Cambridge Isotope Laboratories, Inc.
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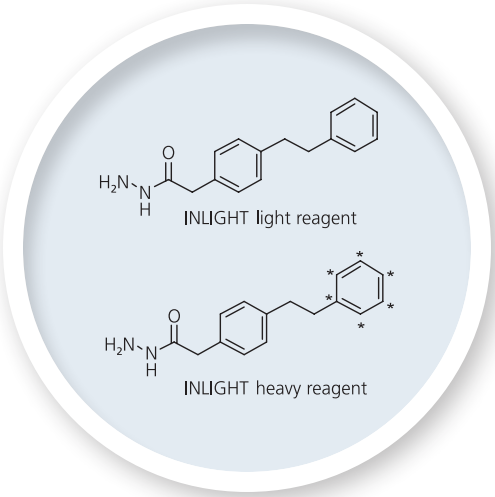
RESEARCH PRODUCTS

INLIGHT® Strategy

N-Linked Glycan Cleavage and Derivatization via Hydrazone Formation



Cambridge Isotope Laboratories, Inc.'s INLIGHT® Glycan Tagging Kit contains natural (light or NAT) and stable ^{13}C-labeled (heavy or SIL) hydrazide reagents for derivatization of free *N*-glycans. The INLIGHT protocol was developed for tagging *N*-glycans isolated from glycoproteins for LC-MS relative quantification.[1-7] This kit features an updated sample preparation protocol that reduces the necessary amount of tagging reagent resulting in simpler sample preparation and an increase in MS ion sensitivity.[8] This updated protocol also includes the parameters for analyzing the *N*-linked glycans using both nano-liquid chromatography (nano-LC) and ultra-high-pressure liquid chromatography (UHPLC) separation techniques. Maltoheptaose is provided for use as a quality-control reagent or internal oligosaccharide standard. The INLIGHT Glycan Tagging Kit (GTK-1000) contains five vials of light reagent and five vials of heavy reagent, which in total provides sufficient tagging for approximately 125 relative quantification experiments (equates to 25 reactions per vial).

Instruction for Use

The following pages contain step-by-step instructions for "INLIGHT Strategy: *N*-Linked Glycan Cleavage and Derivatization via Hydrazone Formation" including the derivatization and analysis of maltoheptaose and *N*-linked glycans from the glycoprotein bovine fetuin A. To ensure appropriate LC-MS conditions, the analysis of maltoheptaose (Malto) and glycoprotein fetuin A (Fet) is highly recommended as a first step in using the INLIGHT strategy for glycan analysis. Maltoheptaose is a linear oligosaccharide (comprises 7 hexose units), and fetuin A is a glycoprotein (comprises 3 glycosites). This will allow proper optimization and troubleshooting of each step, paying particular attention to:

a) time and resource constraints as well as

b) LC-MS platform verification/optimization.

After the INLIGHT strategy has been successfully applied to these well-characterized samples (see **Figure 1**), the strategy may be applied with confidence to complex biological samples (e.g., human plasma[3,8]). The INLIGHT strategy has also been successfully applied to the analysis of *O*-linked glycans.[9]

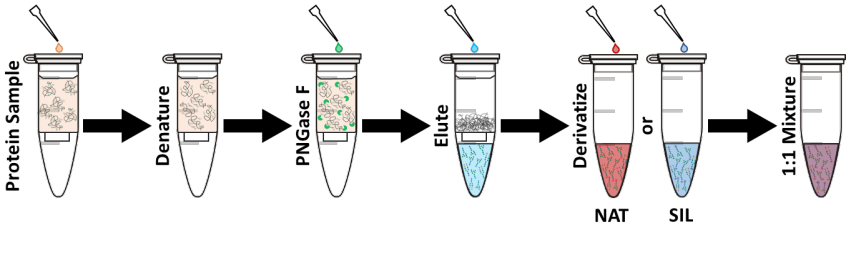


Figure 1. INLIGHT strategy workflow. Overview of glycan sample preparation, which concludes with 1:1 mixing of NAT and SIL pairs for LC-MS/MS analysis. See Kalmar JG et al. 2020 ABC manuscript (**PMID: 32844281**)[8] for additional information.

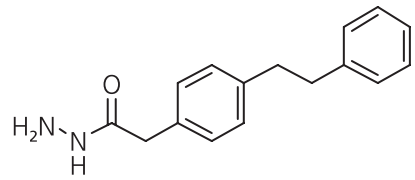
Continued ➤

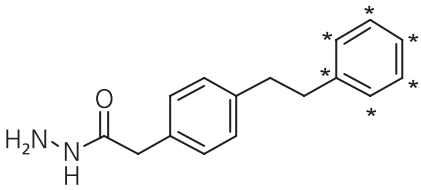
Kit Contents

- Light phenyl 2-GPN INLIGHT reagent (5 × 0.25 mg)
- Heavy phenyl 2-GPN INLIGHT reagent ($^{13}C_6$; 5 × 0.25 mg)
- Unlabeled maltoheptaose (5 × 10 µg)

All kit components are to be stored at room temperature away from light and moisture.

Note: Phenyl 2-GPN, as structurally illustrated here, refers to 2-(4-phenethylphenyl) acetohydrazide.





Required Reagents

Solvents	Manufacturer	Part Number/Catalog Number
Acetonitrile (LC-MS grade)	Fisher Scientific	A955-4
Methanol (LC-MS grade)	Fisher Scientific	A456-4
Water (LC-MS grade)	Fisher Scientific	W6-4
Formic acid	Fisher Scientific	PI85178
Acetic acid	Fisher Scientific	A11350
Reagents and Other Supplies	**Manufacturer**	**Part Number/Catalog Number**
10 kDa MW cut off (MWCO) filters	Sigma Aldrich	UFC5003BK
1 M Dithiothreitol (DTT)	Sigma Aldrich	646563-10VL
Iodoacetimide (IAA)	Sigma Aldrich	A3221-10VL
Bovine Fetuin A	Sigma Aldrich	F3385-1G
Ammonium bicarbonate	Fisher Scientific	A643500
Glycerol-free PNGase F	Bulldog Bio	NZPP010LY

Instruction for Use

A. Solution and Buffer Preparation

1. Digest buffer – 100 mM ammonium bicarbonate – dissolve 1.97 g in 250 mL LC-MS grade water
2. 1 M Iodoacetimide – dissolve 56 g in 303 µL of digest buffer
3. 25 µg/µL Maltoheptaose solution – dissolve 50 µg in 100 uL of LC-MS grade water

Standard Samples: Maltoheptaose and Bovine Glycoprotein Fetuin A

Sample Name	Sample Description	Tagging Reagent
Malto L	Maltoheptaose (50 µg)	Light
Malto H	Maltoheptaose (50 µg)	Heavy
Fet L	Bovine Fetuin (250 µg)	Light
Fet H	Bovine Fetuin (250 µg)	Heavy

B. Glycoprotein Sample Preparation

1. Add 250 µg of Fet from each sample to separate 10 kDa MWCO filters. Place a centrifuge tube beneath each to capture the flow through.
   i. For Malto analysis, begin at step D2 using 50 µg material.
2. Denature the proteins by adding 2 µL of 1 M DTT to each filter.
3. Dilute with 200 µL of 100 mM ammonium bicarbonate (digest buffer).
4. Incubate the samples for 30 minutes at 56°C.
5. To denature the proteins and keep them from reforming their secondary structures, alkylate the free thiol groups of the proteins by adding 50 µL of 1 M IAA to each filter.
6. Incubate for 60 minutes at 37°C.
7. Concentrate the proteins by centrifuging the samples at 14,000 × g for 40 minutes.
8. Wash the samples by adding 100 µL of 100 mM ammonium bicarbonate (digest buffer) to each filter then centrifuge for 20 minutes.
9. Repeat Step 8 twice more for a total of three washes.
10. Remove filters and place onto new centrifuge tubes. Discard old centrifuge tubes containing the flow through.

C. PNGase F Enzymatic Digestion and Glycan Elution

1. ***CRITICAL STEP*** Enzymatically cleave the *N*-linked glycans from the proteins by adding 2 µL of PNGase F (1,000 units) to the filters.
2. Dilute each sample with 98 µL of 100 mM ammonium bicarbonate (digest buffer) and mix by pipetting up and down.
3. Incubate the samples in an oven for 18 hours at 37°C.
4. Elute the cleaved glycans by centrifuging at 14,000 × g for 20 minutes.
5. Continue elution by adding 100 µL of 100 mM ammonium bicarbonate (digest buffer) and centrifuging at 14,000 × g for 20 minutes.
6. Repeat Step 5 above twice more for a total of three times.
7. Discard the filters.
8. Place the centrifuge tubes in -80°C freezer until frozen (~30 minutes).
9. Once the samples are completely frozen, place centrifuge tubes in vacuum concentrator at room temperature for 4 hours or until completely dry.

PAUSE POINT At this point, the samples can be stored at -20°C for up to six months. !

D. Glycan Derivatization

1. Reconstitute the NAT and SIL INLIGHT reagents in 250 µL of LC-MS grade methanol (final concentration of each reagent will be 0.25 µg/µL) and vortex for 10 minutes to ensure complete solubilization of the reagents.
2. ***CRITICAL STEP*** Derivatize each glycosample by adding 10 µL of NAT reagent to one and 10 µL of SIL reagent to the other.
3. ***CRITICAL STEP*** Dilute each sample with 45 µL of LC-MS grade methanol and 45 µL acetic acid, resulting in a tagging solution of 0.1 µg/µL in 55:45 (v/v) of methanol to acetic acid.
4. Incubate the samples at 37°C for 1 hour and 45 minutes.
5. Immediately place the samples in a vacuum concentrator and set to a temperature of 55°C.
6. ***CRITICAL STEP*** Dry for 1 hour or until completely dry. As this quenches the derivatization reaction, all solvent must be evaporated before LC-MS analysis.

PAUSE POINT The completely dry samples can be stored at -20°C for up to six months. !

E. LC-MS Preparation

1. Reconstitute the NAT derivatized sample and the SIL derivatized sample in LC-MS grade water. Pipet up and down to fully suspend the labeled glycans. For optimal ion abundance:
   i. Reconstitute the samples in 50 µL of LC-MS grade water for nanoLC-MS/MS.
   ii. Reconstitute the samples in 25 µL of LC-MS grade water for UHPLC-MS/MS.
2. ***CRITICAL STEP*** Centrifuge samples for 5 minutes at 14,000 × g to remove excess tag. The unreacted tag will be at the bottom of the tube, but this may not be visible to the eye.
3. Carefully pipet the supernatant (containing derivatized glycans) into a new sample tube, avoiding the pellet at the bottom of the centrifuge tube.
4. Combine the pair of NAT and SIL derivatized samples 1:1 v/v for relative quantification. Vortex the sample for 3 minutes.
   i. For nanoLC MS/MS, inject 5 µL on column.
   ii. For UHPLC MS/MS, inject 10 µL on column.
5. Use the maltoheptaose to assess the derivatization technique and the bovine fetuin results to further verify the derivatization technique, as well as to help establish a separation technique for more complex samples.

F. LC-MS Data Processing

Nano Reversed-Phase HPLC Analysis

Trap Column: C_{18} Acclaim™ PepMap™ trap column (2 cm × 75 µm, 3 µm particles)
Analytical Column: EASY-Spray Column (25 cm × 75 µm, 2 µm particles)
Autosampler Temperature: 4°C
Column Temperature: 35°C
Injection Volume: 5 µL
Flow rate: 300 nL/min
Mobile Phase A: 98% water, 2% acetonitrile, 0.1% formic acid
Mobile Phase B: 80% acetonitrile, 20% water, 0.1% formic acid

Nano Reversed-Phase HPLC (C_{18}) Gradient		
Time (min)	Flow Rate (nL/min)	%B
0.0	300	5.0
2.0	300	35.0
42.0	300	70.0
43.0	300	95.0
49.0	300	95.0
50.0	300	5.0
60.0	300	5.0

Reversed-Phase UHPLC Analysis

Analytical Column: Waters UPLC BEH C_{18} (100 × 2.1 mm, 1.7 µm particles)
Autosampler Temperature: 4°C
Column Temperature: 35°C
Injection Volume: 10 µL
Flow rate: 0.1 mL/min
Mobile Phase A: 98% water, 2% acetonitrile, 0.1% formic acid
Mobile Phase B: 98% acetonitrile, 2% water, 0.1% formic acid

Reversed-Phase UHPLC (C_{18}) Gradient		
Time (min)	Flow Rate (mL/min)	%B
0.0	0.1	5.0
1.0	0.1	5.0
3.0	0.1	35.5
23.0	0.1	37.0
24.0	0.1	95.0
26.0	0.1	95.0
27.0	0.1	5.0
30.0	0.1	5.0

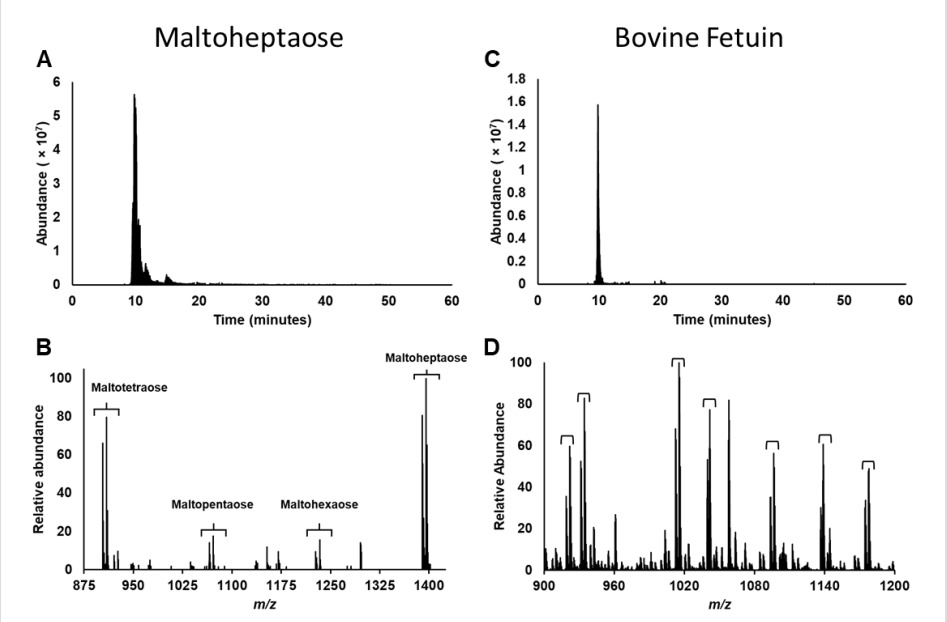


Figure 2. LC-MS examples of INLIGHT derivatized maltoheptaose and *N*-linked glycans from bovine fetuin.

A. Extracted ion chromatogram of $[NAT + H]^+$ maltoheptaose.

B. Mass spectrum at 10 minutes illustrating NAT and SIL derivatized maltoheptaose, maltohexaose, maltopentaose, and maltotetraose.

C. Extracted ion chromatogram of a fetuin-based glycan at *m/z* 1012.3980 corresponding to $[NAT + 2H + (Fuc)_1(Gal)_2(GlcNAc)_4(Man)_3]^{2+}$.

D. Average mass spectrum of a fetuin analysis showing the NAT and SIL derivatized *N*-linked glycan pairs in the LC window 8-11 min.

Note: The NAT/SIL *m/z* values for maltoheptaose and fetuin glycans are tabulated (see link below), while mzXML files are also available for cross-check purposes. Click on the title for the INLIGHT Data Report:

NAT/SIS *m/z* Values of Maltoheptaose and Fetuin Glycans

For details about data processing and analysis options, please see reference #8 for the current INLIGHT Strategy and reference #10 for the GlycoHunter Analysis. For additional information on GlycoHunter see **https://glycohunter.wordpress.ncsu.edu/**.

Example References

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10. Kalmar, J.G.; Garrard, K.P.; Muddiman, D.C. **2021**. GlycoHunter: An open-source software for the detection and relative quantification of INLIGHT®-labeled *N*-linked glycans. *J Proteome Res, 20(4)*, 1855-1863.