# Robust and Sensitive Relative Quantification of Isobarically Labeled Glycans by LTQ Orbitrap Velos Hybrid Mass Spectrometer

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## Overview

**Purpose:** Evaluate the performance of a new hybrid ion trap – Orbitrap mass spectrometer featuring a dual-pressure ion trap and an improved HCD collision cell for MS and $MS^n$-based QUIBL quantification of *N*-linked glycans.

**Methods:** The *N*-linked glycans were permethylated with $^{13}CH_3I$ or $^{12}CH_2DI$, mixed in different ratios, analyzed by $MS/MS^n$ on a LTQ Orbitrap Velos, and quantified**.**

**Results:** Isobarically labeled glycans mixed in different ratios were successfully quantified using full MS, HCD or CID/HCD $MS^n$ spectra over 2 orders of magnitude. The maximum resolution of the Orbitrap mass spectrometer,100000 at *m/z* 400, was required to resolve and quantify the $^{13}CH_3$ and $^{12}CH_2D$ species.

## Introduction

A current challenge in the field of glycomics is determining how to quantify changes in glycan expression between different cells, tissues, or biological fluids. Atwood *et al* [1]described a novel strategy for relative quantification of glycans by isobaric labeling (QUIBL) that requires high resolution and mass accuracy to distinguish isobaric pairs. In this report, the performance of a new hybrid ion trap – Orbitrap mass spectrometer featuring a dual-pressure ion trap and an improved HCD collision cell[2] is evaluated for MS and $MS^n$-based QUIBL quantification of fetuin *N*-linked glycans and lacto-*N*-fucopentaoses isomers.

## Methods

### Sample Preparation

Bovine fetuin *N*-linked glycans were released by overnight incubation with PNGase F (Sigma), separated from peptides by reverse phase chromatography, permethylated with $^{13}CH_3I$ or $^{12}CH_2DI$ (Cambridge Isotopes), and then dried and redissolved in 50% MeOH and 1mM NaOH for MS analysis[1]. Isobarically labeled Lacto-N-fucopentaose I (LNFP I), Lacto-N-fucopentaose III (LNFP III), Lacto-N-difucohexaose I (LNDFH I), and Lacto-N-difucohexaose II (LNDFH II) from V-labs were prepared as described for the fetuin glycans. Mixtures of glycans were prepared with concentration ratios ranging from 20:1 to 1:10.

### MS Analysis

Glycans at pmol concentrations were analyzed on a Thermo Scientific LTQ Orbitrap Velos hybrid mass spectrometer via direct infusion using static nanospray. Data were acquired in full MS, HCD, and FT CID $MS^n$ scan modes at 60,000-100,000 resolving power (RP) at *m/z* 400.

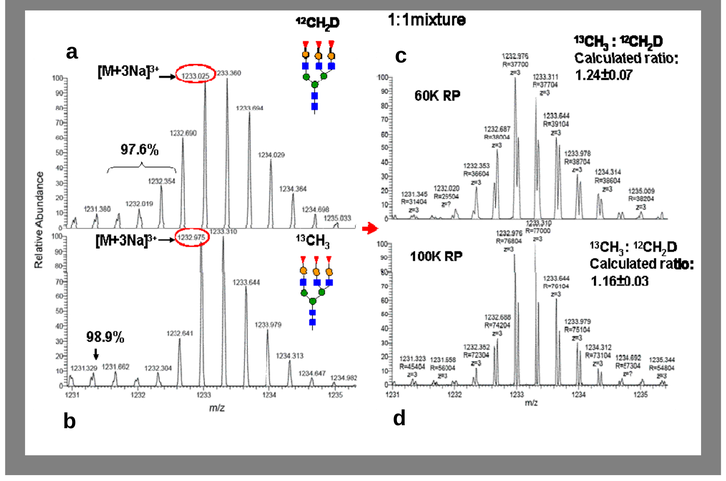
### Data Processing

Thermo Scientific Xtract software was used for spectral deconvolution and monoisotopic mass determination. Quantification was performed by summing the $^{13}CH_3$ or $^{12}CH_2D$-labeled total ion intensities for each glycan and applying correction factors for permethylation completeness.

## Results

Figure 1 illustrates the QUIBL method. Bovine fetuin *N*-linked glycans are permethylated with either $^{12}CH_2DI$ or $^{13}CH_3I$ (Figure 1a, 1b) to generate isobaric pairs. The permethylated samples were mixed in different ratios and analyzed using an LTQ Orbitrap Velos™ mass spectrometer, as high resolution mass spectrometer is required. The mass difference between the isobaric labels is small (0.00292 Da) but glycans contain multiple permethylation sites (shown in parentheses in Figure 2) that together produce a mass difference that is completely resolved using 100,000 RP, but not 60000 at *m/z* 400 (Figure 1d versus 1c). Relative quantification by QUIBL can be performed either in MS mode (Figure 1) or by MS/MS performed at high resolution for analysis of isomeric glycan mixtures (Figure 5b). For accurate relative quantification by QUIBL, correction factors for each label need to be calculated. As shown in Figure 1a versus 1b, the $^{12}CH_2D$ labeled sample shows lower level isotopic of isotopic enrichment than the $^{13}CH_3$ labeled sample (97.6% versus 98.9%) plus more incomplete permethylation due to a kinetic isotope effect [3].

**FIGURE 1. The QUIBL method is demonstrated for the fetuin tri-antennary glycan permethylated with $^{12}CH_2DI$ (a) or $^{13}CH_3I$ (b), and its 1:1 mixture. FTMS spectra were acquired at 60K (a, b, c) or 100K (d).**




Nevertheless, it is still possible to perform QUIBL quantification using the sum of all fragment isotope ion abundances as described in *Methods*. For the 1:1 mixture for fetuin triantennary glycan at 100,000 RP (Figure 1d), a ratio of 1.16 was observed.

The dynamic range and linearity of QUIBL on the LTQ Orbitrap Velos mass spectrometer were evaluated using 4 main species of bovine fetuin N-glycans (Figure 2), mixed in 5 different ratios (20:1-1:10) using 100K resolving power in the MS and MS/MS modes using HCD as shown in Figures 3 and 4. These results demonstrate that quantification using the QUIBL method is linear over at least 2 orders of magnitude, even with incomplete isotopic incorporation. Sialic acid ions generated by HCD provided the most accurate results for quantification for the higher ratios due to highest resolution achieved (>100 K, Figure 4). The overall maximum error was below 15%, which is comparable to other isotopic based quantification techniques.

**FIGURE 2. Orbitrap MS spectrum (a) and HCD spectra (b) of bovine fetuin *N*-linked permethylated glycans mixture and its compositions acquired at 100K RP at *m/z* 400.**

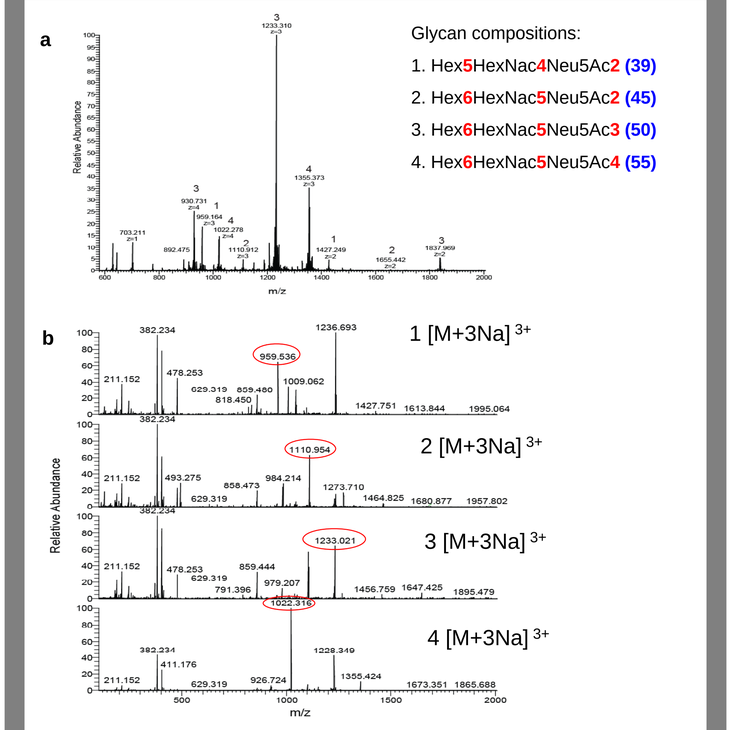



**FIGURE 3. FTMS QUIBL quantification of the fetuin bi-antennary glycan. Orbitrap spectra were acquired at 100K RP. *Inset* shows correlation between the theoretical and experimental ratios for mixtures at 20:1, 10:1, 1:1, 0.5:1, and 1:10 ($^{13}CH_3$:$CH_2D$).**

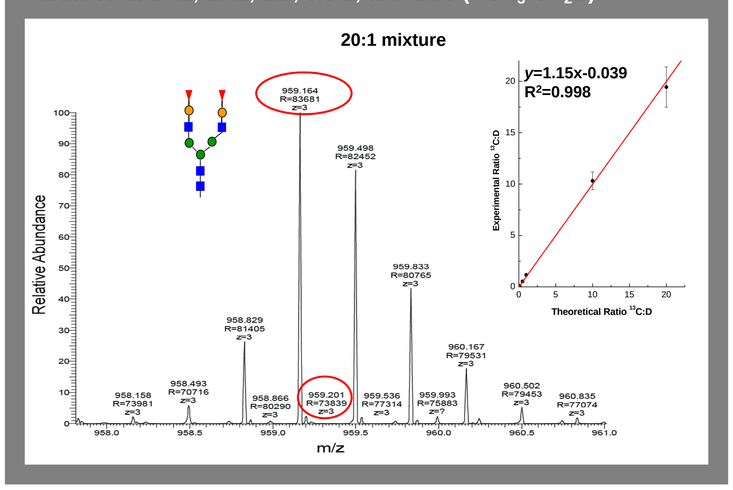
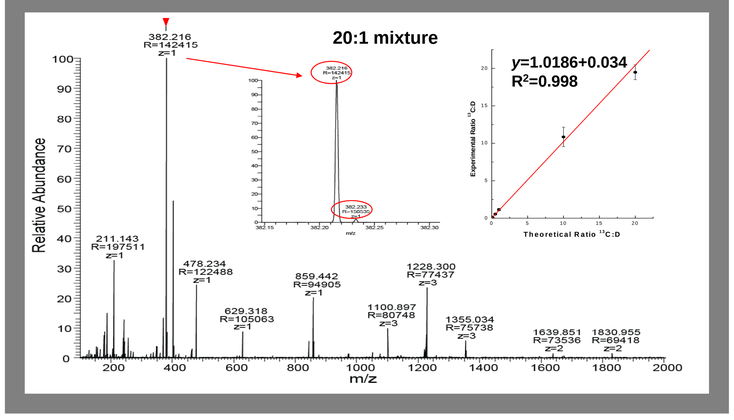


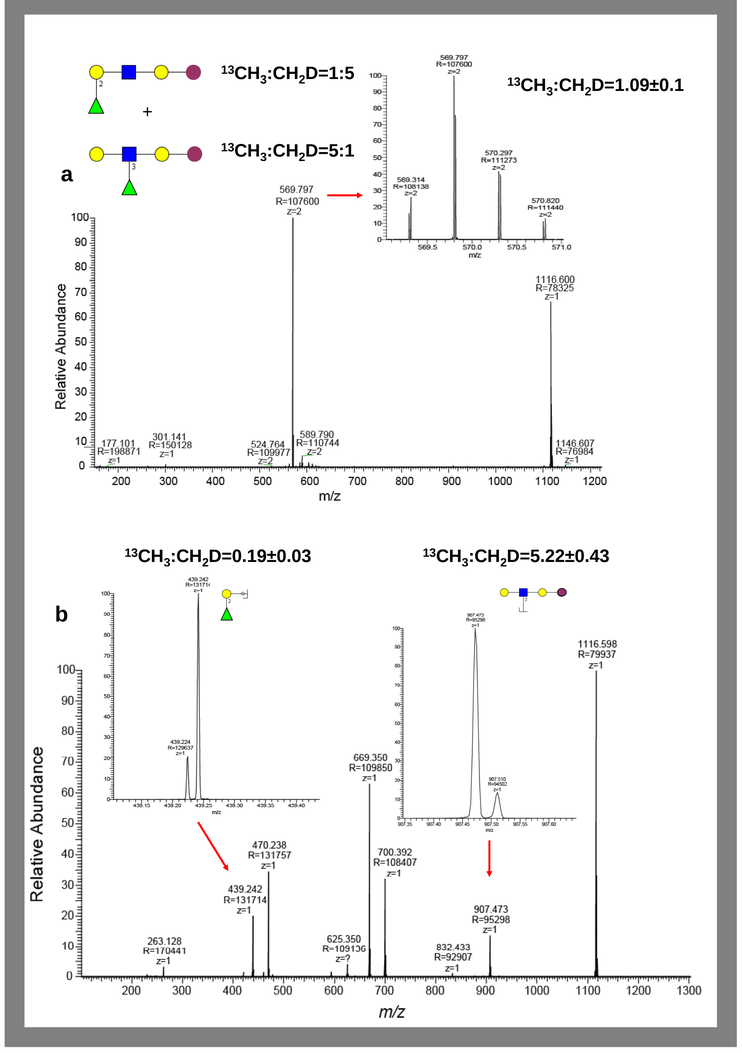

**FIGURE 4. HCD QUIBL quantification of the fetuin tetra-sialylated glycan using sialic acid fragment ion at *m/z* 382. Orbitrap spectra were acquired at 100K RP. *Inset* shows correlation between the theoretical and experimental ratios for mixtures at 20:1, 10:1, 1:1, and 0.5:1 ($^{13}CH_3$:$CH_2D$).**




One of the unique aspects of QUIBL is that simultaneous quantification of isomeric glycans can be performed using $MS^n$ mode. Two pairs of glycan isomers, LNFP I and LNFP III, LNDFH I and LNDFH II, were used to demonstrate such quantification. Each glycan was permethylated with $^{13}CH_3I$ or $^{12}CH_2DI$ and mixed in different ratios. Results for the LNFP I and LNFP III pair mixed in 1:5:5:1 ratio are shown in Figure 5. Isotope enrichment for these experiments was calculated as 98.9% for $^{13}CH_3$ and 98.1% for $CH_2D$. LNFP I and LNFP III have exactly the same molecular mass ($[M+2Na]^{2+}$= 569.8) and, as expected, the ratio calculated using the full MS spectra was close to 1 (Figure 5a).

An isomeric mixture such as LNFP I and LNFP III requires MS/MS performed at high resolution for correct quantification. Introduction of an integrated C-trap/HCD collision cell combination[2] for the LTQ Velos Orbitrap mass spectrometer significantly increases the sensitivity of the instrument in HCD mode, facilitating QUIBL analysis. The ion at *m/z* 569.8 ($M+2Na]^{2+}$) was selected and fragmented by HCD. The ion at *m/z* 439.2 was used for relative quantification of LNFP I. The signature ion at *m/z* 907.4 was used for quantification of LNFP III as shown in Figure 5b (*Insets*). Both calculated glycan ratios were within 10% of their theoretical values. LNDFH I and LNDFH II demonstrated the same relative performance (data not shown). Overall similar dynamic range and linearity of the QUIBL method was obtained for isomer mixtures quantification as for fetuin *N*-glycans.

**FIGURE 5. Orbitrap MS (a) and HCD (b) spectra of isomeric mixture of isobarically labeled LNFP I and LNFP III in 1:5:5:1 ratio. Orbitrap spectra were acquired at 100K RP. *Insets* show zoom in of ions used for quantification.**




## Conclusion

- The QUIBL method was successfully used for relative quantification of glycans in MS or MS/MS mode on a LTQ Orbitrap Velos mass spectrometer over 2 orders of magnitude within <15% error.
- Optimization of the reaction conditions for permethylation and correction factors for rate of incorporation $^{13}CH_3I$ versus $12CH_2DI$ is required.
- Successful quantification of isomer mixtures by HCD at 100,000 FWHM was demonstrated.
- QUIBL was validated as a powerful quantitative glycomics approach.

## References

1. Atwood, J.; Cheng, L.; Alvarez-Manilla, G.; *et al.*, (2007) Quantitation by isobaric labeling: applications to glycomics. *Journal of Proteome Research* 7, 367-374.
2. Olsen, J.; Schwartz, J.C.; Griep-Raming, J.; *et al.*, (2009) A dual pressure linear ion trap – Orbitrap instrument with very high sequencing speed. *Mol. Cell. Proteomics* 8, 2759-2769.
3. Botelho, J.; Atwood, J.; Cheng, L.; *et al.*, (2008) Quantification by isobaric labeling(QUIBL) for the comparative glycomic study of O-linked glycans. *Int. Journal of Mass Spectrometry* 278, 137-142.