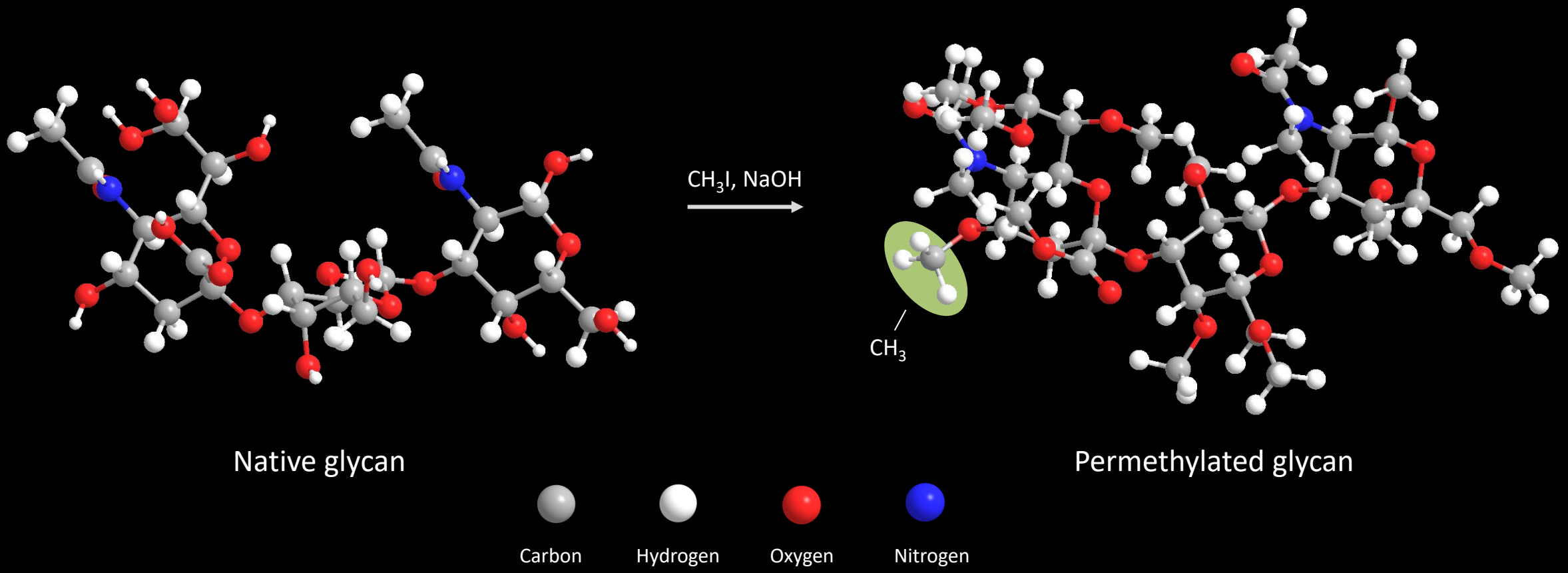
Permethylation of Glycans

LudgerTag™ technology to enable rapid, reliable, high-throughput (HT) MALDI-TOF-MS analysis

Ludger

Glycan Permethylation



Permethylation involves the addition of methyl groups (CH_3) to **all** of the hydroxyl and *N*-acetyl groups, and also methyl esterifies the carboxy function on the sialic acid.

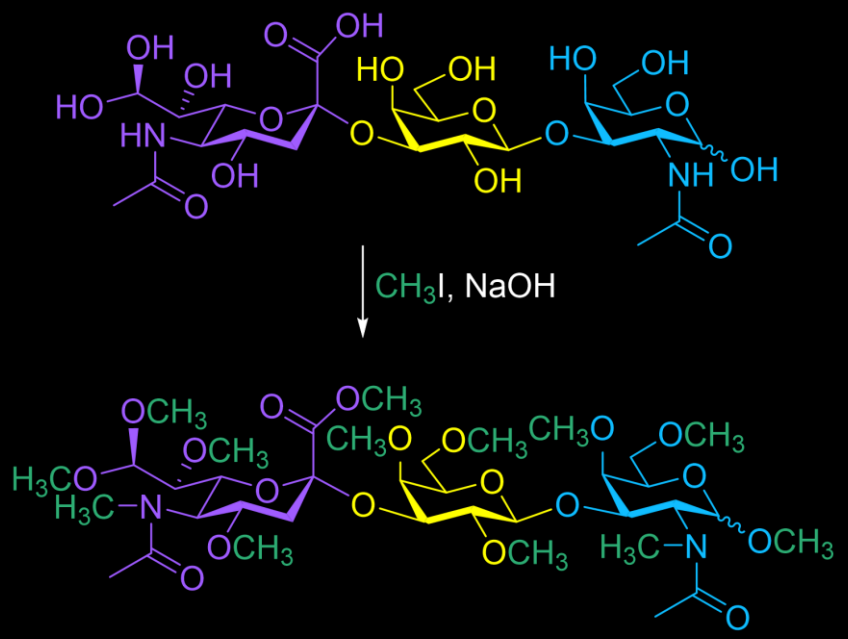
Why Permethylate?

(released N- and O-glycans)

Improves and enhances Ionization efficiency of glycans on mass spec (MALDI-MS, ESI-MS)

When compared to non-derivatized oligosaccharides

Increased glycan hydrophobicity enables LC-MS analysis



Easier determination of branching and glycosidic linkage positions

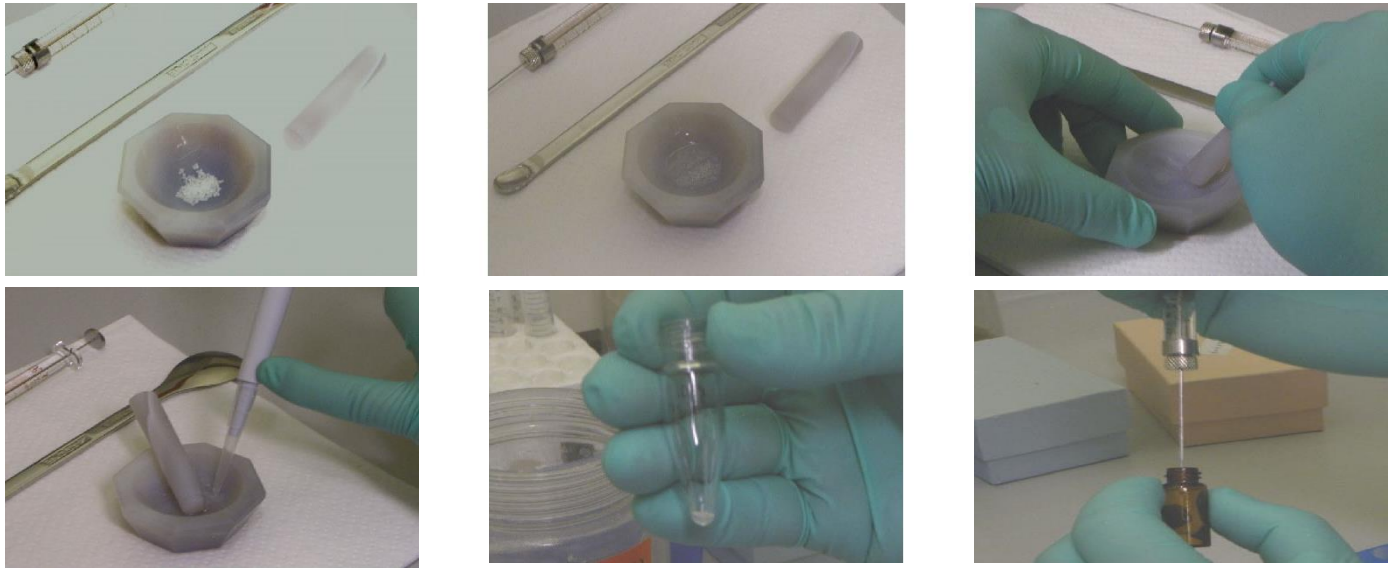
Relative and absolute quantitation can be performed

By introducing isotope labelled internal standards

Enables detection of both neutral and acidic glycans in positive ion mode using MALDI-TOF-MS

Stabilizes the labile sialic acid moieties

Conventional Permethylation Methods are Labour Intensive

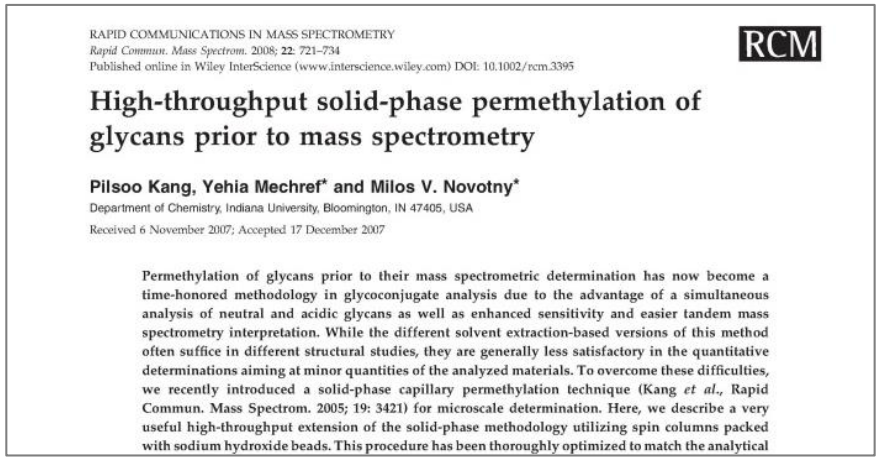


Picture taken from: Akihiko Kameyama, (2014). GlycoPOD http://jcggdb.jp/GlycoPOD. Web.15,8,2014

Slurry method

A slurry of sodium hydroxide in dimethyl sulfoxide needs to be crushed and prepared as shown in the illustration and this slurry needs to be constantly vortexed before adding it to individual sample vials.

This approach is **slow, low throughput** and **labour intensive**.



RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2008; **22**: 721–734
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.3395

RCM

High-throughput solid-phase permethylation of glycans prior to mass spectrometry

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Received 6 November 2007; Accepted 17 December 2007

Permethylation of glycans prior to their mass spectrometric determination has now become a time-honored methodology in glycoconjugate analysis due to the advantage of a simultaneous analysis of neutral and acidic glycans as well as enhanced sensitivity and easier tandem mass spectrometry interpretation. While the different solvent extraction-based versions of this method often suffice in different structural studies, they are generally less satisfactory in the quantitative determinations aiming at minor quantities of the analyzed materials. To overcome these difficulties, we recently introduced a solid-phase capillary permethylation technique (Kang *et al.*, Rapid Commun. Mass Spectrom. 2005; 19: 3421) for microscale determination. Here, we describe a very useful high-throughput extension of the solid-phase methodology utilizing spin columns packed with sodium hydroxide beads. This procedure has been thoroughly optimized to match the analytical

Solid phase methods

Solid phase permethylation techniques have been developed more recently however, many of these **techniques are still labour intensive and repetitive** which are **not practical** for large sample numbers (e.g. for the characterization of biopharmaceuticals).

Reference:
(1) Pilsoo Kang, Y. M. and M. V. N. RAPID Commun. MASS Spectrom. 2008, 22, 721–734.
(2) Jeong, H.-J.; Kim, Y.-G.; Yang, Y.-H.; Kim, B.-G. Anal. Chem. 2012, 84 (7), 3453–3460.
(3) Gao, X.; Zhang, L.; Zhang, W.; Zhao, L. Analyst 2015, 140 (5), 1566–1571.

Ludger Glycan Permethylation Kit (LT-PERMET-96)

Simple to use

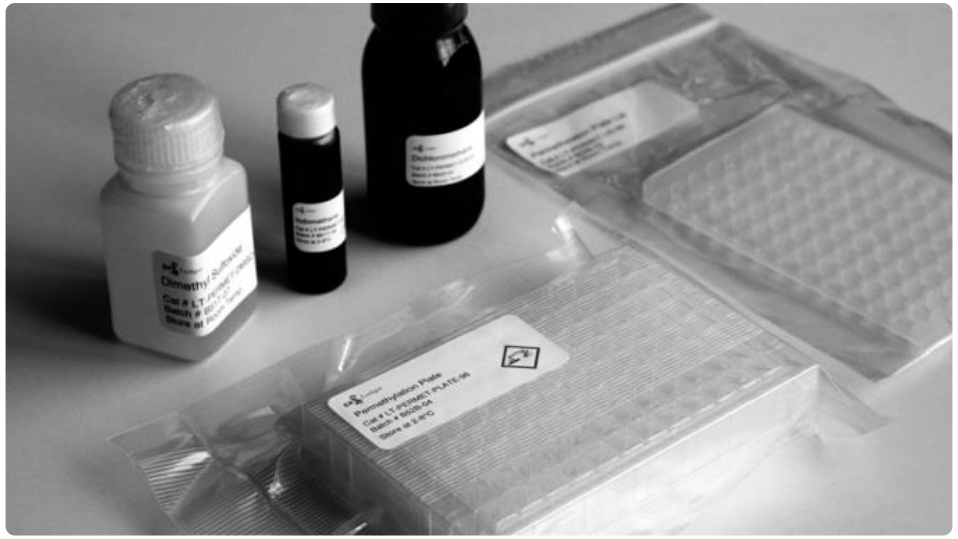
Kit format is less labour intensive than conventional in-solution permethylation

Data is comparable to gold standard HILIC UHPLC data

(2AB / Procainamide labelling)

A microplate based 96-well plate

Format of kit is convenient as it is scalable between 1 to 96 samples.



Reliable and validated according to EMA ICH Q2 (R1) guidelines

Compatible with MALDI-TOF-MS and LC-MS

Absolute quantitation can be achieved by introducing Isotope labelled internal standards.

Method can be automated for HT, rapid sample prep

Workflow can be adapted to a liquid handling robot

Suitable for N- and O-glycan analysis and also aids linkage analysis

Easier determination of branching and glycosidic linkage analysis

Components of the LT-PERMET-96 Kit

1. Derivatisation

Addition of 300µL DMSO to LT-PERMET-96 plate (15 min incubation)

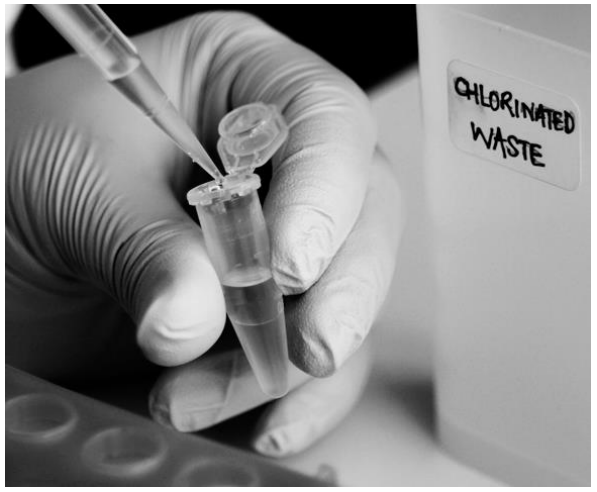
Dimethyl sulfoxide (DMSO)
LT-PERMET-DMSO-96

Addition of 55µL methyl Iodide (1 hour incubation)

Methyl Iodide (MeI)
LT-PERMET-MeI-96

2. Liquid Liquid Extraction

Addition of 400µL DCM and 1ml water

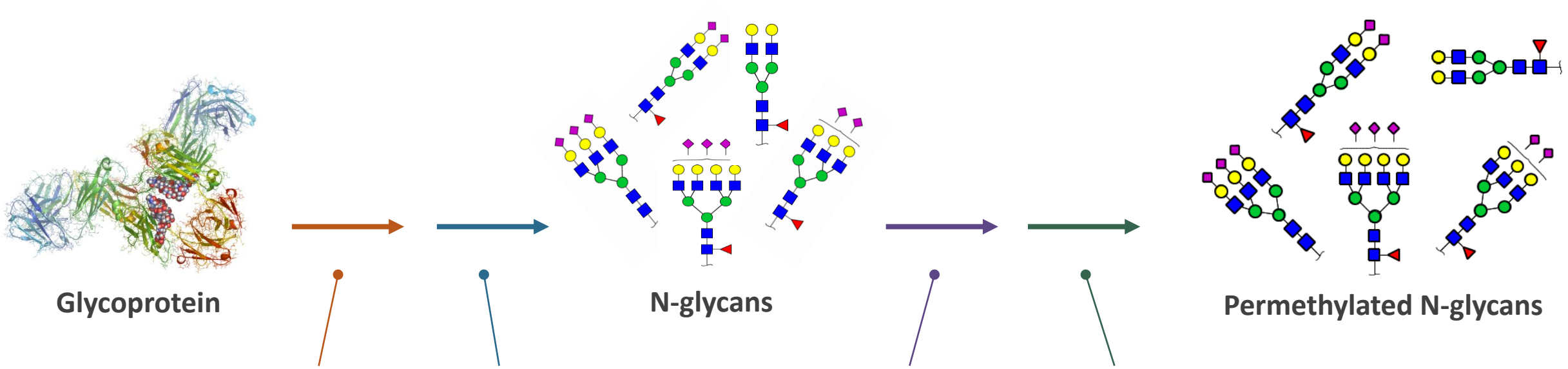


Performing LLE to render the pH neutral prior to MALDI-TOF-MS measurement

Dichloromethane (DCM)
LT-PERMET-DCM-96

Permethylation Workflow using the LT-PERMET-96 kit

Workflow - LudgerTag Permethylation for N-glycan Profiling and Identification



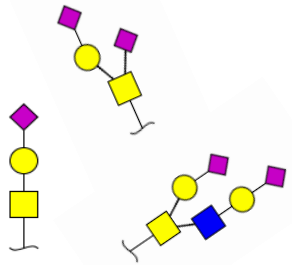
Native Glycans	Fucose	Galactose	Mannose	*N*-Acetylglucosamine	*N*-Acetylneuraminic acid
Permethylated Glycans	Fucose	Galactose	Mannose	*N*-Acetylglucosamine	*N*-Acetylneuraminic acid

Workflow - LudgerTag Permethylation for O-glycan Profiling and Identification

Glycoprotein

LL-HYDRAZ-A2
Hydrazine release

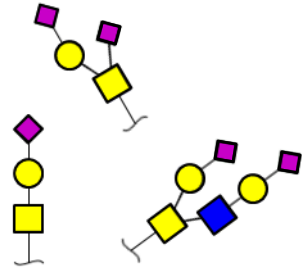
LC-CEX-A6
CEX cartridge clean-up



O-glycans

LT-PERMET-96
Permethylation

LT-PERMET-96
Liquid-liquid extraction



Permethylated O-glycans

Native Glycans	Fucose	Galactose	*N*-Acetylglucosamine	*N*-Acetylgalactosamine	*N*-Acetylneuraminic acid
Permethylated Glycans	Fucose	Galactose	*N*-Acetylglucosamine	*N*-Acetylgalactosamine	*N*-Acetylneuraminic acid

Recommended Components for Workflow – LudgerTag Permethylation for N- and O-glycan Profiling

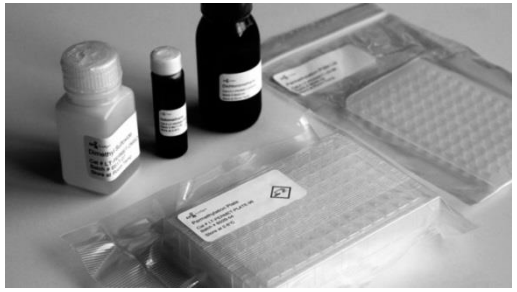
Product	Product code	N- glycans	O- glycans
PNGase F N-glycan release	E-PNG-01	●	
Hydrazinolysis or Orela O-glycan release kit	LL-HYDRAZ-A2* Technical guide		●
	LL-ORELA-A2 Technical guide		●
Enrichment plate	LC-PERMET-96 Technical guide	●	
Cation exchange clean-up cartridges	LC-CEX-A6 Technical guide		●
Permethylation kit	LT-PERMET-96 Technical guide	●	●

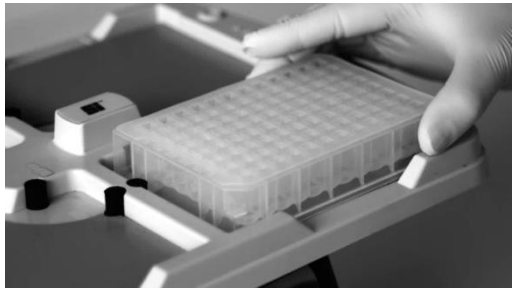
*Note: although hydrazinolysis can also be used for N glycan release, we recommend PNGase F release for the workflow

LT-PERMET-96: Manual Workflow

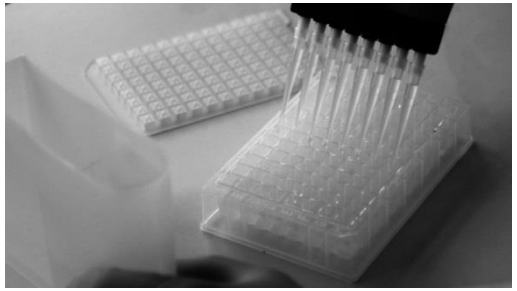
LT-PERMET-96: Manual Procedure

Addition of DMSO, MeI and Incubation

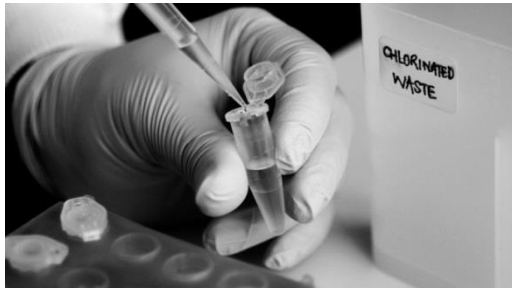


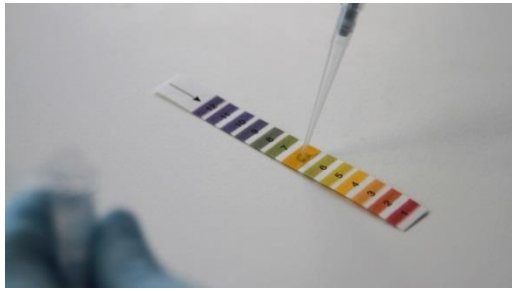



Addition of DCM and water

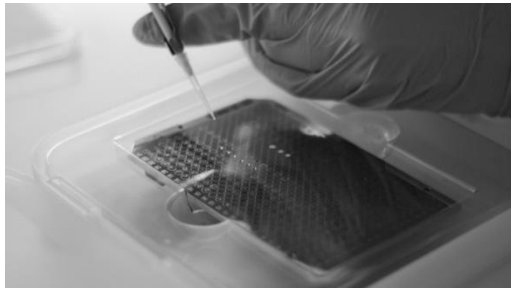
Liquid-liquid extraction

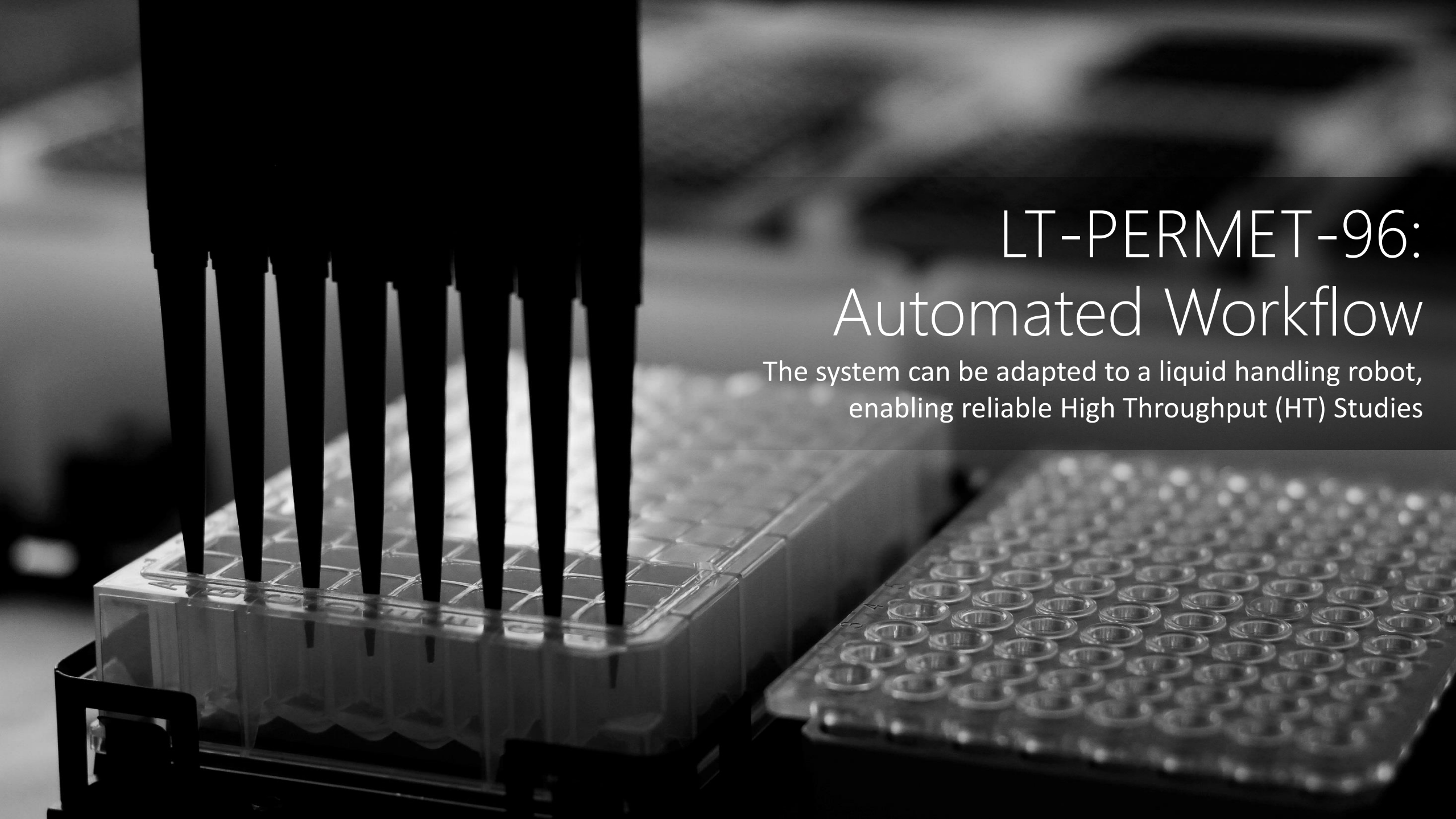


Ready for Analysis



Click for our detailed procedure: LT-Permet-96 product guide



LT-PERMET-96: Automated Workflow

The system can be adapted to a liquid handling robot, enabling reliable High Throughput (HT) Studies

LT-PERMET-96: Automated HT Workflow

Typical timeline for 96 samples

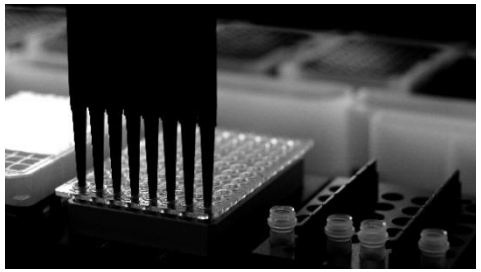
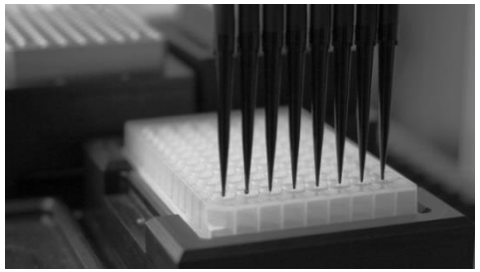
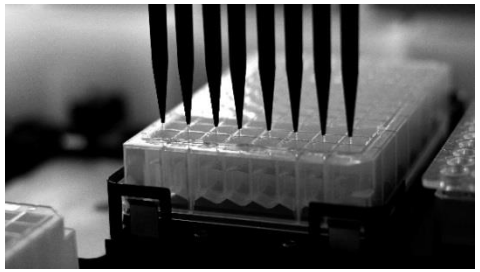
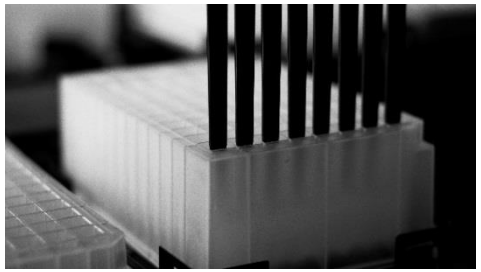
2h
(16h incubation 37C°)

2.5h
(Plus drying time)

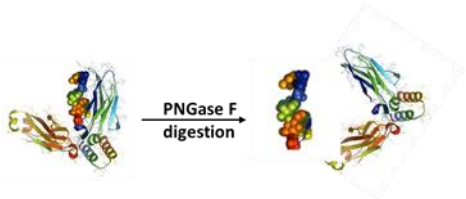
1h
(Plus 1.5h incubation)

3h
(Plus drying time)

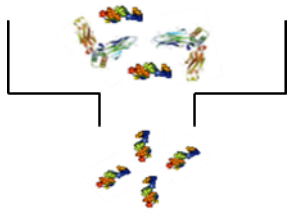
~1min/sample = 1.6h
for data acquisition

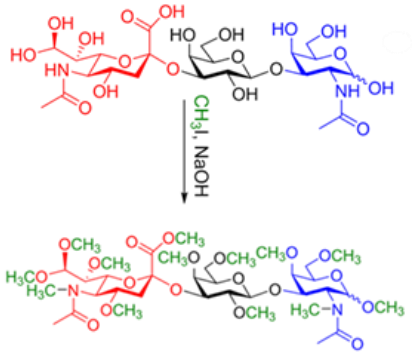

1. Automated PNGase-F release



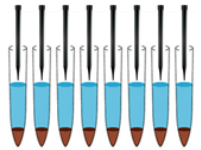
2. Automated HILIC-SPE enrichment



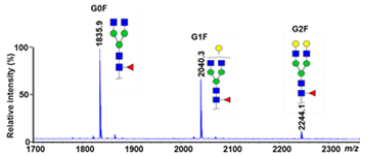
3a. Automated Permethylation



3b. Automated Liquid-Liquid Extraction (LLE)



4. Data acquisition (MALDI-TOF-MS)



Examples of how we're using the LT-PERMET-96 system at Ludger

Permethylated and 2-AB Labelled Human IgG N-glycan Profiles are Comparable

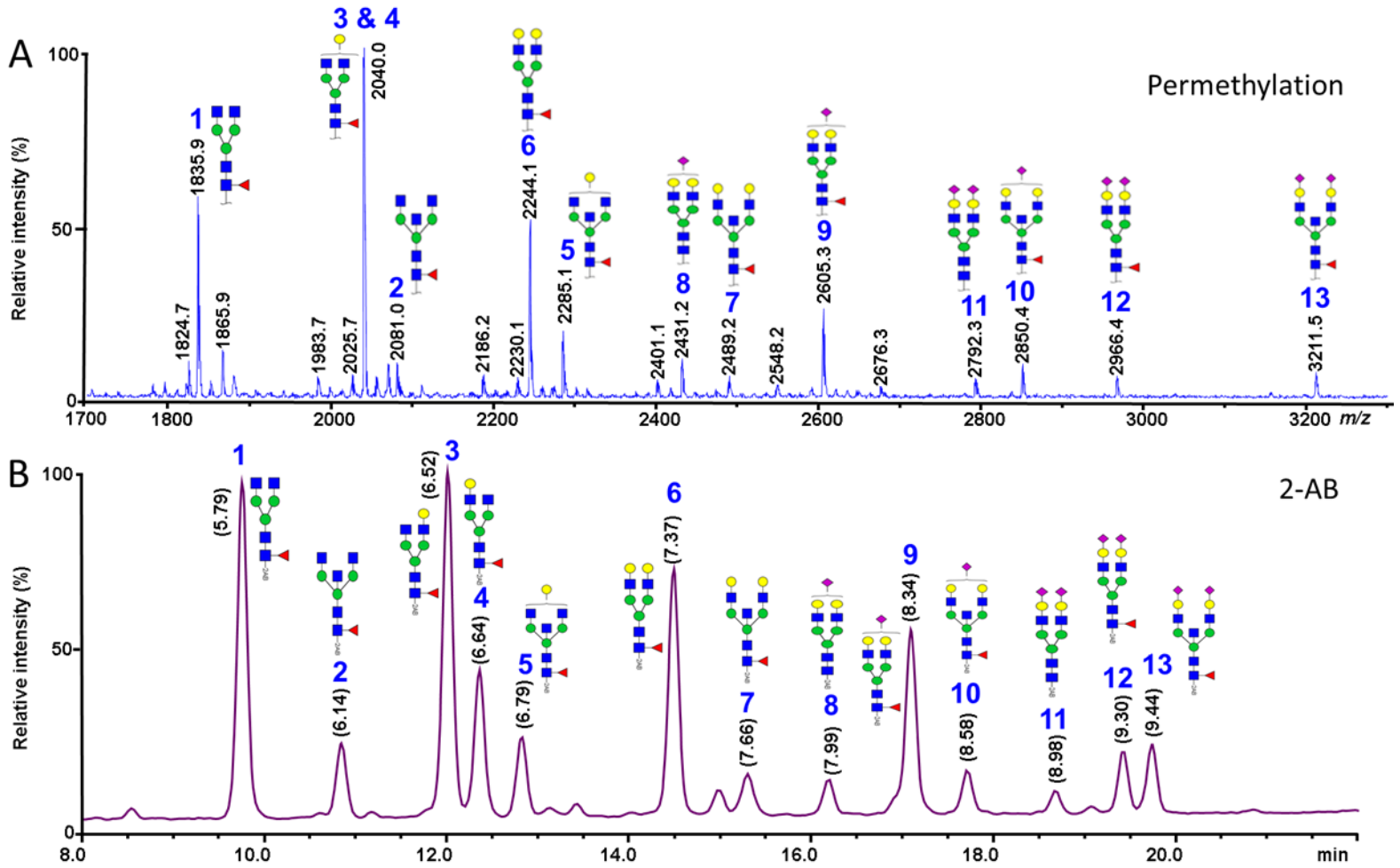


Figure 1. Comparison of PNGase F released and purified human IgG N-glycans analyzed with orthogonal methods (A) MALDI-TOF-MS spectrum of permethylated human-IgG N-glycans (B) 2-AB labelled HILIC UHPLC chromatogram.

Fucose Galactose Mannose *N*-Acetylglucosamine *N*-Acetylneuraminic acid

- We analysed human IgG N-glycans using the automated HT permethylation method and compared the data to those obtained from UHPLC analysis by fluorescence detection as shown in Figure 1 Section A and B.
- Glycan signals were integrated, normalized and the relative intensities and standard deviation was calculated for the 13 major N-glycan peaks.
- The analysis confirmed that peaks with higher relative intensities (above 4%) showed good correlation between the two methods.
- Therefore we conclude that the HT permethylation technique is comparable to UHPLC results and that it gives a reliable overview of the glycosylation profile in a short timespan.

Permethylation Stabilises Fragile Sialic Acids

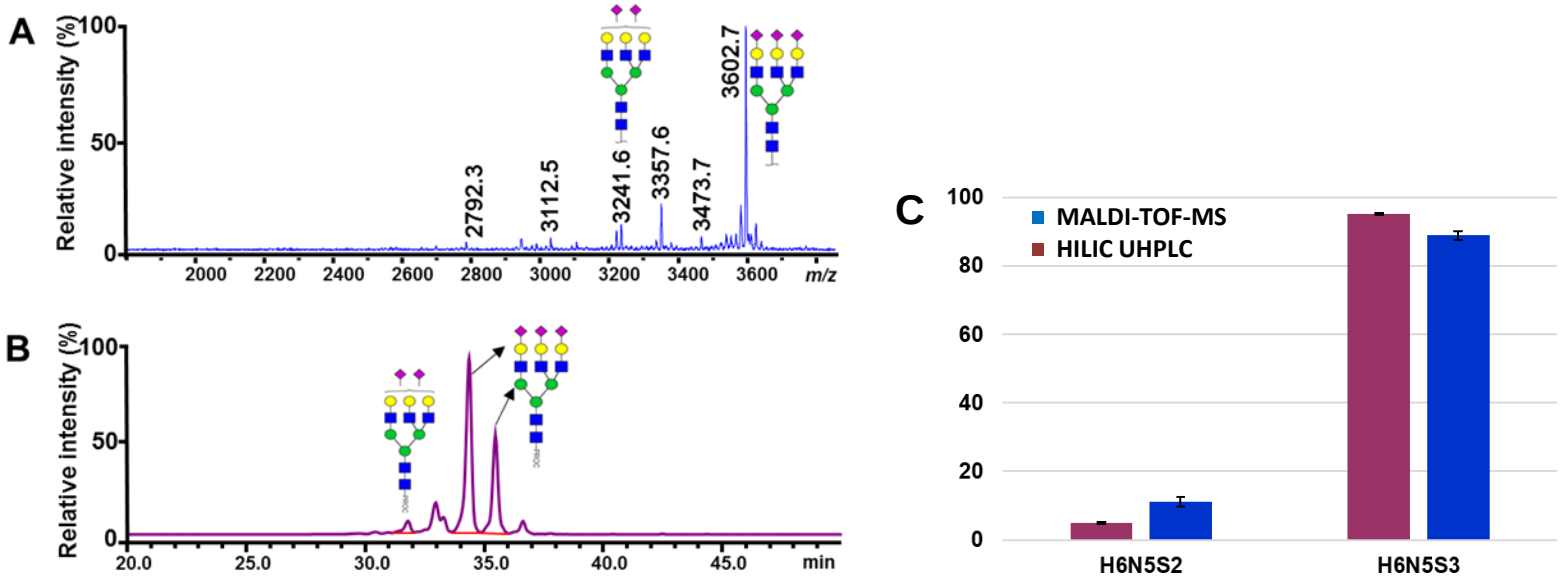
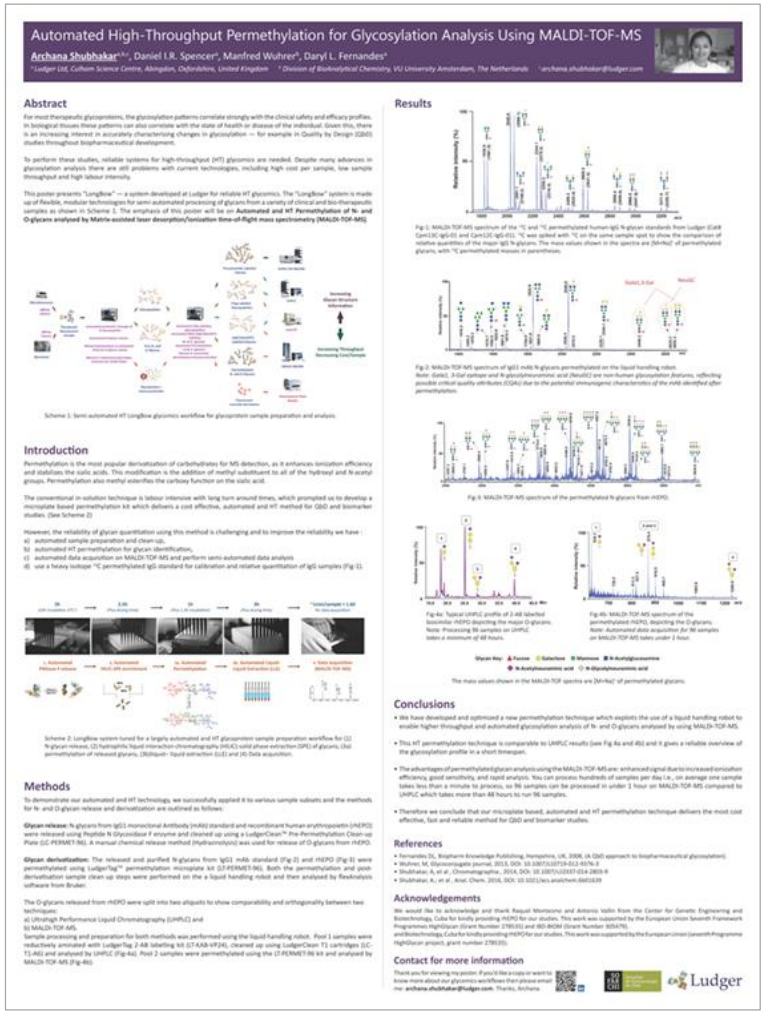


Figure 2. Comparison of the glycosylation profiles of the A3G3S3 N-glycan standard analyzed after sample preparation using the liquid handling robot. (A) MALDI-TOF-MS spectrum after permethylation, (B) HILIC UHPLC chromatogram with fluorescence detection after procainamide labelling. (C) Histogram comparing the relative peak intensities of triantennary, disialylated structures (H6N5S2) and triantennary, trisialylated structures (H6N5S3) after triplicate analysis. The histogram shows comparable relative signal intensities between MALDI-TOF-MS and HILIC UHPLC analysis. The error bars depict standard deviation.

- The relative intensities of triantennary, disialylated structures (H6N5S2) and triantennary, trisialylated structures (H6N5S3) from the A3G3S3 glycan standard were determined by MALDI-TOF-MS after automated HT permethylation.
- This data was then compared to the ratios obtained after procainamide labeling followed by HILIC UHPLC with fluorescence detection as shown in Figure 2.
- Triplicate analysis and relative quantitation was performed for both fluorescent labeling and permethylation.
- The analysis confirmed that the MALDI-TOF-MS data from the sialylated N-glycan standard gave similar and comparable results to that of the UHPLC data.

The LT-PERMET-96 System is Reliable for Semi-automated, HT MS based glycomics



Automated High-Throughput Permethylation for Glycosylation Analysis Using MALDI-TOF-MS

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[a]Ludger Ltd, Culham Science Centre, Abingdon, Oxfordshire, United Kingdom [b]Division of Bioanalytical Chemistry, VU University Amsterdam, The Netherlands [c]archana.shubhakar@ludger.com

Abstract

For most therapeutic glycoproteins, the glycosylation patterns correlate strongly with the clinical safety and efficacy profiles. In biological tissues these patterns can also correlate with the state of health or disease of the individual. Given this, there is an increasing interest in accurately characterising changes in glycosylation — for example in Quality by Design (QbD) studies throughout biopharmaceutical development.

To perform these studies, reliable systems for high-throughput (HT) glycomics are needed. Despite many advances in glycosylation analysis there are still problems with current technologies, including high cost per sample, low sample throughput and high labour intensity.

This poster presents "LongBow" — a system developed at Ludger for reliable HT glycomics. The "LongBow" system is made up of flexible, modular technologies for semi-automated processing of glycans from a variety of clinical and bio-therapeutic samples as shown in Scheme 1. The emphasis of this poster will be on **Automated and HT Permethylation of N- and O-glycans analysed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).**

Scheme 1: Semi-automated HT LongBow glycomics workflow for glycoprotein sample preparation and analysis.

Introduction

Permethylation is the most popular derivatization of carbohydrates for MS detection, as it enhances ionization efficiency and stabilises the sialic acids. This modification is the addition of methyl substituent to all of the hydroxyl and N-acetyl groups. Permethylation also methyl esterifies the carboxy function on the sialic acid.

The conventional in-solution technique is labour intensive with long turn around times, which prompted us to develop a microplate based permethylation kit which delivers a cost effective, automated and HT method for QbD and biomarker studies. (See Scheme 2)

However, the reliability of glycan quantitation using this method is challenging and to improve the reliability we have :

a) automated sample preparation and clean-up,
b) automated HT permethylation for glycan identification,
c) automated data acquisition on MALDI-TOF-MS and perform semi-automated data analysis
d) use a heavy isotope ^{13}C permethylated IgG standard for calibration and relative quantitation of IgG samples (Fig-1).

Scheme 2: LongBow system tuned for a largely automated and HT glycoprotein sample preparation workflow for (1) N-glycan release, (2) hydrophilic liquid interaction chromatography (HILIC) solid phase extraction (SPE) of glycans, (3a) permethylation of released glycans, (3b)liquid - liquid extraction (LLE) and (4) Data acquisition.

Methods

To demonstrate our automated and HT technology, we successfully applied it to various sample subsets and the methods for N- and O-glycan release and derivatization are outlined as follows:

Glycan release: N-glycans from IgG1 monoclonal Antibody (mAb) standard and recombinant human erythropoietin (rHEPO) were released using PeptideN Glycosidase F enzyme and cleaned up using a LudgerClean™ Pre-Permethylation Clean-up Plate (LC-PERMET-96). A manual chemical release method (Hydrazinolysis) was used for release of O-glycans from rHEPO.

Glycan derivatization: The released and purified N-glycans from IgG1 mAb standard (Fig-2) and rHEPO (Fig-3) were permethylated using LudgerTag™ permethylation microplate kit (LT-PERMET-96). Both the permethylation and post-derivatisation sample clean up steps were performed on the a liquid handling robot and then analysed by flexAnalysis software from Bruker.

The O-glycans released from rHEPO were split into two aliquots to show comparability and orthogonality between two techniques:

a) Ultrahigh Performance Liquid Chromatography (UHPLC) and
b) MALDI-TOF-MS.

Sample processing and preparation for both methods was performed using the liquid handling robot. Pool 1 samples were reductively aminated with LudgerTag 2-AB labelling kit (LT-KAB-VP24), cleaned up using LudgerClean T1 cartridges (LC-T1-AG) and analysed by UHPLC (Fig-4a). Pool 2 samples were permethylated using the LT-PERMET-96 kit and analysed by MALDI-TOF-MS (Fig-4b).

Results

Fig-1: MALDI-TOF-MS spectrum of the ^{12}C and ^{13}C permethylated human IgG N-glycan standards from Ludger (Cat# Cpm12C-IgG-01 and Cpm12C-IgG-01). ^{13}C was spiked with ^{13}C on the same sample spot to show the comparison of relative quantities of the major IgG N-glycans. The mass values shown in the spectra are [M+Na]+ of permethylated glycans, with ^{13}C permethylated masses in parentheses.

Fig-2: MALDI-TOF-MS spectrum of IgG1 mAb N-glycans permethylated on the liquid handling robot.
Note: Galα1, 3-Gal epitope and N-glycolylneuraminic acid (Neu5Gc) are non-human glycosylation features, reflecting possible critical quality attributes (CQAs) due to the potential immunogenic characteristics of the mAb identified after permethylation.

Fig-3: MALDI-TOF-MS spectrum of the permethylated N-glycans from rHEPO.

Fig-4a: Typical UHPLC profile of 2-AB labelled bisimilar rHEPO depicting the major O-glycans.
Note: Processing 96 samples on UHPLC takes a minimum of 48 hours.

Fig-4b: MALDI-TOF-MS spectrum of the permethylated rHEPO, depicting the O-glycans.
Note: Automated data acquisition for 96 samples on MALDI-TOF-MS takes under 1 hour.

Glycan Key: Fucose, Galactose, Mannose, N-Acetylglucosamine, N-Acetylneuraminic acid, N-Glycolylneuraminic acid

The mass values shown in the MALDI-TOF spectra are [M+Na]+ of permethylated glycans.

Conclusions

- We have developed and optimized a new permethylation technique which exploits the use of a liquid handling robot to enable higher throughput and automated glycosylation analysis of N- and O-glycans analysed by using MALDI-TOF-MS.
- This HT permethylation technique is comparable to UHPLC results (see Fig 4a and 4b) and it gives a reliable overview of the glycosylation profile in a short timespan.
- The advantages of permethylated glycan analysis using the MALDI-TOF-MS are: enhanced signal due to increased ionization efficiency, good sensitivity, and rapid analysis. You can process hundreds of samples per day i.e., on average one sample takes less than a minute to process, so 96 samples can be processed in under 1 hour on MALDI-TOF-MS compared to UHPLC which takes more than 48 hours to run 96 samples.
- Therefore we conclude that our microplate based, automated and HT permethylation technique delivers the most cost effective, fast and reliable method for QbD and biomarker studies.

References

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Acknowledgements

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and Biotechnology, Cuba for kindly providing rHEPO for our studies. This work was supported by the European Union Seventh Programme HighGlycan project, grant number 278535).

Contact for more information

Thank you for viewing my poster. If you'd like a copy or want to know more about our glycomics workflows then please email me: archana.shubhakar@ludger.com. Thanks, Archana

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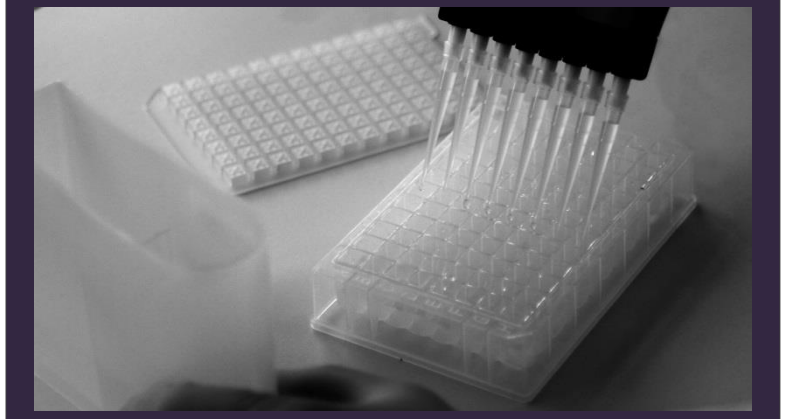

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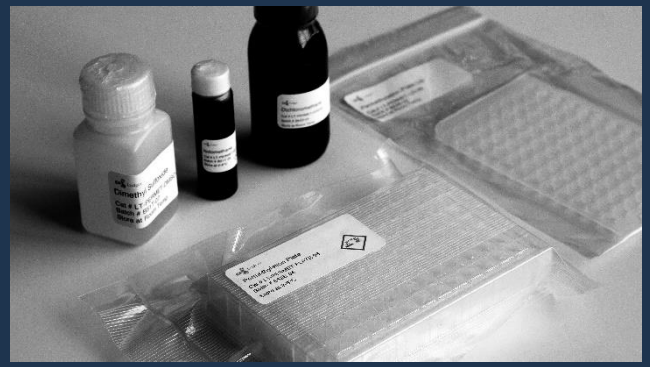

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