

Carbonyl-Reactive Tandem Mass Tag (TMT) Reagents for Mass Spectrometry-Based Quantitative Glycomics

Sergei I. Snovida,¹ Rosa Viner,² John C. Rogers¹

¹Thermo Fisher Scientific, Rockford, IL; ²Thermo Fisher Scientific, San Jose, CA



Overview

Purpose: To demonstrate applications of the new carbonyl-reactive aminoxyTMT reagent in quantitative analysis of carbohydrates by direct infusion and liquid chromatography-coupled mass spectrometry.

Methods: *N*-glycans released from standard glycoproteins, including several monoclonal antibodies, were individually labeled with the isobaric aminoxyTMT reagents, and combined together for quantitative analysis by mass spectrometry.

Results: Relative quantitation of the labeled glycans from different samples was done by measuring relative peak intensities of the TMT[®] reporter ions at MS² or MS³ levels.

Introduction

Aberrant glycosylation profiles may reflect abnormal physiological state of a cell, an organ, or an organism as a whole, and may be indicative of a disease state or cancer. Thus, glycans may be used as biomarkers. Additionally, many biotherapeutic drugs are glycoproteins and their activity and efficacy greatly depends on the type and extent of glycosylation. These compounds must be characterized in terms of their glycosylation profiles in both the development and quality control phases of drug development and manufacture.

Accurate quantitation of glycans remains elusive due to the lack of a comprehensive selection of available standards, poor ionization efficiency of carbohydrates relative to other classes of biomolecules, and broad structural heterogeneity of glycomic samples. Recently, we introduced a set of isobaric carbonyl-reactive TMT reagents, Thermo Scientific[™] aminoxyTMT[™] (Tandem Mass Tag[®]) Label Reagents (Figure 1), which can react with the reducing end of glycans to form a stable oxime product. The six compounds of the Thermo Scientific[™] aminoxyTMTsixplex[™] Reagent Set have the same mass (i.e., isobaric) and chemical structure (carbonyl-reactive aminoxy group, spacer arm, and mass reporter). However, the specific distribution of ¹³C and ¹⁵N isotopes on either side of the MS/MS fragmentation site in each reagent results in a unique reporter mass (126–131 Da) in the low *m/z* region of MS² spectra. This set of reporter ions is used to measure the relative abundance of labeled molecules in a combined (multiplexed) MS sample representing six different treatment conditions, time points, or replicates. For glycobiology MS applications, the reagents enable quantitative profiling of glycoforms and discovery of glycan biomarkers; they provide improved ionization of glycans for increased sensitivity and increased analytical throughput by sample multiplexing.

In this work, we showcase the use of these reagents for quantitative glycomics by combining our multiplexed TMT-based approach with HILIC LC-MS technique to enable more-sensitive analysis with improved glycome coverage.

Methods

Sample Preparation

Bovine thyroglobulin (Sigma-Aldrich) and several monoclonal antibodies (Thermo Fisher Scientific) were reduced, alkylated, and digested with MS-grade trypsin (Thermo Fisher Scientific). Digest mixture was then treated with PNGase F (New England Biolabs) to release *N*-linked glycans. Following deglycosylation, released glycans were separated from peptides using Oasis[®] HLB (Waters) solid phase extraction columns. After drying, glycan samples were labeled with aminoxyTMT reagents (Thermo Fisher Scientific) according to manufacturer's protocol.

Liquid Chromatography

LC-MS experiments were performed in HILIC mode on a Thermo Scientific[™] Accucore[™] Amide column (2.6 μ m, 150 \AA ; 75 μ m \times 150mm). Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Separation was carried out with a 400 nL/min flowrate using different, sample-dependent gradients.

Mass Spectrometry

For direct infusion experiments, labeled glycan samples were dissolved in 50% acetonitrile solution containing 50 μ M NaOH. Samples were analyzed on Thermo Scientific[™] Orbitrap Velos Pro[™] ion trap and Thermo Scientific[™] Orbitrap Fusion[™] Tribrid[™] mass spectrometers in the positive ion mode.

Data Analysis

All data was processed manually.

Results

General Features of aminoxyTMT-labeled Glycans

Two types of the reagent are available, Thermo Scientific™ aminoxyTMTzero™ Label Reagents, the light version of the reagent recommended for method development work, and the heavy, isobaric aminoxyTMT sixplex set (Figure 1). When a labeled glycan is subjected to MS/MS fragmentation by HCD, mass reporter region of the label molecule is cleaved, leading to an intense peak in the low m/z region of MS/MS spectrum. For the aminoxyTMTsixplex set, each member of the set produces a mass reporter ion with a unique m/z , thus allowing for relative quantitation of glycoforms across up to six different samples (Figure 3). The carbonyl-reactive alkoxyamine group reacts rapidly with aldehydes and ketones to yield a stable oxime product, which is resistant to hydrolysis over a broad range of experimental conditions.

Comparison of the fragmentation spectra of the sodiated precursors of unlabeled and aminoxyTMT-labeled glycans (Figure 2) shows that the same structurally relevant product ions are produced for both the labeled and the unlabeled glycans, suggesting that structural information is not lost or diminished after labeling with the aminoxyTMT reagent. Therefore, in addition to the mass reporter ions relevant for quantitation, information which can be derived from the MS/MS spectra of the unlabeled glycans is still present here.

FIGURE 1. Chemical structure of aminoxyTMT reagents and labeling reaction scheme.

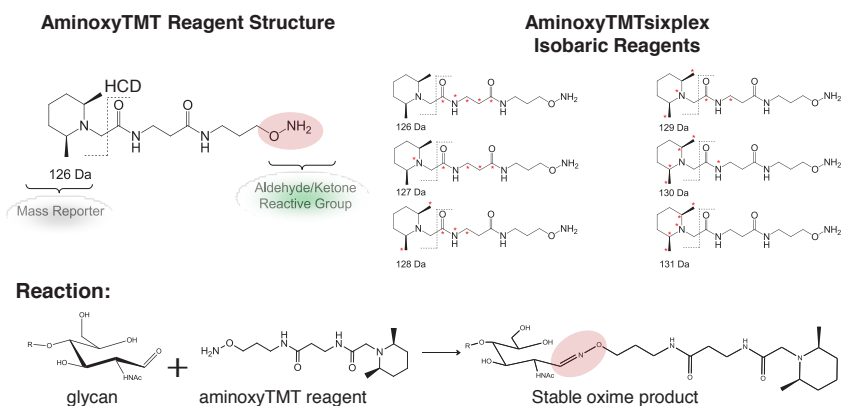


FIGURE 2. Positive ion mode HCD fragmentation of native unlabeled and aminoxyTMT-labeled high-mannose glycans.

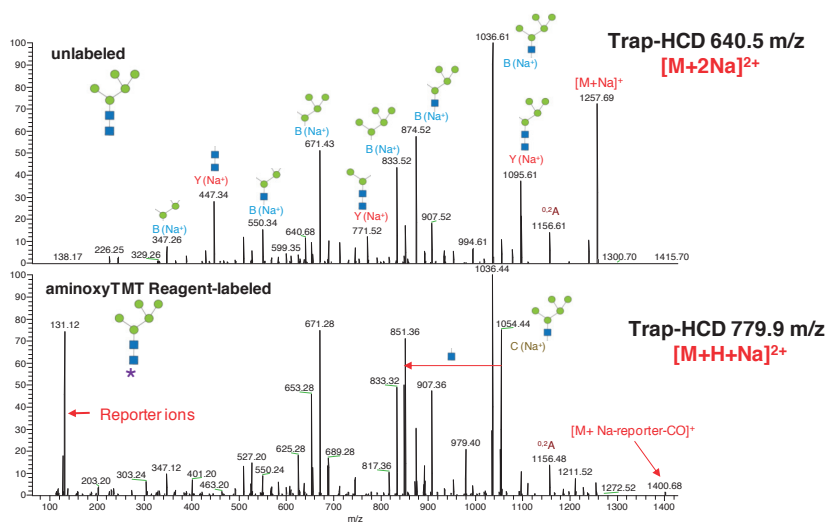
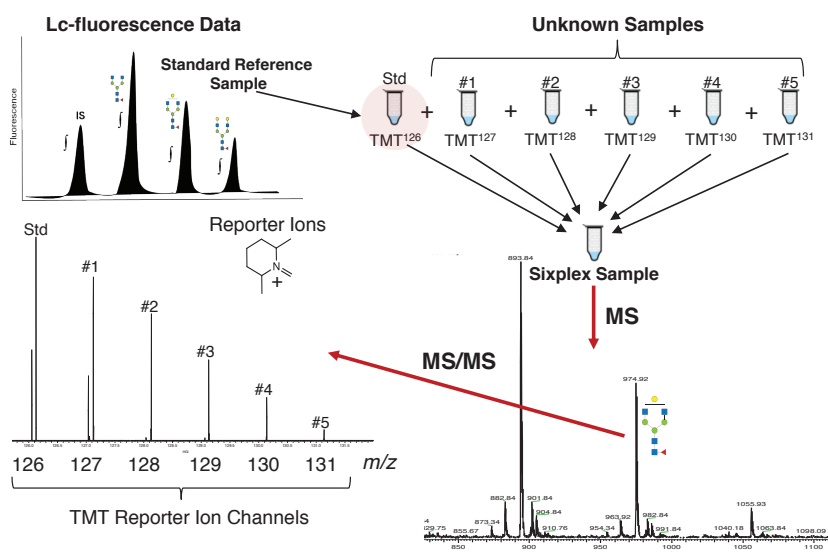


FIGURE 3. Workflow for quantitative glycomics multiplexed experiment.



Multiplexing Glycomic Samples - Overview

In a typical experiment, up to six separate samples may be analyzed together, which allows lower overall analysis time and minimizes the variations in quantitative reproducibility, as the number of independent sample handling steps is reduced in this workflow (Figure 3). After labeling with aminoxyTMT reagents, unreacted reagents are quenched with acetone and samples are combined. An additional clean-up step using HILIC solid phase extraction material is used to separate the quenched reagent from the labeled glycans. During MS analysis, precursor ions corresponding to the glycoforms of interest are identified and are subjected to MS/MS analysis by HCD fragmentation. Relative peak intensities of the reporter ions observed in the MS/MS spectra are proportional to the relative abundance levels of the selected glycoform in different samples in the set.

Several peaks observed in the low m/z region of MS/MS spectra of the labeled glycans may interfere with accurate quantitative analysis if a low resolution mass spectrometer is used (Figure 3). These peaks are some of the product ions of HexNAc fragmentation, and are thus present in MS/MS spectra of most glycoforms.

Although this may be an issue for low resolution instruments, these interferences are easily resolved and do not pose a problem for accurate quantitation at the lowest resolution settings of a Thermo Scientific™ Orbitrap™ mass spectrometer.

If a Velos Pro ion trap mass spectrometer is used for the analysis, application of a targeted MS³ method (Figure 4) solves this interference problem. First, precursor of interest is isolated and fragmented using CID or HCD fragmentation mode. In the second step, abundant Y₁ ions, which still contain intact aminoxyTMT label, are isolated for MS³ HCD. MS³ spectra contain “clean” mass reporter ions and their ratios are now as expected for the sample set.

Multiplexing Glycomic Samples - Examples

Several experiments showcasing the use of these reagents for quantitative analysis of glycans were performed using antibodies as glycan sources. In the first experiment (Figure 5), 100 μg of two anti c-Myc monoclonal antibodies, each coming from a different source, were separately deglycosylated using PNGase F glycosidase, glycans were isolated and each sample was labeled with a different heavy version of the isotopic aminoxyTMT reagent. After quenching and clean-up, the samples were combined and analyzed by direct infusion using a Velos Pro mass spectrometer.

FIGURE 4. Targeted MS³ acquisition approach using a Velos Pro ion trap instrument.

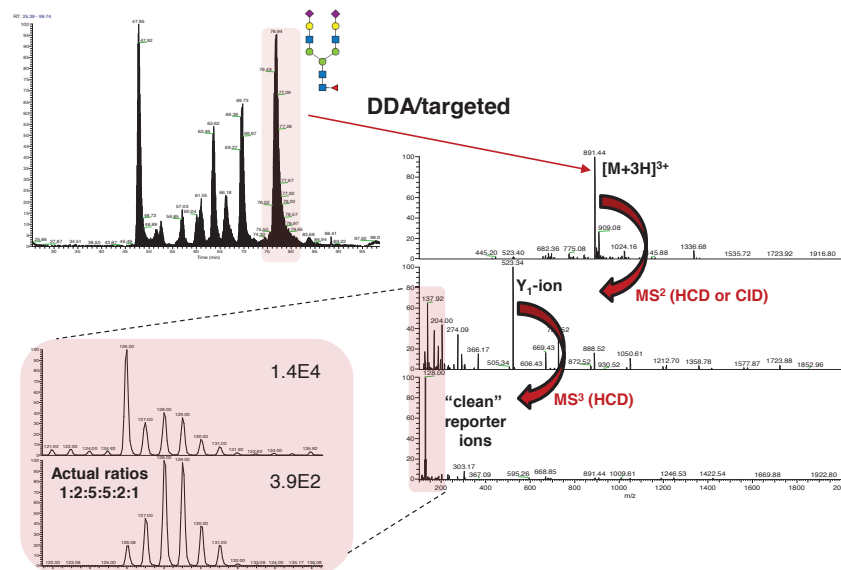
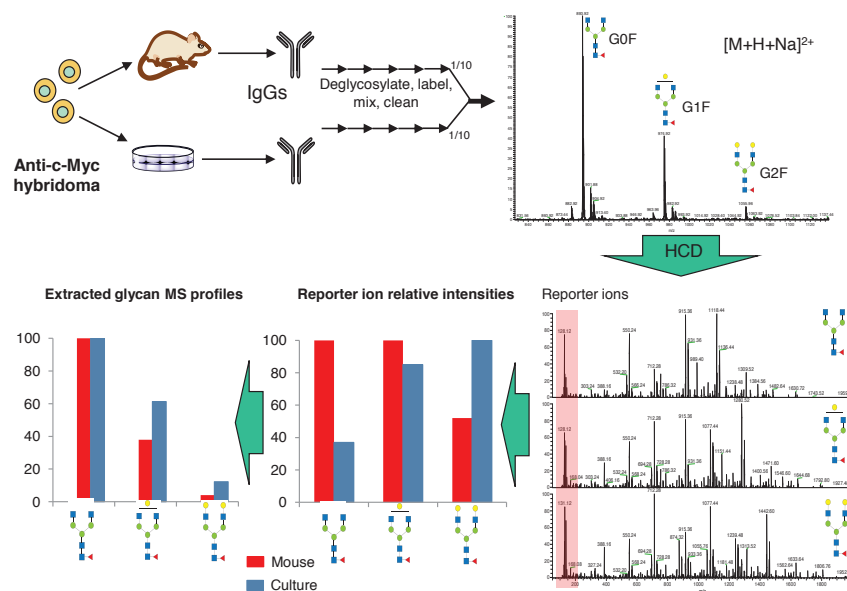


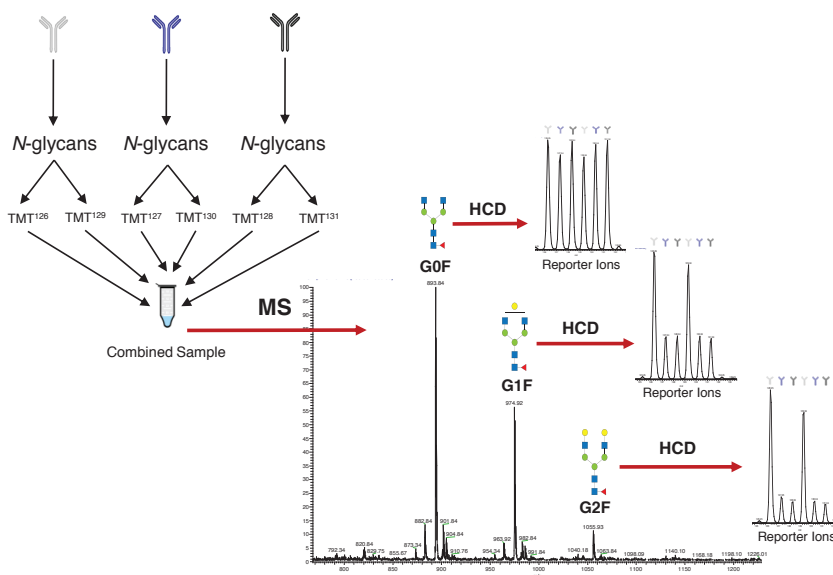
FIGURE 5. Comparing glycosylation profiles of two monoclonal antibodies from different sources.



Relative quantitation of the three main glycoforms, G0F, G1F, and G2F, was performed by comparing relative peak intensities of the mass reporter peaks in the MS/MS spectra for each glycoform. After signal normalization, we found that the antibody produced in the cell culture contained higher amounts of the G1F and G2F glycoforms than the antibodies obtained from the mouse.

In another example (Figure 6), 100 μg of three different monoclonal antibodies were deglycosylated using PNGase F glycosidase, glycans were isolated, and each sample pool was divided into two equal parts. Each part was labeled with a different version of the heavy isobaric aminoxyTMTsixplex reagent, and after a quenching and clean-up, the individual samples were combined to yield a single sixplex sample consisting of three separate samples in a duplicate. The sample was then analyzed by direct infusion MS to reveal that two out of three different antibodies analyzed were identical with respect to glycosylation profiles of the three main glycoforms, with the third antibody having substantially higher abundance of the G1F and G2F glycoforms.

FIGURE 6. Quantitative comparison of glycosylation of several monoclonal antibodies with replicates.



Conclusion

- Increase sample analysis throughput with aminoxyTMT reagents
- Overall improvement in MS sensitivity with aminoxyTMT label approach
- Structural elements are preserved in labeled glycans
- LC-UV/fluorescence and TMT-based quantitation can be complementary
- Ideal for analysis of biotherapeutics and biomarker discovery

Acknowledgements

We would like to thank the following colleagues and collaborators for critical advice and helpful discussions: Dr. Ryan Bomgarden, Dr. Chris Etienne, Dr. Kay Opperman, Dr. Venky Shivalingappa, and Dr. Julian Saba (Thermo Fisher Scientific); Dr. Kay-Hooi Khoo, Dr. Ming-Yi Ho, Dr. Shui-Hua Wang, and Dr. Chia-Wei Lin (Academia Sinica, Taipei, Taiwan); Dr. Ian Pike and Dr. Karsten Kuhn (Proteome Sciences); Dr. Bernhard Küster, Dr. Hannes Hahne, and Dr. Patrick Neubert (Technische Universität München, Munich, Germany); Dr. Lingjun Li, Dr. Xuefei Zhong, and Dr. Yan Liu (University of Wisconsin, Madison, WI, USA); Dr. Yehia Mechref, Shiyue Zhou, and Yunli Hu (Texas Tech University, Lubbock, TX, USA); Dr. Shonali Paul and Dr. Sanjib Meitei (Premier Biosoft).

www.thermoscientific.com

©2014 Thermo Fisher Scientific Inc. All rights reserved. ISO is a trademark of the International Standards Organization. Tandem Mass Tag and TMT are registered trademarks of Proteome Sciences plc. Sigma-Aldrich is a registered trademark of Sigma-Aldrich Co. LLC. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.



Thermo Fisher Scientific,
San Jose, CA USA is
ISO 9001:2008 Certified.

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China 800 810 5118 (free call domestic)
400 650 5118

Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 9 3291 0200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591

Japan +81 45 453 9100
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0

Singapore +65 6289 1190
Spain +34 914 845 965
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

Thermo
S C I E N T I F I C

A Thermo Fisher Scientific Brand