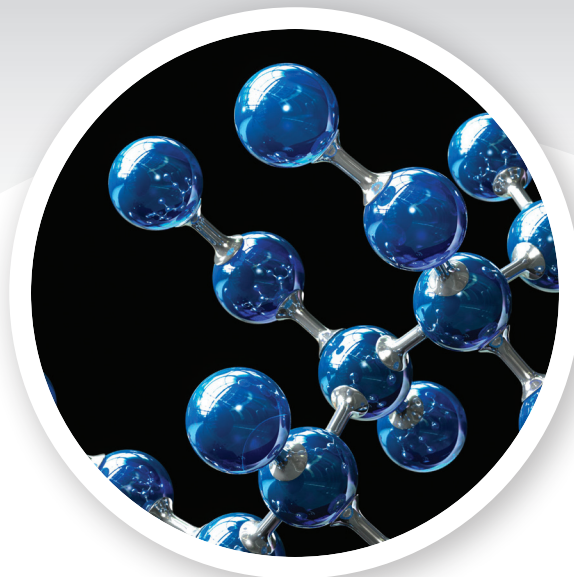


# Metabolic Incorporation of Stable Isotope Labels into Glycans

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Glycosylation is one of the most common post-translational protein modifications in eukaryotic systems.<sup>1-3</sup> It has been estimated that 60-90% of all mammalian proteins are glycosylated at some point during their existence<sup>1,3</sup> and virtually all membrane and secreted proteins are glycosylated.<sup>2</sup> Glycoprotein glycans often play crucial roles in physiological events such as cell-cell recognition,<sup>4-6</sup> signal transduction,<sup>7</sup> inflammation<sup>8</sup> and tumorigenesis.<sup>9-13</sup> Given the important physiological roles of protein glycosylation, numerous research groups have devoted significant effort to the characterization of specific glycan structures, the identification of proteins that express each glycan, and the detailed study of how these structures change, e.g., as cells differentiate or as tumor cells progress. All of these efforts have given rise to the emerging field of glycomics.<sup>14</sup>

Mass spectrometry has developed into the analytical method of choice as it provides a rapid, sensitive and reliable method to analyze complex mixtures.<sup>15</sup> Glycomic studies typically involve the *en toto* release of the glycans followed by MS analysis of the glycans.<sup>16,17</sup> While this is a fruitful approach for qualitative characterization of the glycome, multiple issues arise when MS is used to obtain quantitative results. For instance, matrix effects, caused by phenomenon such as ion suppression from the presence of other compounds competing with or interfering with the ionization of the analyte, can alter the response from a particular analyte even when the analyte's concentration does not change. Other issues that interfere with quantitative MS analyses result from variable instrument response, instrument-to-instrument variability and differential losses during sample handling/processing. The success of the different approaches for relative quantitation depends on how well each of these sources of error is addressed.

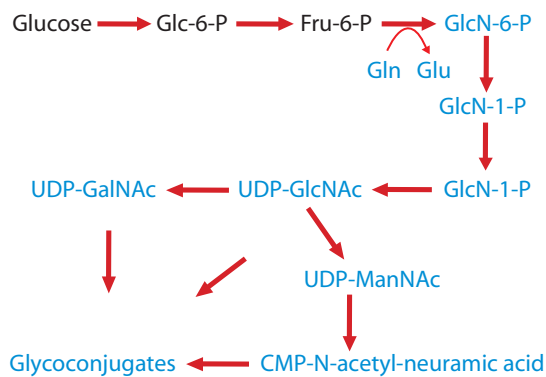
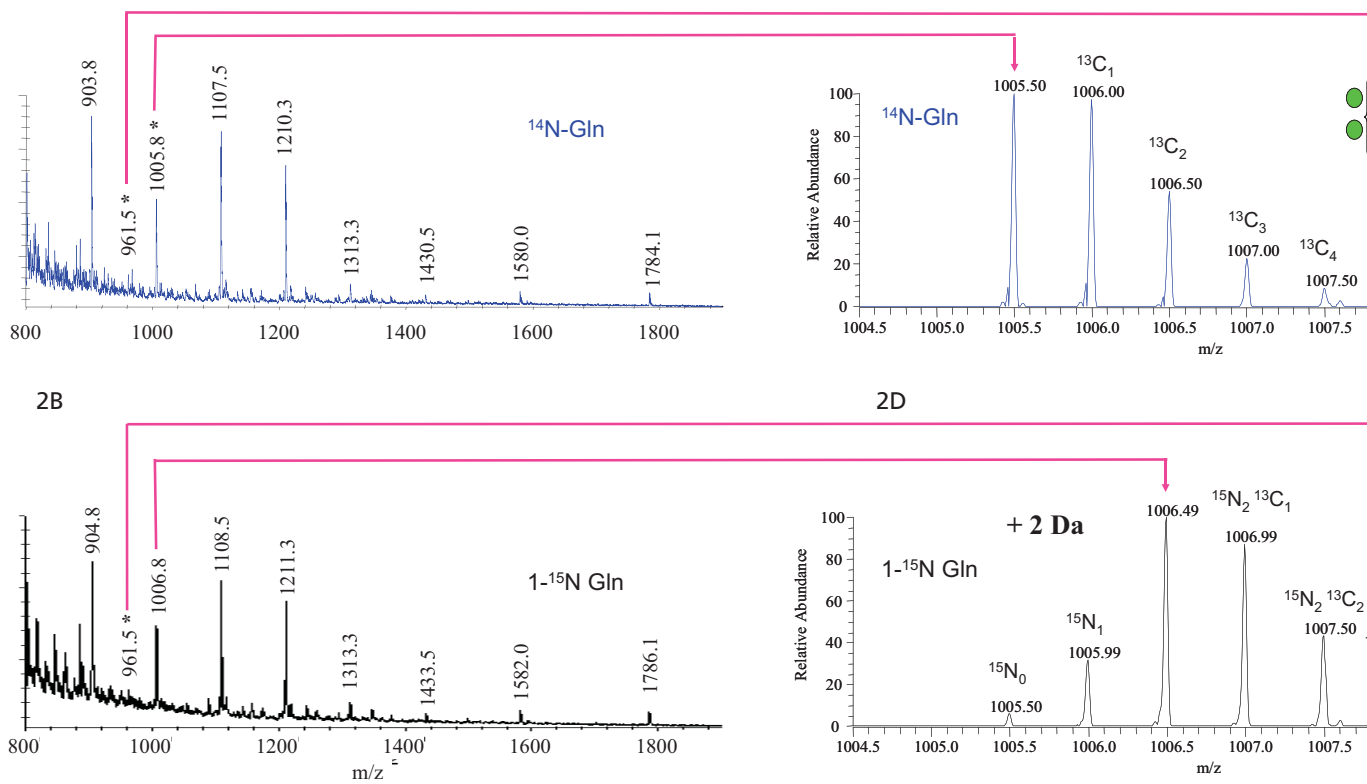
The use of isotopic labeling can compensate for these quantitative issues, and thus has gained widespread use in the various -omics fields, with the techniques used in the field of proteomics probably the most developed. A good example is stable isotope labeling

**“My lab has utilized a range of CIL products, and we have been very pleased with their performance for quantitative/comparative proteomics and glycomics. The CIL sales staff is very helpful and extremely knowledgeable. They have aided us on experimental design numerous times, and without this help we would not have been able to do this work.”**

– Ron Orlando

with amino acids in cell culture (SILAC), which provides an elegant method for the incorporation of an isotopic label into proteins prior to MS-based proteomics.<sup>18</sup> In a SILAC experiment, two cell populations are grown in culture media that are identical except that one of them contains a “light” (natural abundance) and the other a “heavy” (isotopically enriched) form of a particular amino acid (unlabeled and L-Lysine•2HCl (<sup>13</sup>C<sub>6</sub>, 99%) (CIL #CLM-2247-H) and L-Arginine•HCl (<sup>13</sup>C<sub>6</sub>, 99%) (CIL #CLM-2265-H), for example). The isotopically heavy amino acids are supplied to cell cultures in place of the natural amino acids, and they become incorporated into all newly synthesized proteins. After multiple cell divisions, each instance of the particular amino acids is replaced by its “heavy” isotope-containing analog. An advantage of this approach is that the cells are mixed together immediately after cell lysis, thereby proteins from both cell types are subjected to the exact same experimental conditions during the sample handling, digestion, purification and separation steps. For this reason, SILAC is often considered the “gold standard” for comparative quantitative proteomic analyses.<sup>19</sup>

(continued)



**Figure 1.** The hexosamine biosynthetic pathway demonstrating that the side-chain of glutamine is the sole donor source of nitrogen for amino sugars in the production of sugar nucleotides, which allows the introduction of an  $^{15}\text{N}$  isotopic tag into all aminosugars, including GlcNAc, GalNAc and sialic acids. Species containing  $^{15}\text{N}$  are indicated by blue type.

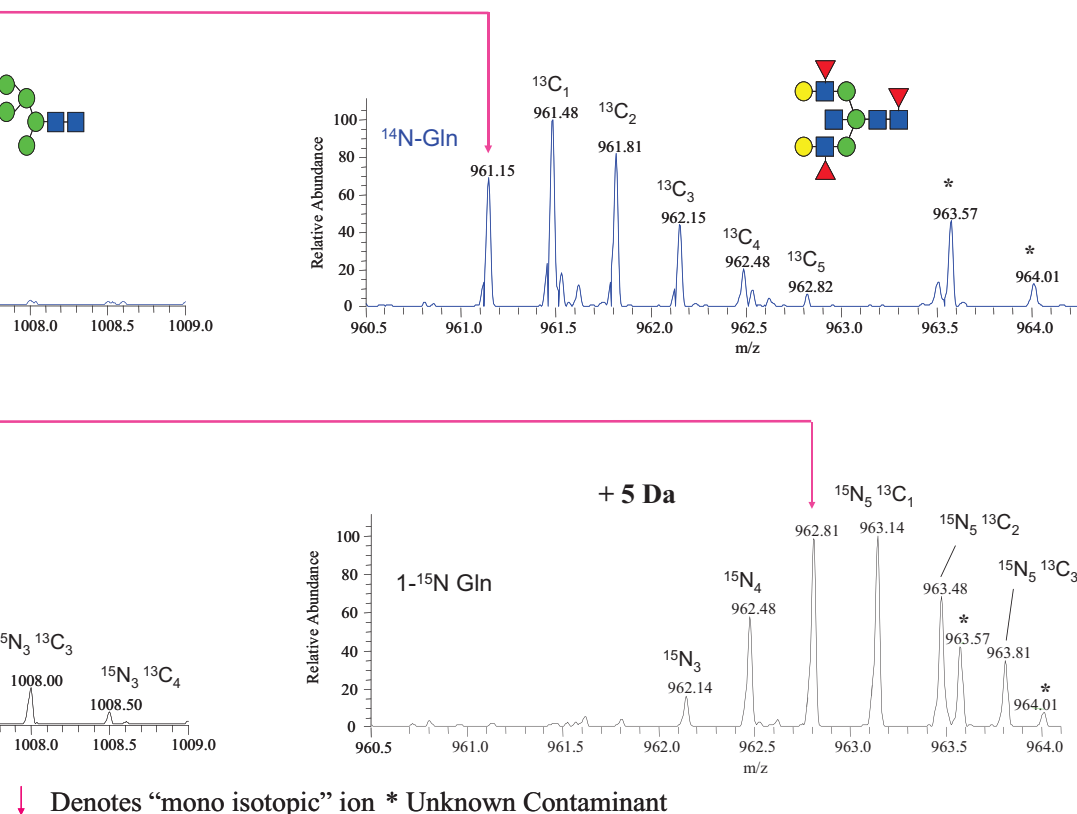
In this note, an *in vivo* labeling strategy for glycomic studies is described which is analogous to the SILAC approach used for proteomics. This glycomic methodology is called IDAWG – Isotopic Detection of Aminosugars With Glutamine – and is based on the side-chain of glutamine being the sole nitrogen donor source in the biosynthesis of aminosugar nucleotides

(Figure 1). Thus, introduction of L-Glutamine (amide- $^{15}\text{N}$ , 98%+), (CIL #NLM-557) into Gln-free media allows for all aminosugars, including GlcNAc, GalNAc and sialic acids, to become labeled with  $^{15}\text{N}$ . This leads to the mass of *N*- and *O*-linked glycans, glycolipids and extracellular matrix polysaccharides to be increased by +1 Da per aminosugar. The utility of this approach is demonstrated by the analysis of *N*-linked glycans released from proteins of murine embryonic stem cells grown in both the presence of light and amide- $^{15}\text{N}$ -Gln. The success of these experiments leads us to predict that the IDAWG technology will be useful for a variety of future comparative glycomic studies in cell culture.

## Results

The first and rate-limiting step in the hexosamine biosynthetic pathway (Figure 1) is the conversion of the glycolysis intermediate fructose-6-phosphate into glucosamine-6-phosphate.<sup>20</sup> The amino nitrogen introduced during this step is provided solely by the side-chain amide of Gln, which is converted to Glu.<sup>21</sup> Glucosamine-6-phosphate is the precursor of UDP-GlcNAc, which in turn leads to the other major aminosugar-containing sugar nucleotides, UDP-GalNAc and CMP-sialic acid<sup>22, 23</sup>. Thus, all GlcNAc, GalNAc and sialic acid-containing molecules are targets for isotopic labeling by supplementation of cell culture media with amide- $^{15}\text{N}$ -Gln.

Initial experiments were performed to evaluate the possibility of using metabolic labeling as a method to incorporate a stable isotope into the glycans of cells grown in culture. In these



**Figure 2.** Isotopically labeled *N*-linked Glycans. Full FT-MS spectra from 850-2000 *m/z* of the permethylated *N*-linked glycans released from cells grown in either unlabeled-Gln (2A) or amide-<sup>15</sup>N-Gln (2B). Expanded regions of the spectra show the expected 2 Da mass shift from <sup>15</sup>N incorporation into the two core GlcNAc residues of the GlcNAc<sub>2</sub>Man<sub>7</sub> glycan (2C, 2D), and the 5 Da increase corresponding to <sup>15</sup>N incorporation into the five amminosugars of the GlcNAc<sub>5</sub>Man<sub>3</sub>Gal<sub>2</sub>Fuc<sub>3</sub> glycan (2E, 2F).

experiments, R1 murine embryonic stem cells (mESCs) were grown using standard conditions. Cell culture media is typically supplied without glutamine, as this amino acid decomposes to glutamic acid and ammonia in aqueous solution. This formulation simplifies the IDAWG-labeling approach, because there is no need to specifically deplete the media of Gln. Either amide-<sup>15</sup>N-Gln or unlabeled Gln was used to supplement the media at the standard concentration (2 mM). This straightforward substitution is the only alteration to the normal cell culturing procedure needed for IDAWG. In this example, murine ES cells were grown for three days with “heavy” or with “light” Gln, and then both *N*- and *O*-linked glycans were isolated from proteins for mass spectrometry-based analysis.

The incorporation of <sup>15</sup>N into the *N*-linked glycans of mESCs was investigated by comparing the FT-MS spectra of the permethylated *N*-linked glycans released from cells grown in either unlabeled-Gln or L-Glutamine (amide-<sup>15</sup>N, 98%+) (Figures 2A and B). The abundant ions observed in these spectra correspond to high mannose glycans (GlcNAc<sub>2</sub>Man<sub>5-9</sub>) that are either singly or doubly charged. Comparison of these two spectra reveals that the glycan ions obtained from the cells grown in the “heavy” media are increased by 1 *m/z* unit for the doubly charged ions and 2 *m/z* units for the singly charged ions. This is the expected result provided that <sup>15</sup>N has been incorporated into the two core GlcNAcs contained in these glycans, which are the only nitrogens present in high mannose glycans.

This shift in glycan mass can be clearly seen upon closer inspection of the doubly charged molecular ion, (M+2Na)<sup>2+</sup>, resulting from the GlcNAc<sub>2</sub>Man<sub>7</sub> glycan, which appears at 1005.5 and 1006.5 *m/z* units from glycans grown in the <sup>14</sup>N and amide-<sup>15</sup>N, respectively (Figure 2C and D). In these spectra, the most intense ion appears at the monoisotopic mass calculated with either <sup>14</sup>N or two <sup>15</sup>N, demonstrating that the majority of this glycan has <sup>15</sup>N incorporated into both possible sites. Ions are present that correspond to under labeling, i.e., the incorporation of zero and one, <sup>15</sup>N, however the dominant species is fully labeled.

Larger *N*-linked glycans are similarly increased in mass by the incorporation of one <sup>15</sup>N per GlcNAc residue. This can be observed by expanding the spectra shown in Figure 2A and 2B so that only the region from 960.5 to 964.1 is observed (Figure 2E and F, for the glycans released from cells grown in <sup>14</sup>N and amide-<sup>15</sup>N Gln media, respectively). This region shows the molecular ion, (M+3Na)<sup>3+</sup>, for a complex glycan with a composition of GlcNAc<sub>5</sub>Man<sub>3</sub>Gal<sub>2</sub>Fuc<sub>3</sub>. As predicted, the monoisotopic ion from this glycan is increased by 5 Da when it was obtained from cells grown in the amide-<sup>15</sup>N Gln. In addition, the fully labeled glycan is the dominant species, although labeling is not complete as ions are observed from under labeling.

Calculations performed on the ratios of the intensities of the different isotopes in the <sup>15</sup>N spectrum indicate a 98% incorporation of <sup>15</sup>N into this glycan, which happens to equal the extent of <sup>15</sup>N

(continued)

in the glutamine used for this experiment. Similar calculations on the isotopic distributions from other glycans indicate that their  $^{15}\text{N}$  incorporation ranged from 96-98%. Combined, these examples demonstrate that  $^{15}\text{N}$  from L-Glutamine (amide- $^{15}\text{N}$ , 98%+) becomes extensively incorporated into the GlcNAc residues of N-linked glycans, suggesting that this is potentially a useful strategy for introducing a stable isotope into glycans.

## Discussion

Metabolic labeling of glycans provides many new opportunities for assessing the dynamics of glycan turnover during the course of any cellular behavior that can be induced or sustained in culture. For example, labeling cells with L-Glutamine (amide- $^{15}\text{N}$ , 98%+) and then replacing the media supplement with "light" Gln will allow the determination of the half-life of any aminosugar-containing glycans. Previously, glycan turnover studies required incorporation of radioactive monosaccharide and extensive subsequent fractionation to identify specific changes in glycan expression. Generally, these radiotracer techniques allowed for very sensitive detection of glycan classes, but lacked the resolution to follow individual glycan structures or subsets of biosynthetically

related species. The stable isotope incorporation method reported here merges the analytic advantages of high-resolution mass spectrometry to the biological necessity of understanding the dynamics of glycan turnover. Thus, IDAWG appears to be a powerful quantitative tool for exploring the biological role of glycans, glycoproteins and glycolipids in cell culture systems.

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## Related Products

Catalog No.	Description
CLM-2265-H	L-Arginine•HCl ( $^{13}\text{C}_6$ , 99%)
NLM-557	L-Glutamine (amide- $^{15}\text{N}$ , 98%+)
CLM-2247-H	L-Lysine•2HCl ( $^{13}\text{C}_6$ , 99%)

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