930	79-33-4		45858		C00186	LMFA01050410	
Fumarate	C4H4O4	116.01096	☐	☐	5.130	110-17-8		3242	HMDB00134	C00122		
Succinate	C4H6O4	118.02661	☐	☐	5.050	110-15-6		114	HMDB00254	C00042	LMFA01170043	

Use Agilent’s PCDL Manager to curate database:

- Include only detectable metabolites
- Add retention time to metabolite entries

Acquire LC/MS Data for Targeted Metabolites

LC/MS Analysis Considerations

LC

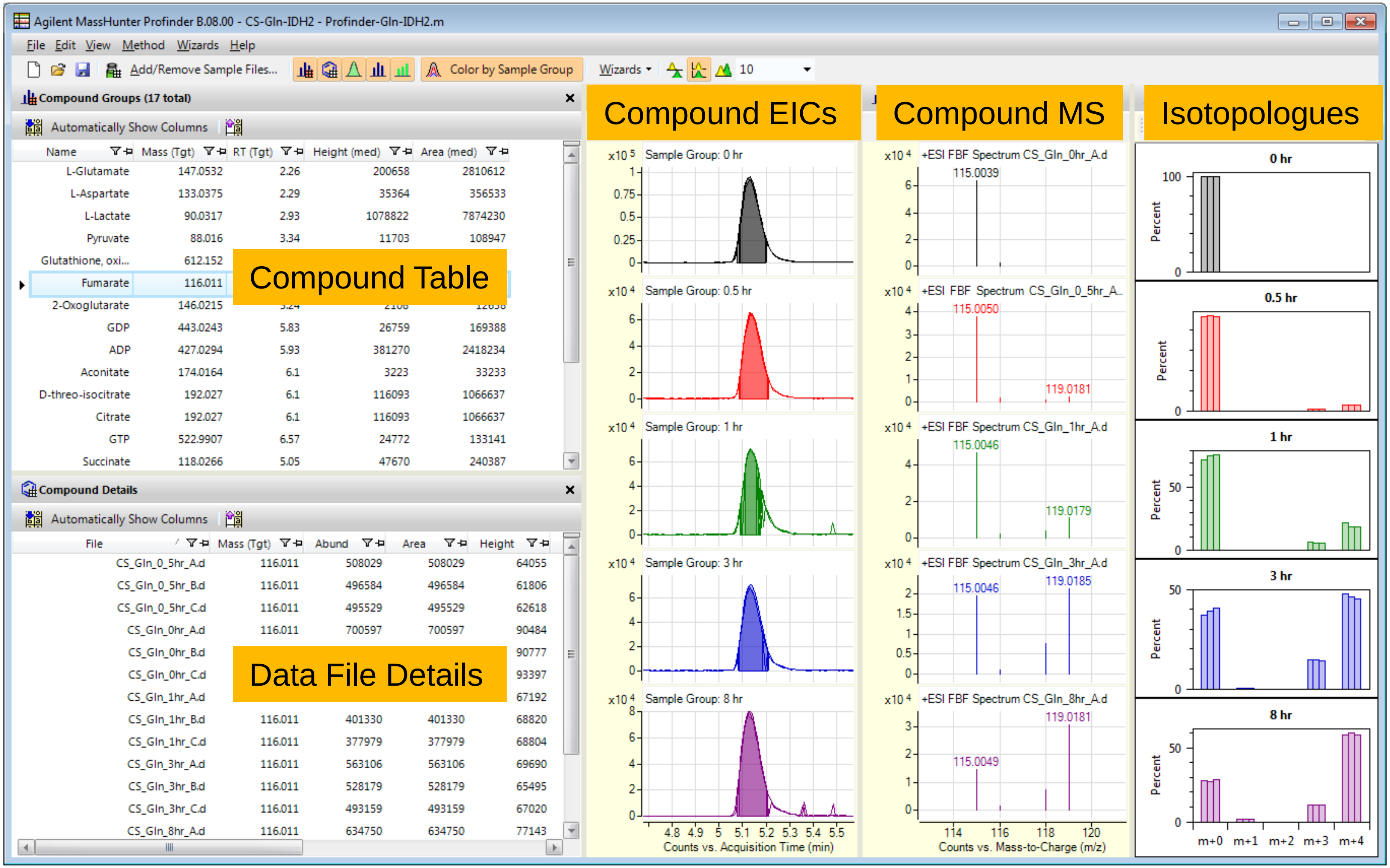
- Separate isomeric metabolites to eliminate isotopologue interference
- Separate metabolites with overlapping isotopologues

MS

- High mass accuracy and resolution to confidently track isotopologues
- Good isotopic fidelity to provide accurate ratios to track incorporation levels
- Wide dynamic range for detection of low and high intensity isotopologues in a single experiment

Targeted Data Mining

MassHunter Profinder Batch Isotopologue Extraction



Compound Groups (17 total)

Automatically Show Columns

Name	Mass (Tgt)	RT (Tgt)	Height (med)	Area (med)
L-Glutamate	147.0532	2.26	200658	2810612
L-Aspartate	133.0375	2.29	35364	356533
L-Lactate	90.0317	2.93	1078822	7874230
Pyruvate	88.016	3.34	11703	108947
Glutathione, oxi...	612.152			
Fumarate	116.011			
2-Oxoglutarate	146.0215	[illegible]	[illegible]	[illegible]
GDP	443.0243	5.83	26759	169388
ADP	427.0294	5.93	381270	2418234
Aconitate	174.0164	6.1	3223	33233
D-threo-isocitrate	192.027	6.1	116093	1066637
Citrate	192.027	6.1	116093	1066637
GTP	522.9907	6.57	24772	133141
Succinate	118.0266	5.05	47670	240387

Compound Table

Compound Details

Automatically Show Columns

File	Mass (Tgt)	Abund	Area	Height
CS_Gln_0_5hr_A.d	116.011	508029	508029	64055
CS_Gln_0_5hr_B.d	116.011	496584	496584	61806
CS_Gln_0_5hr_C.d	116.011	495529	495529	62618
CS_Gln_0hr_A.d	116.011	700597	700597	90484
CS_Gln_0hr_B.d				90777
CS_Gln_0hr_C.d				93397
CS_Gln_1hr_A.d				67192
CS_Gln_1hr_B.d	116.011	401330	401330	68820
CS_Gln_1hr_C.d	116.011	377979	377979	68804
CS_Gln_3hr_A.d	116.011	563106	563106	69690
CS_Gln_3hr_B.d	116.011	528179	528179	65495
CS_Gln_3hr_C.d	116.011	493159	493159	67020
CS_Gln_8hr_A.d	116.011	634750	634750	77143

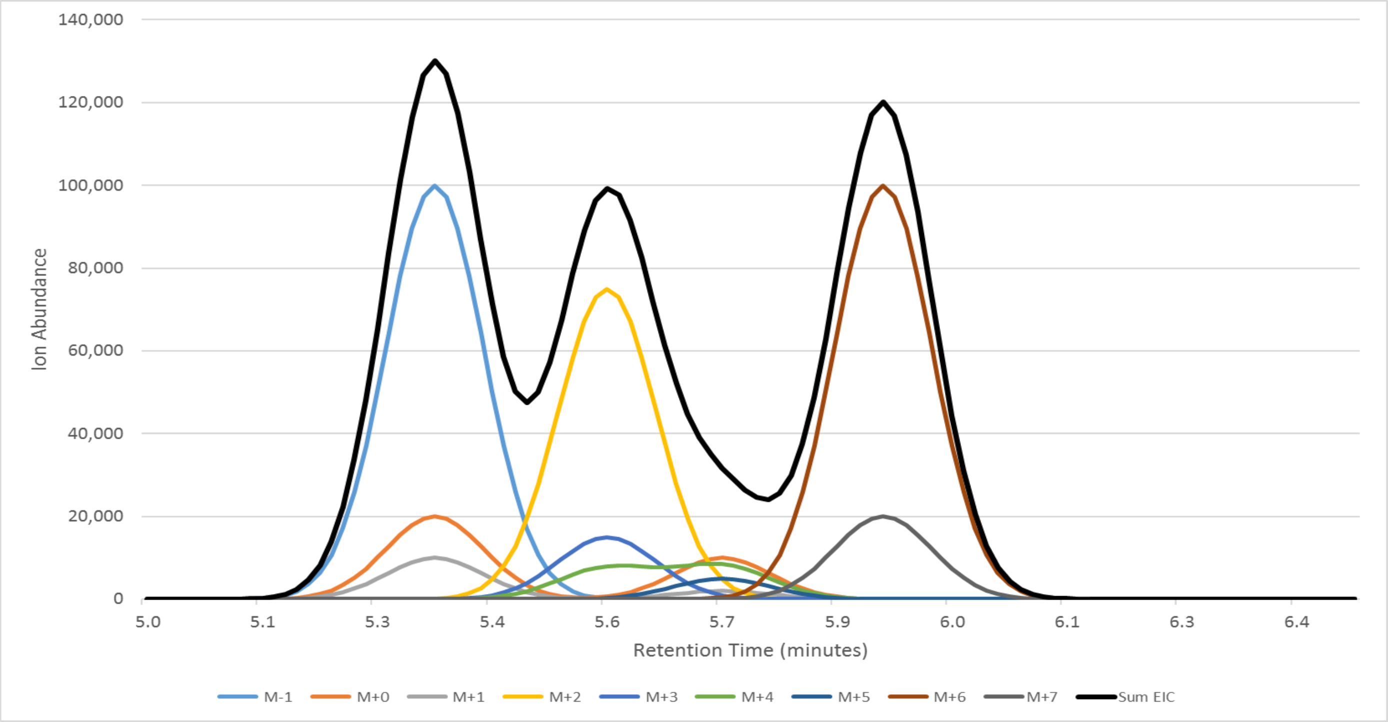
Data File Details

Multi-stage Isotopologue Extraction Algorithm

First Stage

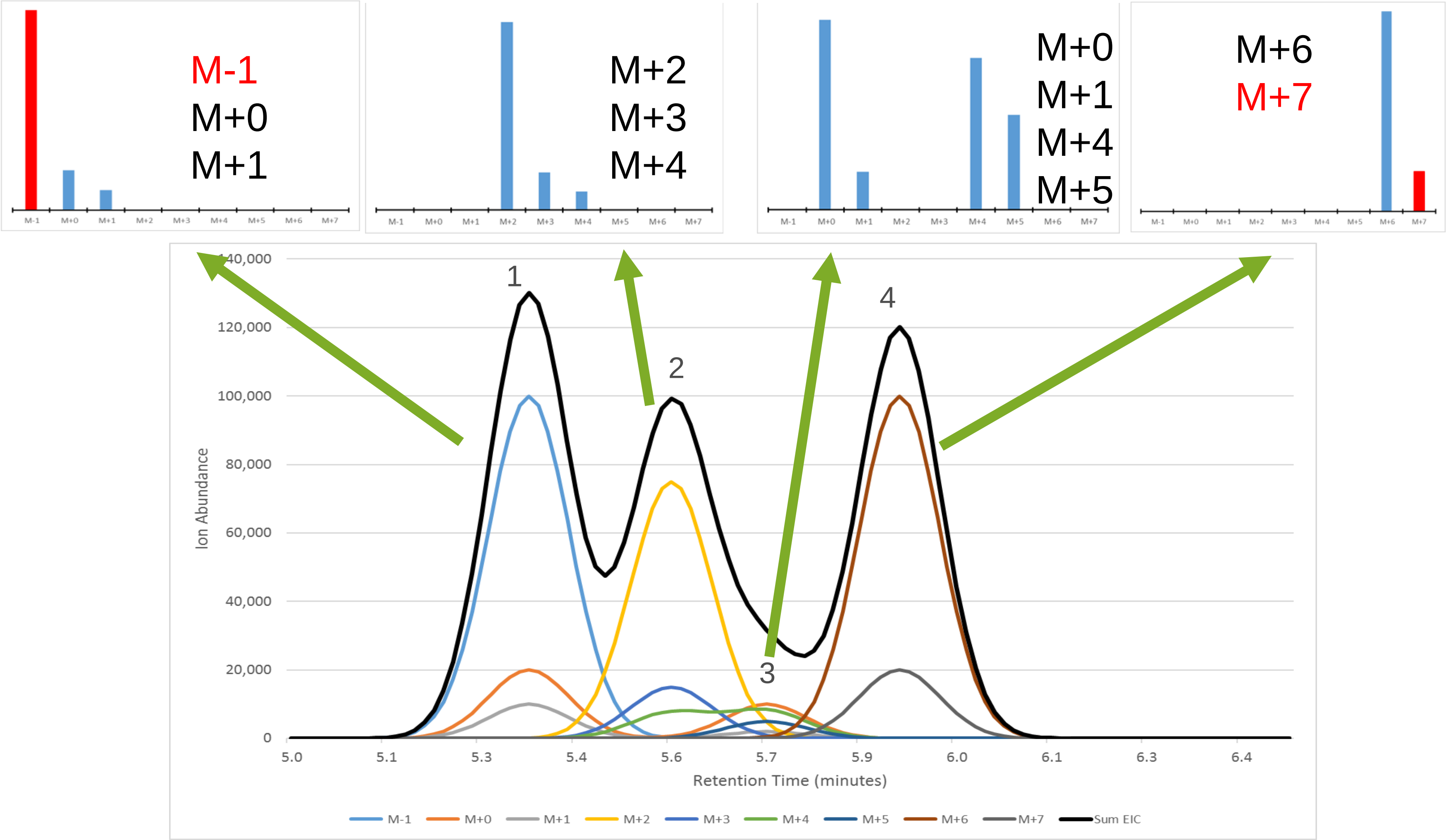
Extracts chromatogram for each possible isotopologue

- Uses retention time and formula specified in database
- Uses a wide tolerance for retention time and *m/z* (“survey scan”)



C_5 Compound Isotopologue Extraction Example

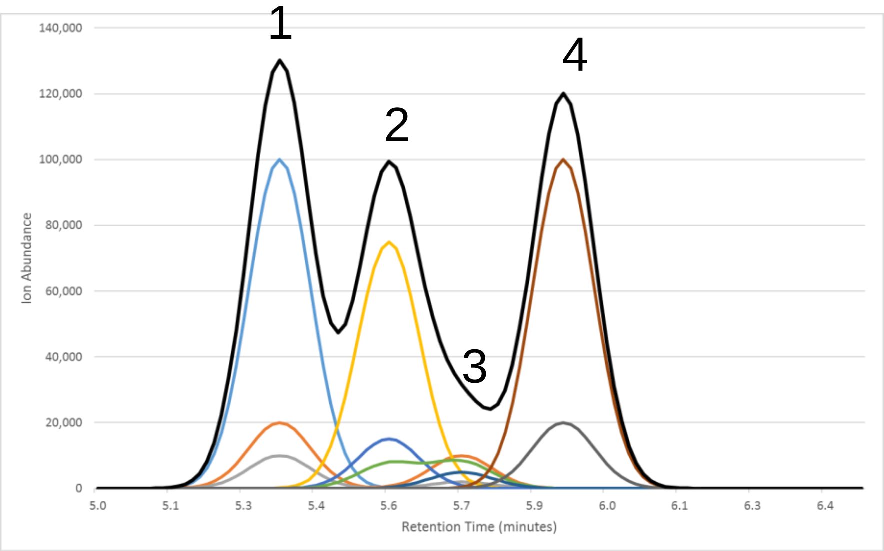
First Stage Extraction – Wide m/z and RT Windows



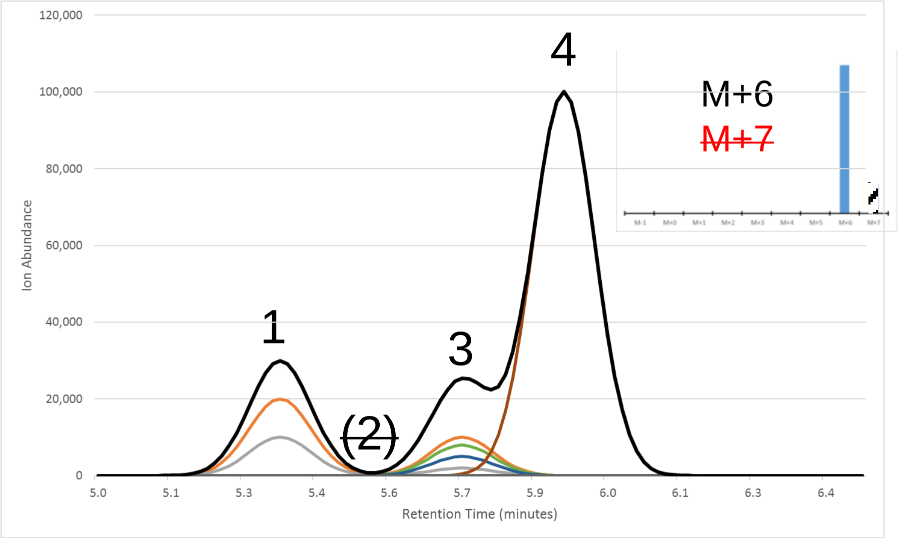
Multi-stage Isotopologue Extraction Algorithm

Second Stage Extraction – Refinement

Wide *m/z* & RT Tolerance



Narrow *m/z* & RT Tolerance



- *m/z* and RT tolerances are dynamically narrowed
- Peaks are scored by # of ions, relative abundance and distance from target RT
- Peak 2 is now completely disqualified by the new *m/z* tolerance thresholds
- The m+7 isotopologue from peak 4 is also rejected by *m/z* tolerance

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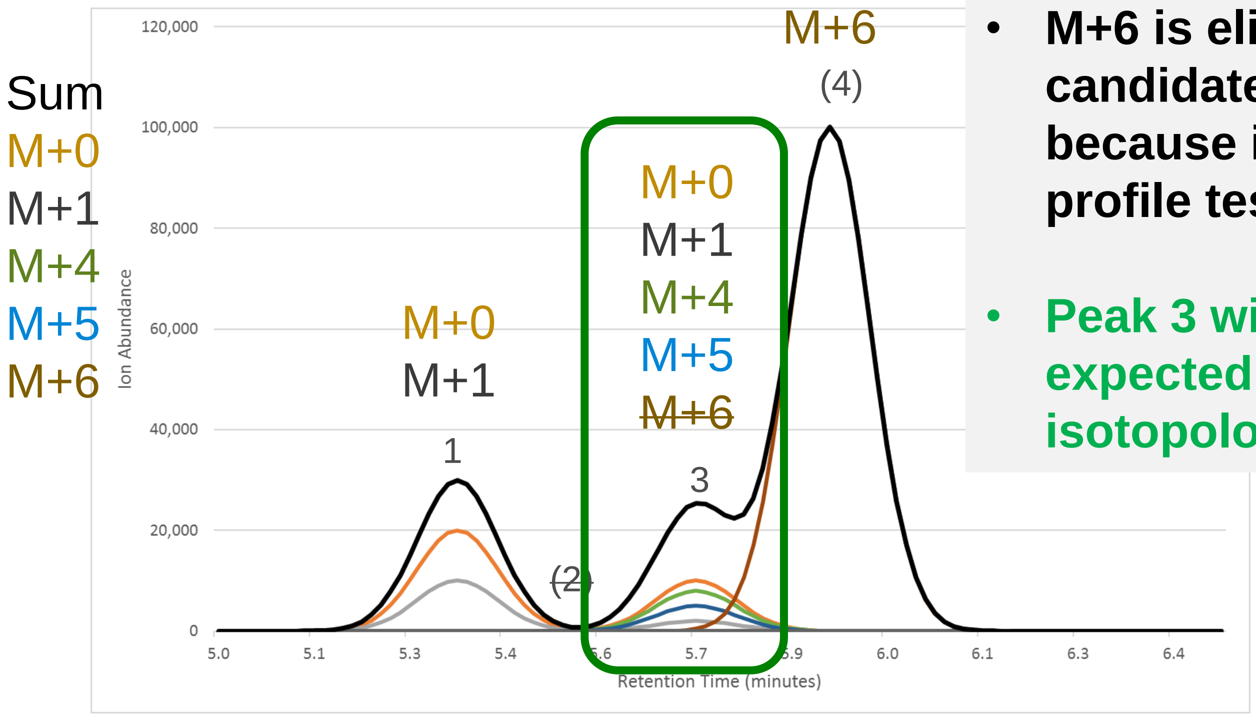
Isotopologue Extraction Example

Second Stage Extraction – Narrow m/z and RT Windows

Qualified Ions After 2nd Stage



- **M+6 is eliminated as a candidate ion for peak 3 because it fails the elution profile test.**

- **Peak 3 wins – closest to expected RT and has the most isotopologue peaks**

Isotopologue Mining Algorithm

Natural Isotope Abundance Correction

Theoretical natural isotope abundances

Isotopologue abundance

Mined raw values

$$\begin{bmatrix} 1.0000 & 0 & 0 & 0 \\ 0.0456 & 1.0000 & 0 & 0 \\ 0.0497 & 0.0348 & 1.0000 & 0 \\ 0.0019 & 0.0493 & 0.0239 & 1.0000 \end{bmatrix} \cdot \begin{bmatrix} b_0 \\ b_1 \\ \dots \\ b_n \end{bmatrix} = \begin{array}{cc} \begin{bmatrix} a_0 \\ a_1 \\ \dots \\ a_n \end{bmatrix} & \begin{array}{c} m+0 \\ m+1 \\ \dots \\ m+n \end{array} \end{array}$$

Solve for this!

Isotopologue Mining Algorithm

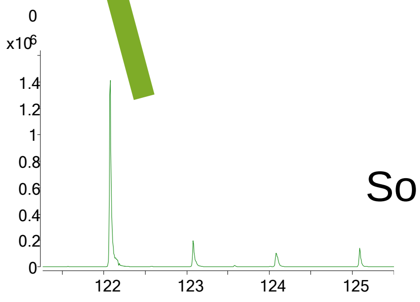
Natural Isotope Abundance Correction

Theoretical natural isotope abundances

Mined raw values

Isotopologue abundance

$$\begin{bmatrix} 1.0000 & 0 & 0 & 0 \\ 0.0456 & 1.0000 & 0 & 0 \\ 0.0497 & 0.0348 & 1.0000 & 0 \\ 0.0019 & 0.0493 & 0.0230 & 1.0000 \end{bmatrix}^{-1} \cdot \begin{bmatrix} a_0 \\ a_1 \\ \dots \\ a_n \end{bmatrix} \begin{array}{l} m+0 \\ m+1 \\ \dots \\ m+n \end{array} = \begin{bmatrix} b_0 \\ b_1 \\ \dots \\ b_n \end{bmatrix}$$



Solve for this!

- Since there is some error in the observed abundances, this is not a simple matrix product
- We thus use a chi-squared minimization to solve for the isotopologue abundance

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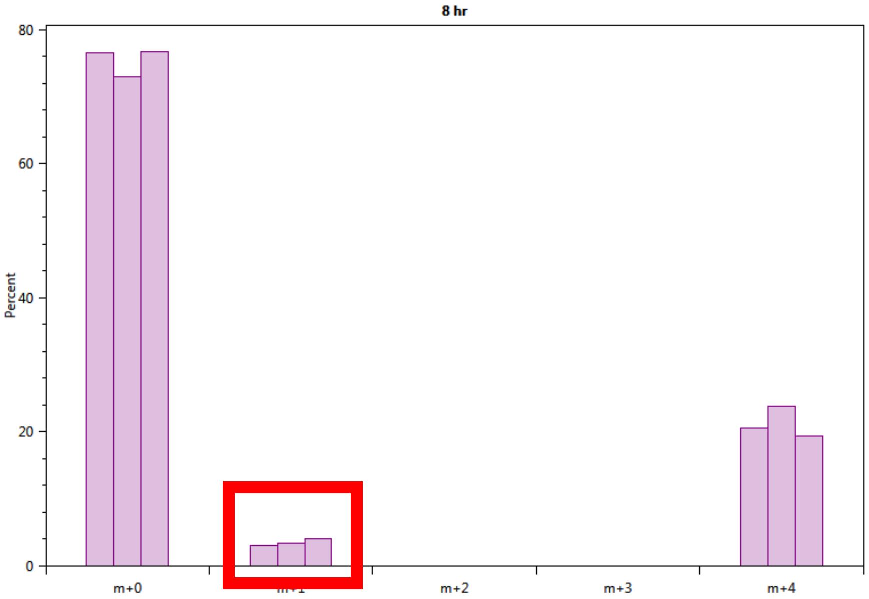
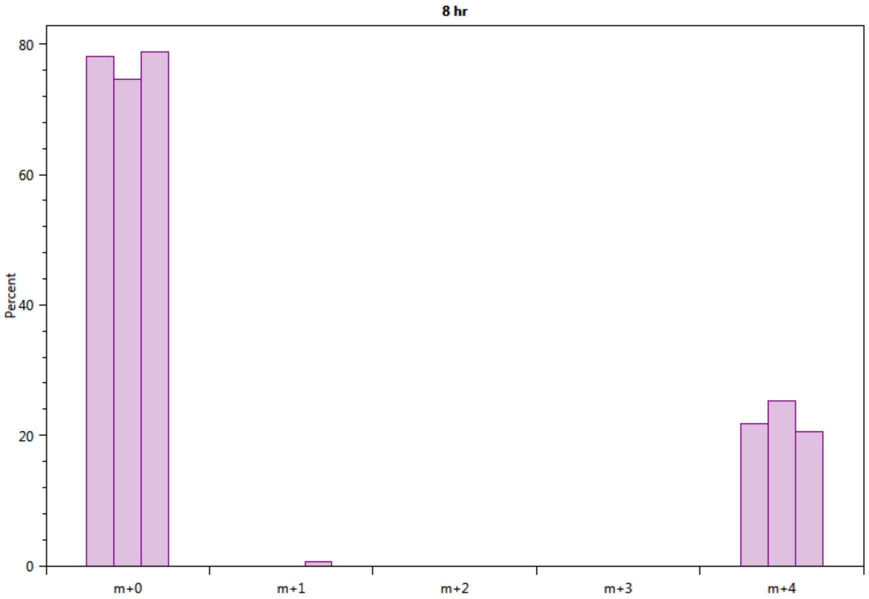
Natural Isotope Abundance Correction

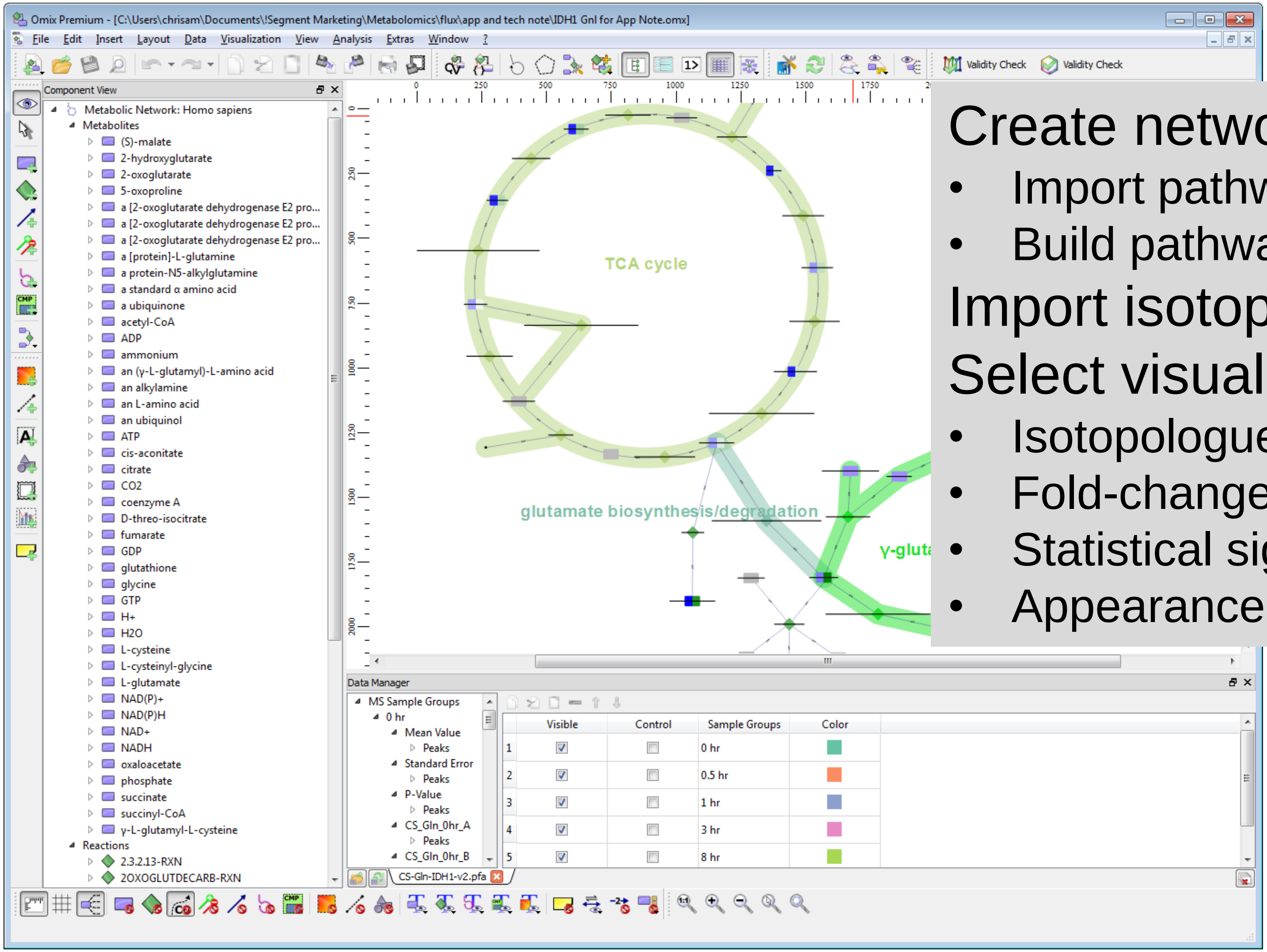
Succinic Acid (raw)



Succinic Acid (corrected)



Visualize Isotopologue Results on Pathways



	Visible	Control	Sample Groups	Color
1	☑	☐	0 hr	🟩
2	☑	☐	0.5 hr	🟧
3	☑	☐	1 hr	🟦
4	☑	☐	3 hr	🟪
5	☑	☐	8 hr	🟩

Create network

- Import pathway(s) or
- Build pathway manually

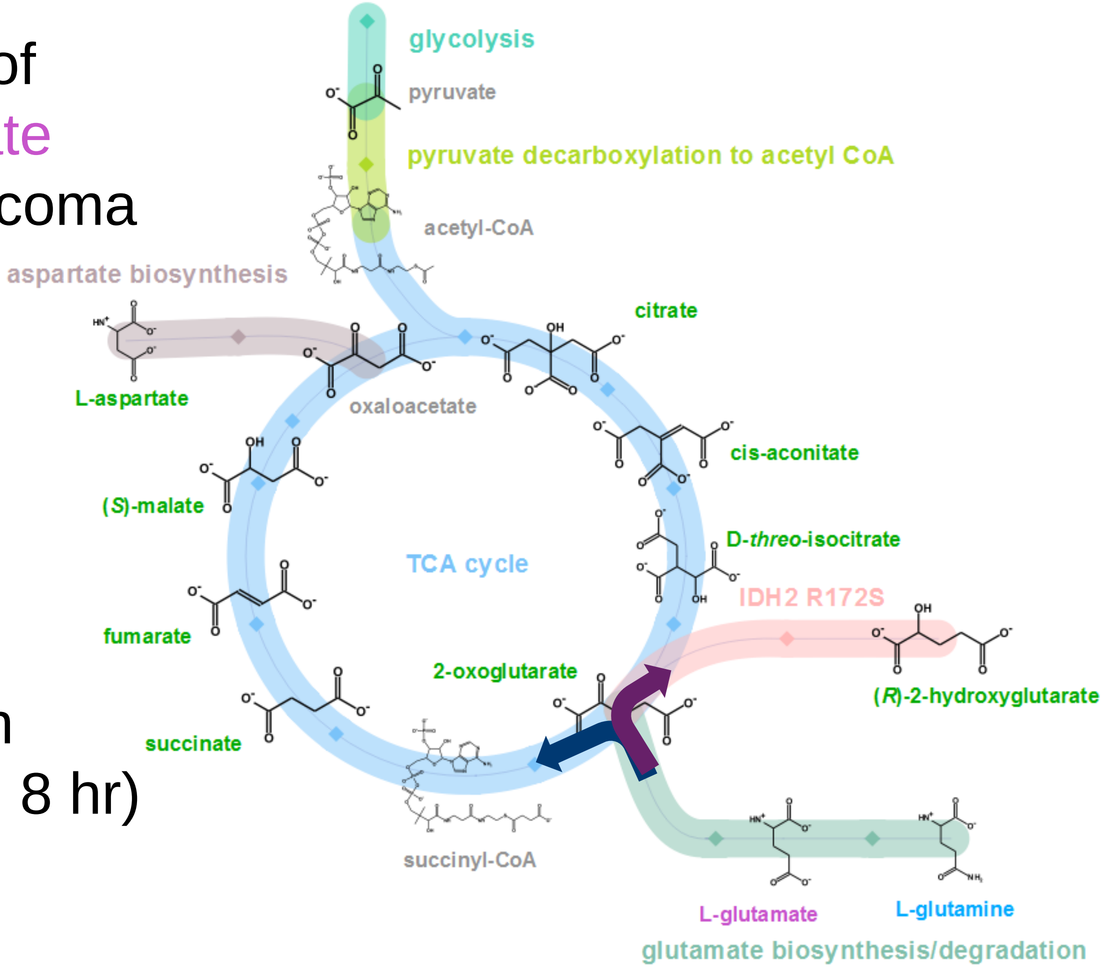
Import isotopologue results

Select visualization(s)

- Isotopologue incorporation
- Fold-change
- Statistical significance
- Appearance of network

Qualitative Flux Analysis of a Cancer Cell Line with Enzyme Mutation (IDH2)

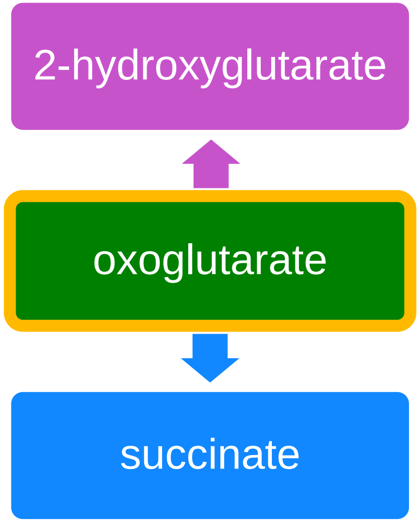
- Goal: Understand impact of IDH2 mutation on glutamate metabolism in chondrosarcoma cell line
- Tracer experiment with U-13C-Glutamine (Gln)
- Monitoring metabolites labeled in green
- Triplicate analysis for each time point (0, 0.5, 1, 3 and 8 hr)



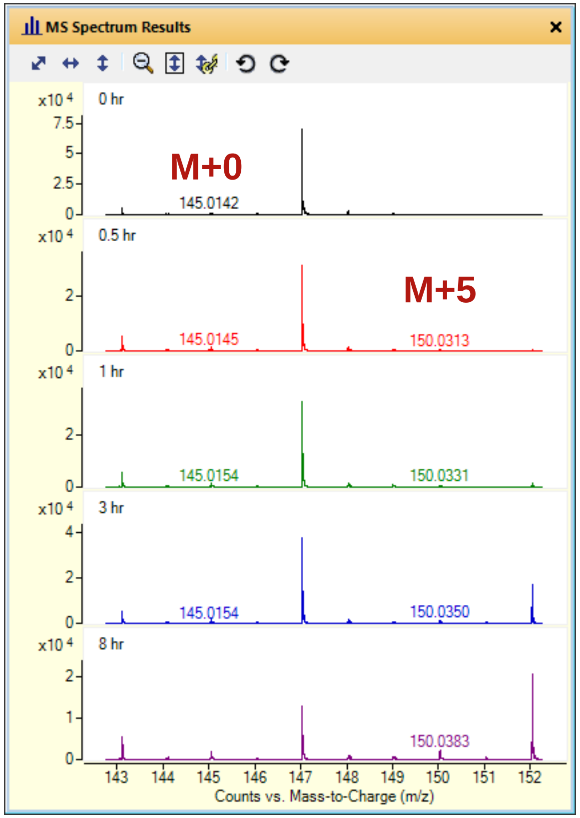
Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Qualitative Flux Results: Isotopologue Extraction

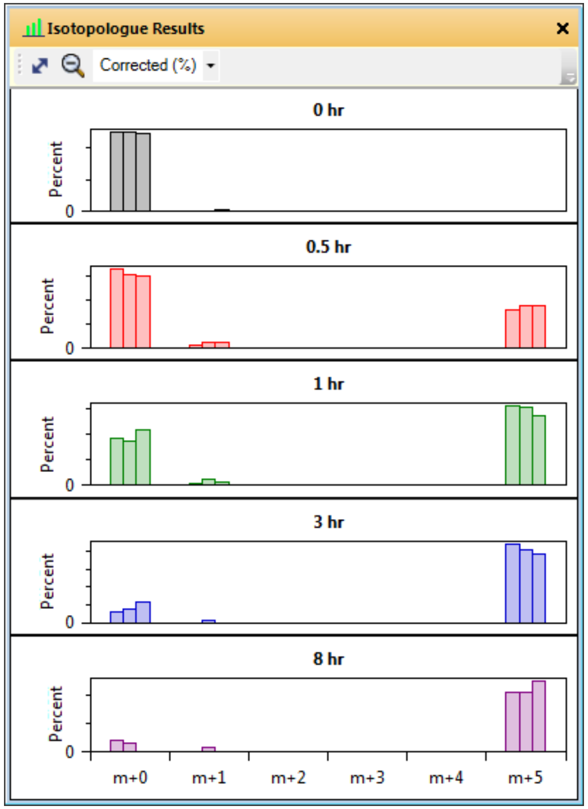
Oxoglutarate



MS Spectra



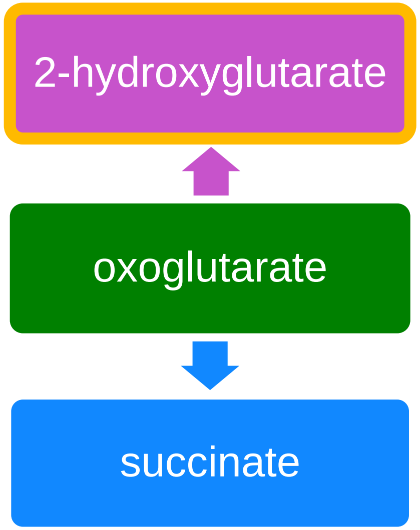
Isotopologue Results



Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Qualitative Flux Results: Isotopologue Extraction

2-Hydroxyglutarate

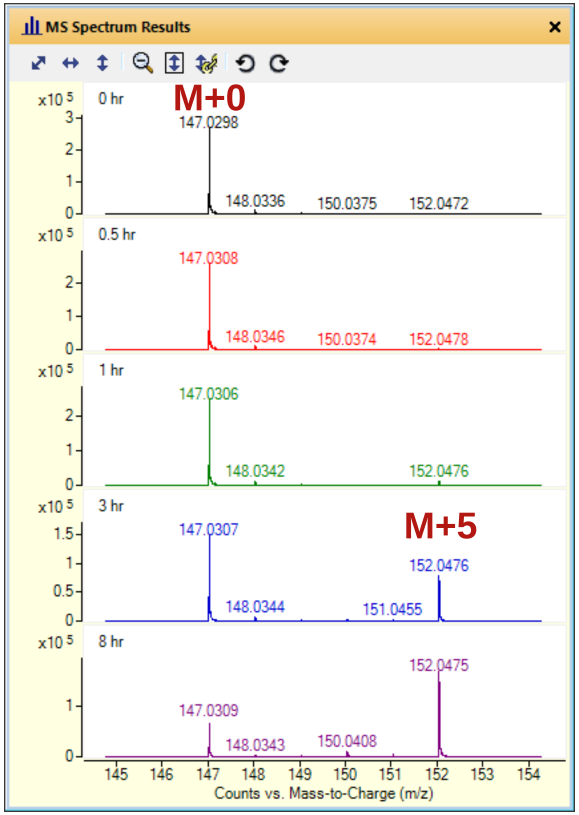


2-hydroxyglutarate

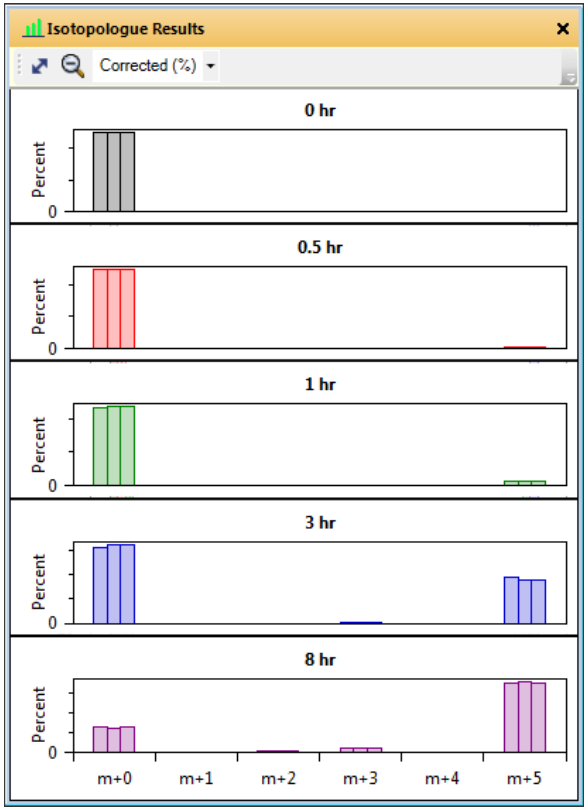
oxoglutarate

succinate

MS Spectra



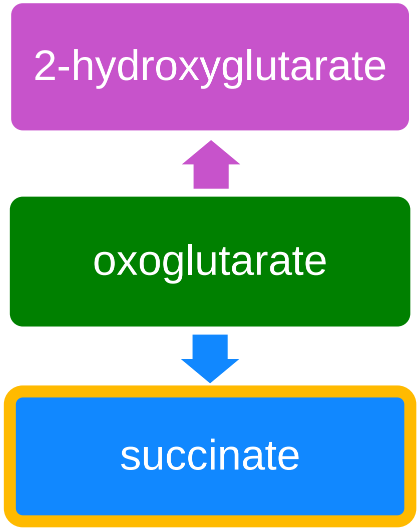
Isotopologue Results



Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Qualitative Flux Results: Isotopologue Extraction

Succinate

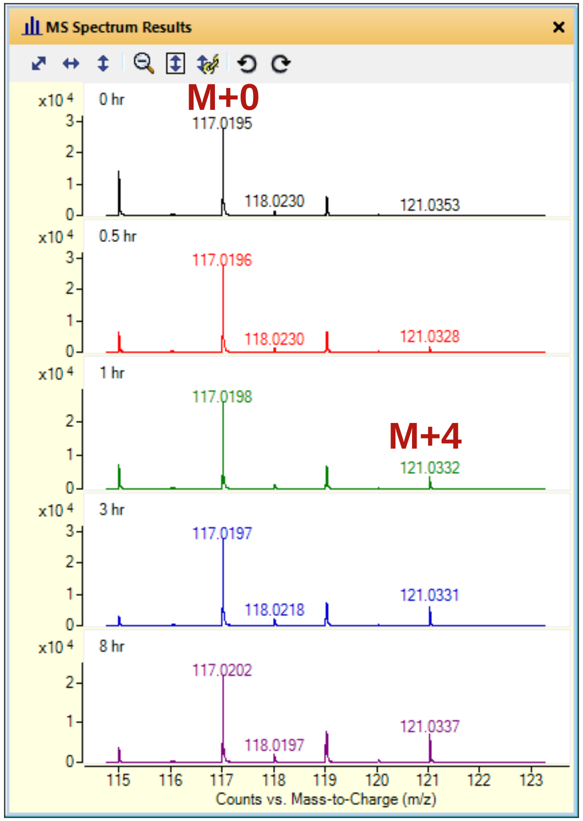


2-hydroxyglutarate

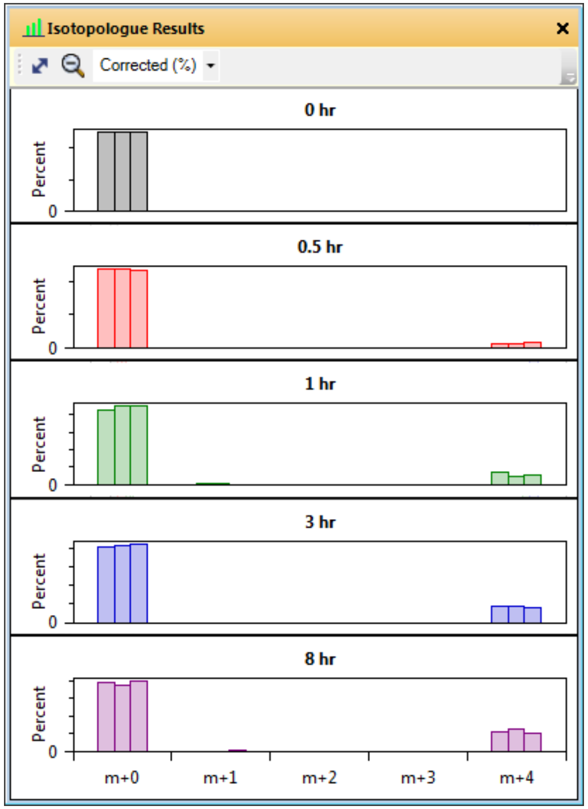
oxoglutarate

succinate

MS Spectra



Isotopologue Results

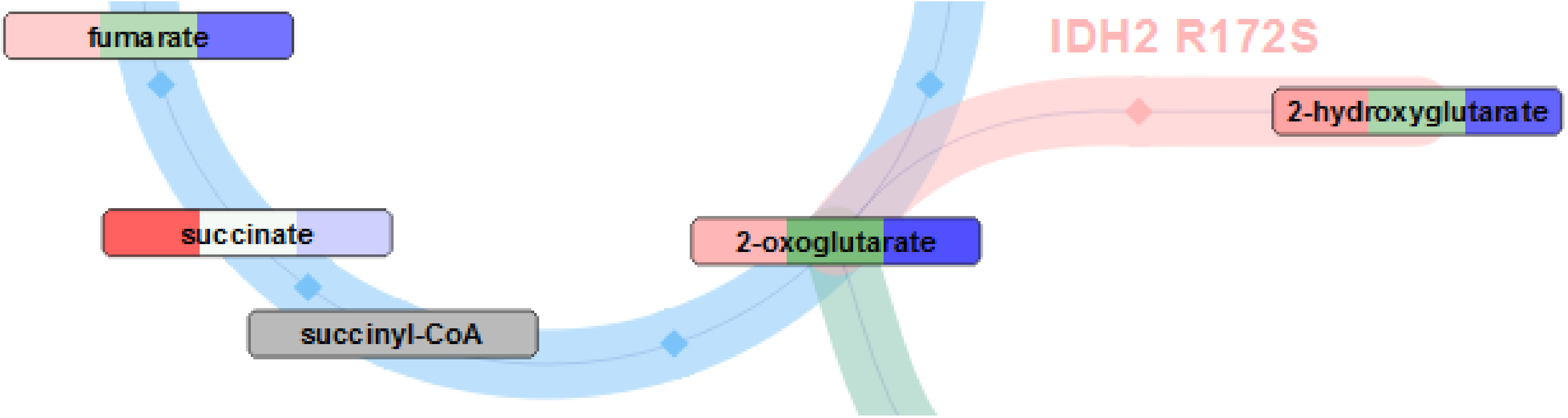


Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Visualizing Qualitative Flux Results

Quick Summary of Isotopologue Results on Network

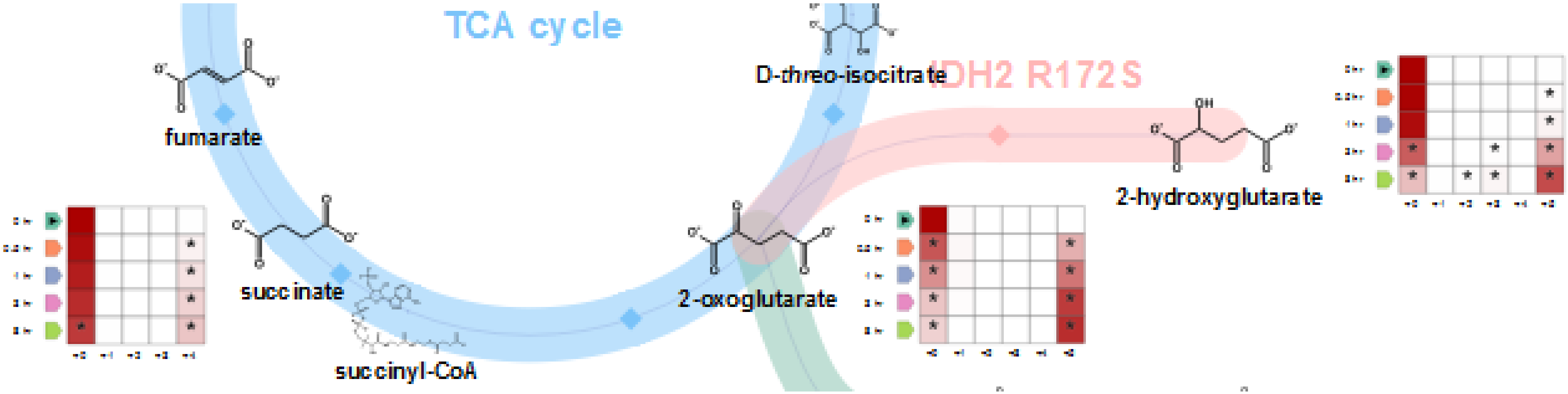
- Metabolite node coloring gives quick summary of results on network
- Fold-change (left), label incorporation (middle) and fractional labeling (right) are shown here
- 2-HG is produced from glutamine by the mutant IDH2 enzyme via oxoglutarate as shown by label incorporation and fractional labeling



Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Visualizing Qualitative Flux Results

Quilt Plot Display of Isotopologue Results

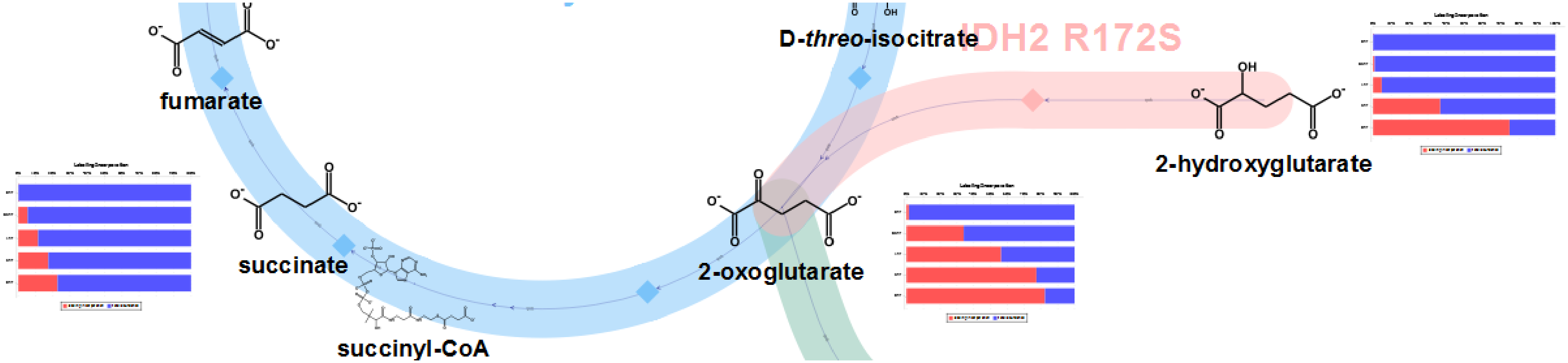


- Quilt plot displays all time points and isotopologue results
- Statistically significant (*) label incorporation in M+5 isotopologue of 2-HG at 3 and 8 hours

Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Visualizing Qualitative Flux Results

Label Incorporation Display of Isotopologue Results

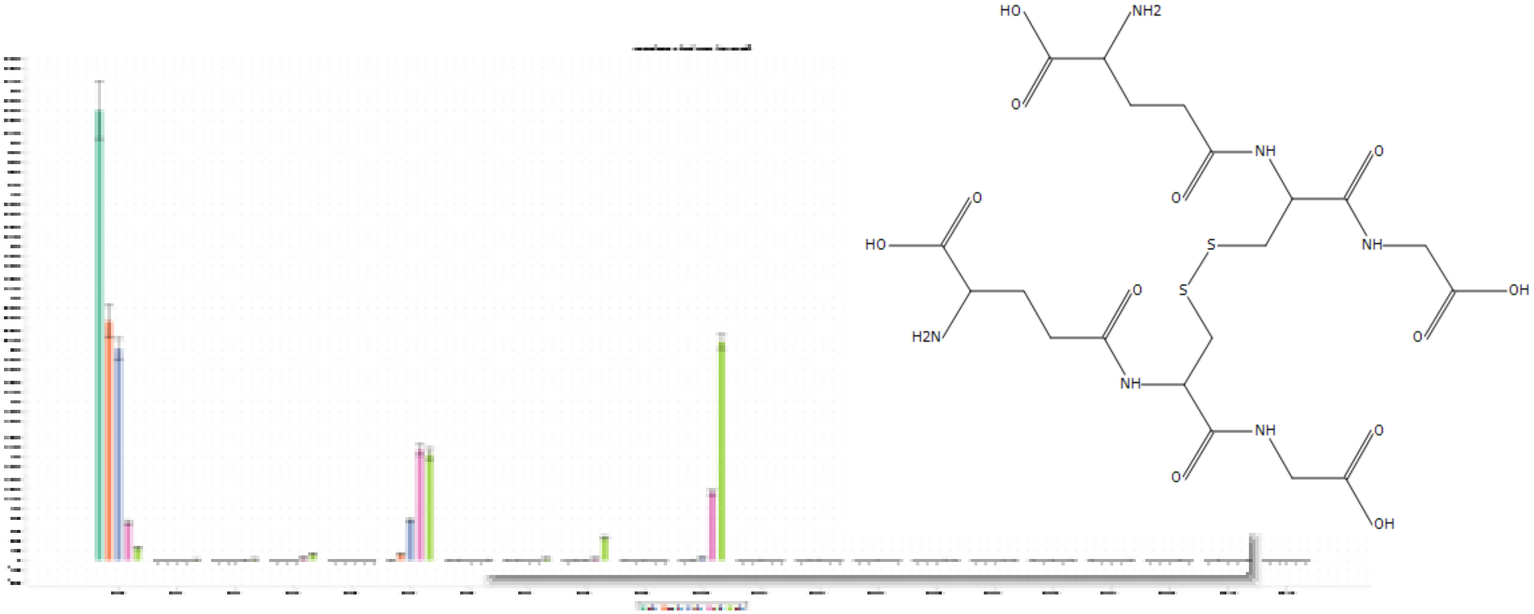


- Percent unlabeled and labeled for each metabolite
- Significant label incorporation into M+5 isotopologue of 2-HG at 3 and 8 hours

Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Qualitative Flux Results

Isotopologue Plot of Oxidized Glutathione

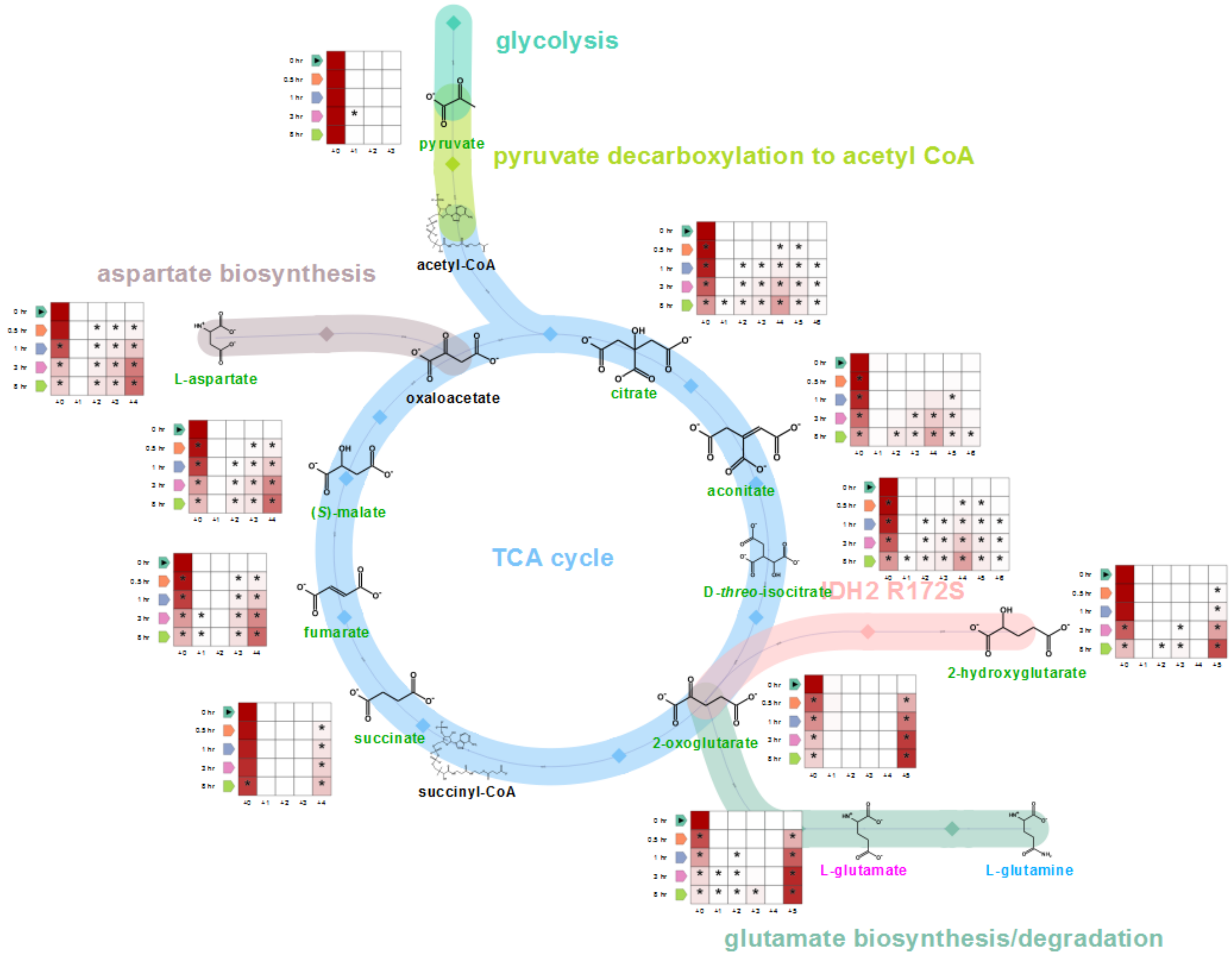


- Shows cellular glutathione pool becomes labeled with first one and then two glutamine-derived 5 carbon building blocks.
- Due to large cellular pool, replacement most apparent at **3** and **8** hours

Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Qualitative Flux Analysis Experiment

Comprehensive View of Isotopologue Results on Network



Courtesy of Justin Cross, PhD., Donald B. and Catherine C. Marron Cancer Metabolism Center, Memorial Sloan Kettering Cancer Center

Summary

- This experiment confirms glutamine is the major substrate used to produce 2-HG in IDH2-mutant CS-1 cells
- For 2-HG, the presence of the M+5 isotopologue instead of the M+2 or M+4 isotopologue demonstrates the direction of the reaction
- The new Agilent VistaFlux workflow easily extracts isotopologue information and visualizes results on pathways
  - Manual EIC extraction for each isotopologue and natural isotope correction for **5** metabolites at all time points required **2 weeks**
  - The VistaFlux workflow performed the same analysis for **17** metabolites in about **2 minutes**!

Acknowledgements

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Justin Cross

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Weige Qin

Hardik Shah

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