



# $^{13}\text{C}$ -Glutamine Qualitative Flux Analysis of a Chondrosarcoma Cell Line Using Agilent VistaFlux

## Application Note

Metabolomics

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### Abstract

Interpretation of metabolomics data is often complicated by a lack of dynamic information since significant changes in flux through a pathway can occur without corresponding changes in metabolite abundances. Stable isotope tracing (qualitative flux analysis) has tremendous potential to help address these situations, and allow a deeper understanding of biological systems. In qualitative flux analysis, a stable isotope tracer (typically containing  $^{13}\text{C}$ ,  $^{15}\text{N}$ , or  $^2\text{H}$ ) is introduced into the biological system, and results in changes in the isotopic pattern (isotopologue distribution) of downstream metabolites. The approach is particularly useful in cell culture models that are amenable to the introduction of stable isotope tracers, and analysis of the resulting labeling pattern and kinetics of tracer incorporation provides insights into enzyme function, pathway dependence, and the effect of changes in gene expression or protein function.

Agilent MassHunter VistaFlux software is designed to be a comprehensive workflow that helps scientists perform qualitative flux analysis, and facilitates the processing and visualization of high-resolution accurate mass TOF and Q-TOF LC/MS data. Tools are provided for feature extraction, analysis of isotope incorporation and isotopologue abundance, natural abundance correction, statistical analysis, and data visualization on pathways.



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This application note demonstrates qualitative flux analysis in a chondrosarcoma cell line, using  $^{13}\text{C}_5$ -glutamine (U- $^{13}\text{C}$ -Gln) as the tracer. Data were acquired on an Agilent LC/TOF system, and MassHunter VistaFlux was used for data analysis and interpretation. The study 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.

## Introduction

Somatic IDH mutations have recently been described in a broad range of solid and hematologic tumors, including glioma, acute myeloid leukemia, and chondrosarcoma. Mutations in isocitrate dehydrogenase enzymes (IDH1/2) occur in the enzyme's active site, and confer a gain-of-function phenotype resulting in an increased ability to produce the metabolite 2-hydroxyglutarate (2-HG). Recent research has shown that 2-HG accumulates to high levels in IDH mutant cells due to saturation of the disposal pathway, whereupon 2-HG becomes a competitive inhibitor for a variety of  $\alpha$ -ketoglutarate ( $\alpha$ -KG) dependent dioxygenase enzymes including the TET family of DNA demethylases, Jumonji C family of histone demethylases, mRNA demethylases FTO and ALKBH5, and prolyl hydroxylase (PHD) enzymes that regulate hypoxia-inducible factors (HIFs)<sup>1</sup>. These changes promote a repressive chromatin environment that lock cells in an undifferentiated state.

Chondrosarcomas, a type of sarcoma arising from the chondrocytes that deposit cartilage, exhibit high incidences of IDH mutations. The availability of tumor-derived cell lines has made chondrosarcoma a useful model system for understanding the consequences of IDH mutations. An important component of this has been the use of qualitative flux analysis to show that 2-HG produced by the mutant enzyme is made from glutamine through  $\alpha$ -KG and the TCA cycle.

## Experimental

### Cell culture and reagents

Human chondrosarcoma CS-1 cell line<sup>2</sup>, was maintained in DMEM containing 10 % FBS, 25 mM glucose, 4 mM L-glutamine, 100 units/mL penicillin, and 100 ug/mL streptomycin under 5 %  $\text{CO}_2$  in a 37 °C incubator. For metabolic tracing experiments, 300,000 cells/well were plated in standard 6-well culture plates 2 days before the start of the labeling period, and triplicate wells were prepared for each time point. The tracer-containing medium was DMEM without glutamine, supplemented with 10 % dialyzed FBS and 4 mM [U- $^{13}\text{C}$ ]-L-glutamine (Isotec 605-166, Sigma). At the start of the labeling period, cells were washed once with PBS, and transferred to tracer-containing medium for 0.5, 1, 3, and 8 hours prior to harvest. Since medium changes can also perturb metabolite pools, the control (unlabeled) cells were harvested 8 hours after changing into DMEM containing 10 % dialyzed FBS and 4 mM L-glutamine (without isotopic enrichment), as the control cells were an exact match for the 8 hours U- $^{13}\text{C}$ -Gln time point.

### Metabolite extraction

Culture medium was gently aspirated, and cells were extracted in 1 mL/well 80 % aqueous methanol (v/v). Plates were incubated at -80 °C for >1 hour to aid protein precipitation, then scraped. The extract was transferred to microcentrifuge tubes for clarification by centrifugation at 21,000 x g for 15 minutes at 4 °C. Then, the extract was transferred to clean tubes, and evaporated to dryness in a vacuum concentrator (Genevac).

Note that since changing the cell culture medium and washes with PBS can perturb rapidly-exchanging metabolite pools, for this qualitative flux experiment, cells were only washed with PBS prior to addition of the stable isotope-containing medium. At harvest, the extraction solvent was added directly after aspirating the medium without additional washing to best preserve

levels of intracellular metabolites and the associated labeling patterns. However, if such a procedure is followed, care must be taken when interpreting results for metabolites also present at high levels in the culture medium, since this approach will result in a higher degree of residual medium contamination in the cell extracts than if a PBS wash had been included immediately prior to harvesting the cells.

### LC/MS analysis

Dried extracts were resuspended in 120  $\mu\text{L}$  of water, and incubated on ice for 20 minutes, vortexing every 5 minutes. Extracts were then centrifuged at 21,000 x g for 15 minutes at 4 °C to remove any residual particulate material. A 100  $\mu\text{L}$  volume was transferred to a reduced-volume autosampler vial, and 10  $\mu\text{L}$  was removed from every sample to assemble a pooled QC 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
- Agilent 1290 Infinity thermostatted column compartment
- Agilent 1290 Infinity HTS autosampler

An Agilent 1260 Infinity isocratic pump with a 100:1 flow 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.

The analytical method was designed for the metabolites of interest in this experiment. 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 was selected for best detection of the target compounds.

### Data analysis

Using Agilent MassHunter Pathways to PCDL software, an Agilent Personal Compound Database and Library file (.cdb) was created for the tricarboxylic acid (TCA) cycle drawn from BioCyc. In addition, lactate, pyruvate, aspartate, glutamate, oxidized glutathione, and 2-HG were appended to the custom PCDL using Agilent METLIN PCDL as the source. This was edited in Agilent PCDL Manager software to remove metabolites not measured by this analytical method, and add retention times for each compound. The PCDL file was then used as a target list for batch isotopologue extraction in Agilent MassHunter Profinder software (Table 2). The Profinder batch results, corrected for  $^{13}\text{C}$  natural abundance, were exported to Omix Premium as a Profinder archive file (.PFA), which includes sample group information, compound identifiers, and isotopologue abundances. Export of the complete dataset as a comma-separated value (.csv) file is also supported.

Table 1. LC/MS analysis parameters.

Parameter	Value
LC conditions	
Column	Waters Cortecs C18+ 150 mm × 2.1 mm, 2.7 μm
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 conditions	
Ionization mode	Dual ESI
Ionization polarity	Negative
Gas temperature	250 °C
Drying gas	13 L/min
Nebulizer pressure	45 psi
Capillary voltage	3,500 V
Octopole 1 RF voltage	600 V
Acquisition range	<i>m/z</i> 50–1,700
Reference masses	<i>m/z</i> 119.0363 and 980.01638

Table 2. Target metabolite list for isotopologue extraction.

Compound	Formula	CAS	RT (min)
L-Glutamate	$\text{C}_5\text{H}_9\text{NO}_4$	56-86-0	2.26
L-Aspartic acid	$\text{C}_4\text{H}_7\text{NO}_4$	56-84-8	2.29
L-Lactic acid	$\text{C}_3\text{H}_6\text{O}_3$	79-33-4	2.93
Glutathione, oxidized	$\text{C}_{20}\text{H}_{32}\text{N}_6\text{O}_{12}\text{S}_2$	27025-41-8	4.84
Succinic acid	$\text{C}_4\text{H}_6\text{O}_4$	110-15-6	5.05
2-Hydroxyglutarate	$\text{C}_5\text{H}_8\text{O}_5$	2889-31-8	5.11
Fumaric acid	$\text{C}_4\text{H}_4\text{O}_4$	110-17-8	5.13
L-Malic acid	$\text{C}_4\text{H}_6\text{O}_5$	97-67-6	5.13
Oxoglutaric acid (α-KG)	$\text{C}_5\text{H}_6\text{O}_5$	328-50-7	5.24
Guanosine diphosphate (GDP)	$\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{11}\text{P}_2$	146-91-8	5.83
Adenosine diphosphate (ADP)	$\text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{10}\text{P}_2$	58-64-0	5.93
Aconitic acid	$\text{C}_6\text{H}_6\text{O}_6$	499-12-7	6.1
<i>threo</i> -Isocitric acid (not resolved from citric acid)	$\text{C}_6\text{H}_8\text{O}_7$	6061-97-8	6.1
Citric acid (not resolved from <i>threo</i> -isocitric acid)	$\text{C}_6\text{H}_8\text{O}_7$	77-92-9	6.1
Guanosine triphosphate (GTP)	$\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{14}\text{P}_3$	86-01-1	6.57
Adenosine triphosphate (ATP)	$\text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{13}\text{P}_3$	987-65-5	6.63

## Results and Discussion

After isotopologue extraction in Profinder, results were reviewed and manually curated as necessary; only glutamate and  $\alpha$ -KG needed to be manually adjusted for more consistent results. The isotopologue display in Profinder provides useful insight into the incorporation of the stable label, as well as reproducibility of replicates within a sample group. This can be viewed as raw values or % corrected values, with and without an additional correction for natural isotope abundance. Figure 1 shows the isotopologue results for 2-HG. Results are displayed as natural abundance-corrected values grouped by time point. In this case, time points demonstrate excellent consistency across the biological replicates but, if necessary, outlier samples could be excluded at this stage. Initial inspection of the data shows that over time a decrease in 2-HG M+0 is observed with a corresponding increase in 2-HG M+5, revealing that all five carbons of 2-HG are being derived from the U- $^{13}\text{C}$ -Gln tracer, and that after 8 hours the majority of the 2-HG pool has become labeled and is, therefore, derived from glutamine.

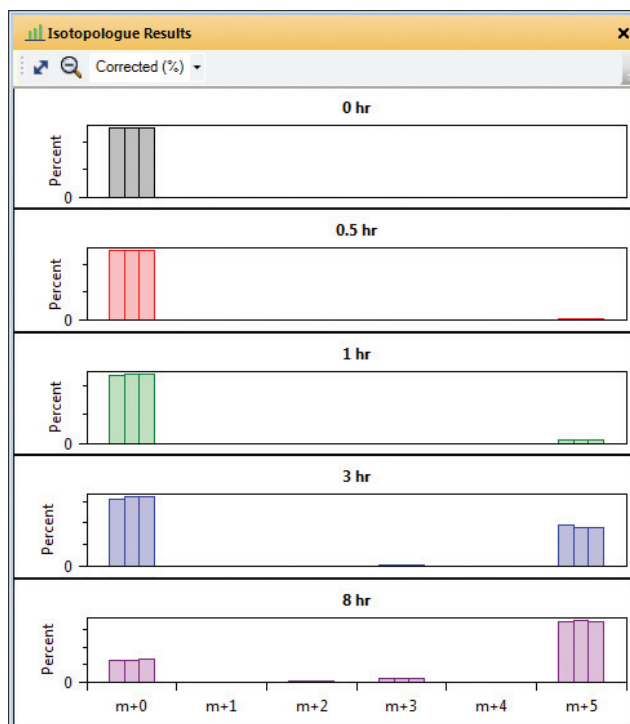


Figure 1. Agilent MassHunter Profinder isotopologue display of 2-HG.

Qualitative flux analysis is most powerful when used to follow the fate of a stable labeled isotope tracer through the context of biological pathways. To visualize the Profinder results in a pathways context, a network was created in Omix Premium from the human BioCyc database. Additional metabolites of interest, not in the canonical TCA cycle (lactate, pyruvate, oxidized glutathione, aspartate, and glutamine) were manually added to the network. Some metabolites such as ADP, GDP, ATP, and GTP are ubiquitous, and the measured

abundance represents the total cellular concentration. To monitor incorporation in these compounds, metabolite nodes are shown separate from a specific pathway with the same options for information visualization. After creating the network, Profinder results were imported into Omix Premium (Figure 2). In the program, the Component View on the left lists all metabolites in the network, the main window displays the network, and the Data Manager window at the bottom allows control of the imported Profinder results.

Omix Premium offers graphical options specifically designed for the visualization and interpretation of qualitative flux data including the ability to change properties of the metabolite node, and to add associated charts and graphics. As networks become more complex, a quick summary of the experiment can be achieved displaying metabolite abundance by coloring the node. In Figure 2, the colored fill intensity reflects the abundance of the metabolites measured (with grey-filled metabolite nodes being compounds not measured in this experiment).

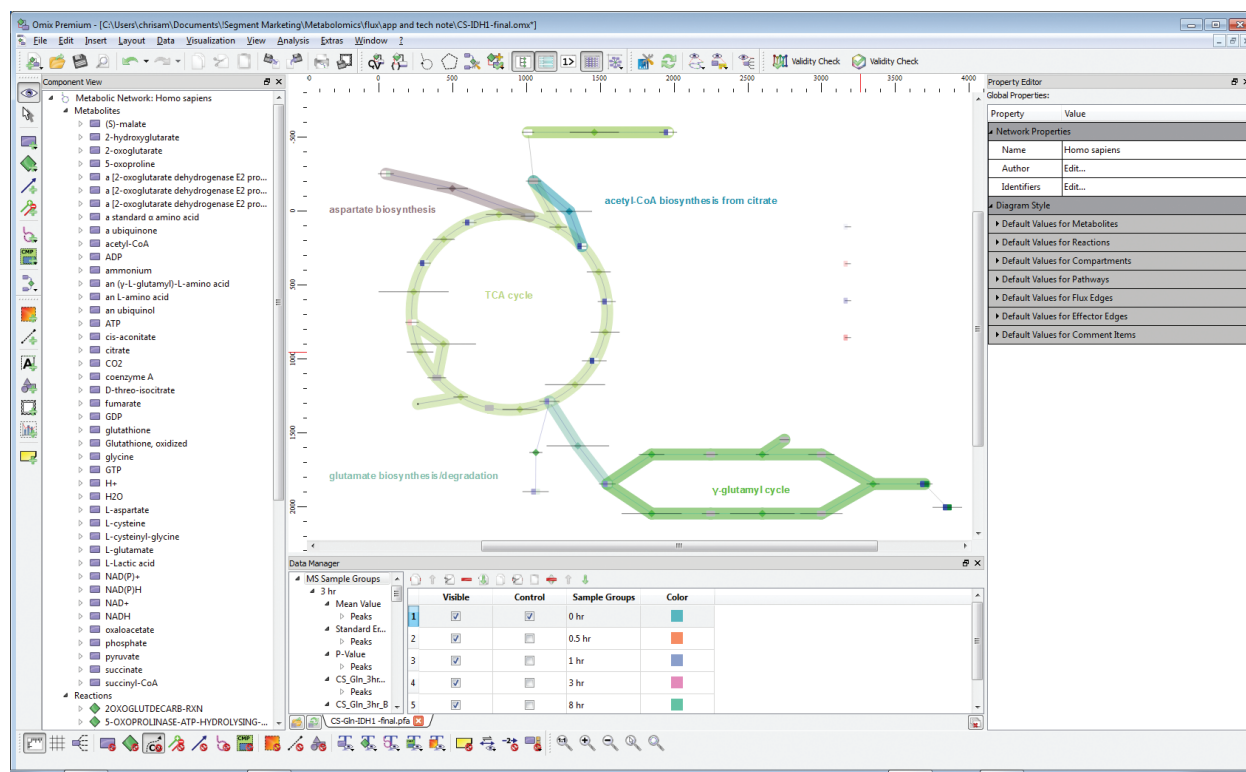


Figure 2. Omix Premium software displaying network and imported Agilent MassHunter Profinder results.

There are three options for the color of the metabolite node, as shown in Figure 3 for 2-HG. The node fill can be used to show that this metabolite is present at a high level (intensity of color in Figure 3A), or that stable label incorporation is occurring (Figure 3B). Figure 3C presents a combined view, showing both pieces of information.

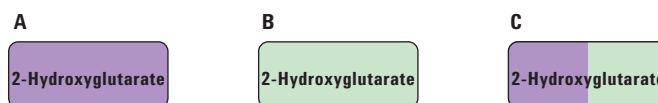


Figure 3. 2-HG node filled as A) abundance of the metabolite relative to other detected metabolites, B) percent stable label incorporation as a color gradient, and C) split fill with abundance on the left side and percent incorporation on the right side.

The information shown on the metabolite nodes is designed to indicate where the maximum fold changes are occurring relative to samples designated as the control group. Once this high level review of the data across the pathway has been completed, a more detailed inspection of the stable isotope incorporation is possible. Figure 4 shows additional information that can be displayed in quilt plots and horizontal bar charts to the side of each metabolite node. These plots summarize isotopologue abundance and tracer incorporation, and expand on summary information displayed on the node. In this example, the time point results are displayed on the y-axis of the quilt plot, and the isotopologues are shown on the x-axis. The fill color intensity indicates relative abundance of tracer incorporation. The bar chart provides an alternative view of the total tracer incorporation as a proportion of the total metabolite abundance (sum of all isotopologues detected). These views confirm the result first observed during manual data inspection of the data in Profinder, namely that after 3 hours and 8 hours of incubation with U-<sup>13</sup>C-Gln there is significant label incorporation of the tracer into the M+5 isotopologue of 2-HG, demonstrating that glutamine is the major substrate used to produce 2-HG in IDH2-mutant CS-1 cells.

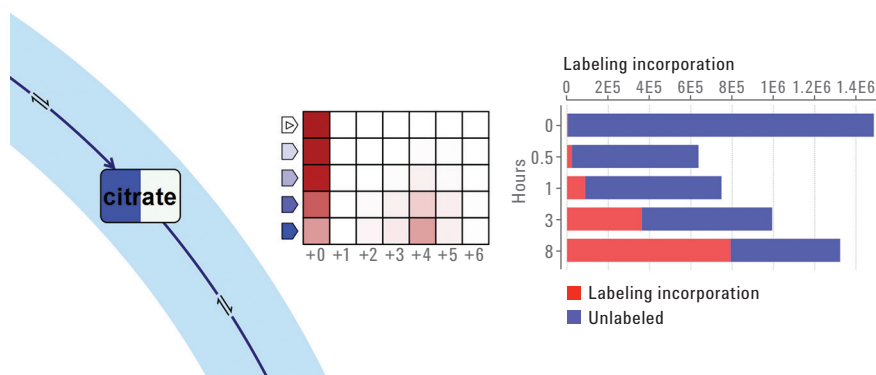


Figure 4. Detailed information about U-<sup>13</sup>C-Gln tracer incorporation into citrate in the CS-1 cell line.

Figure 5 shows another useful view for inspecting detailed information about the isotopologue incorporation. This plot displays the isotopologue results for the different groups (time points), with error bars to indicate the standard deviation observed for replicate measurements. Similar to the isotopologue display in Profinder, this plot provides a numerical view of the raw data. In this example, the plot for oxidized glutathione clearly shows

that, over time, the cellular glutathione pool becomes labeled with first one and then two Gln-derived 5-carbon building blocks. The node fill also indicates that oxidized glutathione is a highly abundant metabolite; thus it takes time for a significant proportion of the total cellular pool to incorporate one and then two Gln-derived subunits.

Metabolite nodes can also be included for compounds such as ADP, GDP, ATP, and GTP to show no incorporation or change in cellular levels throughout the experiment (Figure 6). This implies that Gln does not contribute significantly to biosynthetic pathways for these metabolites within an 8-hour time period. However, including information about the relative abundance of the di- and tri-phosphate nucleotide pools can still be helpful in interpreting cell health and viability.

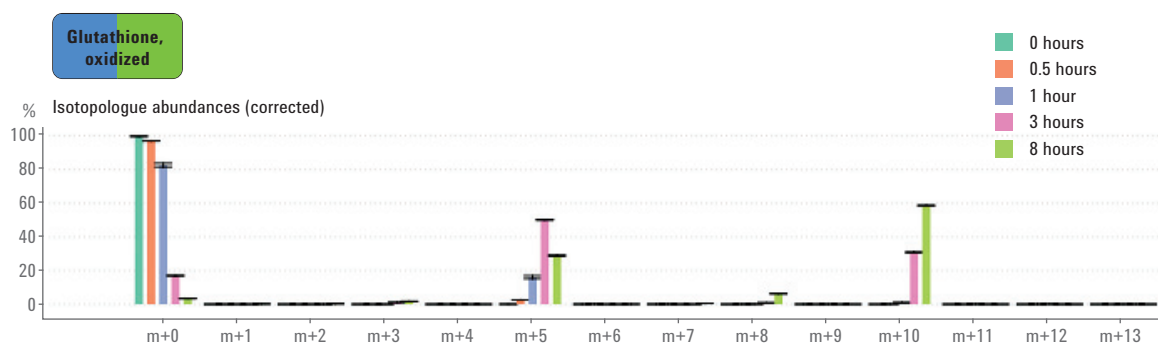


Figure 5. Glutathione isotopologue abundances as a bar chart showing the incorporation of glutamine tracer with time.

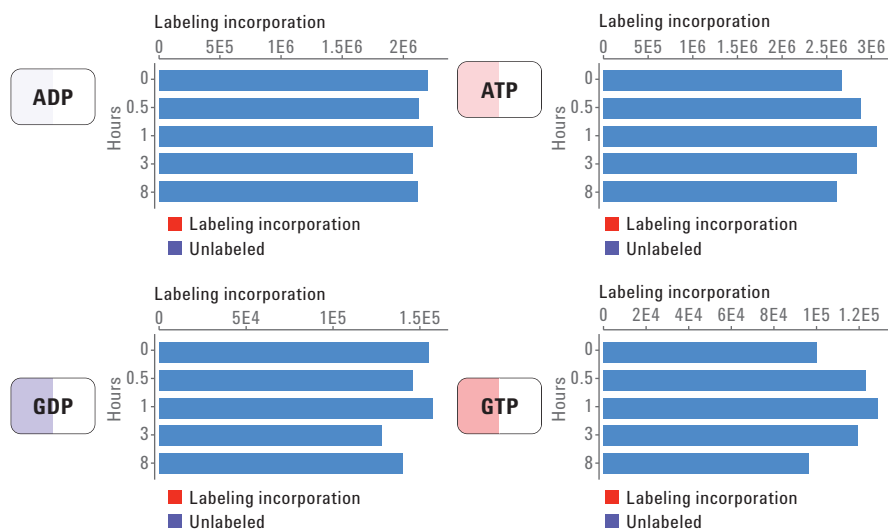


Figure 6. Display of overall flux for ubiquitous metabolites ADP, GDP, ATP, and GTP.



Combining these views, it becomes possible to compare the isotopologue distribution and labeling kinetics across the network (Figure 7). For presentation purposes, metabolite node fills can be replaced with structural formulas imported from BioCyc. Basic statistical information can also be overlaid on the quilt plot. Figure 7 shows the results of Welch's t-test for each isotopologue relative to abundance in the control group (designated as the 0 hour time point samples), with • indicating  $p < 0.05$ .

Inspection of these qualitative flux profiling results clearly reveals that the  $U\text{-}^{13}\text{C}\text{-Gln}$  tracer rapidly labels glutamate (Glu), somewhat faster than labeling of the 2-HG pool. This is reflective of the relatively low activity of the mutant IDH2 enzyme for completing the reductive reaction to produce 2-HG. It can also be inferred that the pathway is likely direct, since Glu,  $\alpha\text{-KG}$  (not shown), and 2-HG all retain all five  $^{13}\text{C}$  atoms from the  $U\text{-}^{13}\text{C}\text{-Gln}$  tracer. The results show that there is also forward (oxidative) consumption

of  $U\text{-}^{13}\text{C}\text{-Gln}$  in the TCA cycle, resulting in rapid labeling of succinate, fumarate, and malate predominantly as the M+4 isotopologue. Oxaloacetate (OAA) is present at low levels in most cells, and is too unstable to reproducibly survive extraction and analysis by LC/MS. However, the OAA pool is in equilibrium with L-aspartic acid (Asp), which is also rapidly labeled by the tracer, predominantly as the M+4 isotopologue.

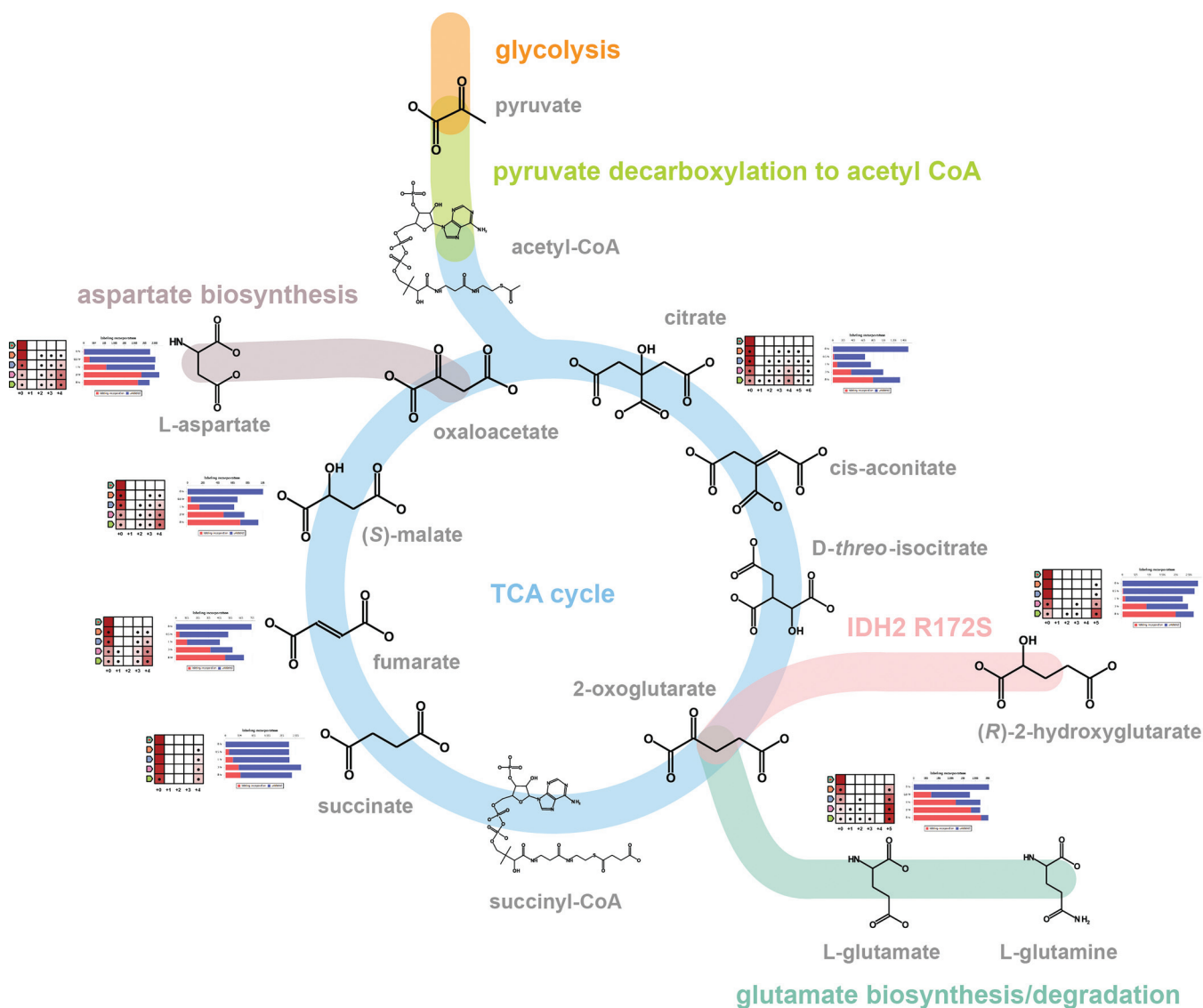


Figure 7. Summary of  $U\text{-}^{13}\text{C}\text{-Gln}$  labeling of 2-HG and the TCA cycle metabolites.



Later, M+3 labeling is seen in 2-HG, succinate, fumarate, malate, and aspartate. This is the result of M+4 labeled OAA combining with two unenriched carbons contributed from acetyl-CoA, and resulting in citrate M+4. The M+3 isotopologue seen in downstream metabolites is the result of decarboxylation by the wild type IDH reaction, acting in the forward (oxidative) direction. There is also evidence of M+5 labeling in citrate, resulting from reductive carboxylation catalyzed by the wild type IDH enzyme acting in reverse. By 8 hours, more complex labeling patterns are observed, resulting from:

- Further rounds of the TCA cycle
- Scrambling of labeling patterns due to the symmetry in succinate, fumarate, and citrate
- Potentially the action of malic enzyme

These patterns could be investigated further using alternative stable isotope tracers, or with Gln labeled at specific carbon positions. Finally, the summary view makes it possible to assess the relative kinetics of metabolite labeling, and it can be seen that Asp and Glu labeling are approaching steady state by 8 hours of labeling. Longer time points would be required for the other metabolite pools to reach metabolic steady state.

## Conclusions

Using the Agilent MassHunter VistaFlux workflow, qualitative flux profiling was performed in CS-1 chondrosarcoma cells with U-<sup>13</sup>C-Gln as the metabolic tracer. This analysis clearly demonstrates the activity of the IDH2 mutant isoform to convert Gln-derived  $\alpha$ -KG into 2-HG. Over 8 hours, the majority of the 2-HG pool becomes labeled as the M+5 isotopologue in cells harboring an IDH mutation. This increased production of 2-HG, driven by the neomorphic activity of the IDH2 R172S mutant enzyme, overwhelms the endogenous 2-HG dehydrogenases, allowing 2-HG to accumulate to high levels. Then, 2-HG becomes a competitive inhibitor for a variety of  $\alpha$ -KG dependent dioxygenase enzymes including TET family DNA demethylases and Jumonji C family histone demethylases, resulting in epigenetic changes including DNA hypermethylation, promoting an undifferentiated chromatin environment. The analysis also shows the consequences of the U-<sup>13</sup>C-Gln tracer entry into the canonical TCA cycle pathway.

Compared to manual data mining, the VistaFlux workflow provides a comprehensive, automated, and accelerated framework for the processing of stable isotope tracing data, including isotopologue extraction and pathway visualization of qualitative flux profiling data.

## References

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