Cambridge Isotope Laboratories, Inc.
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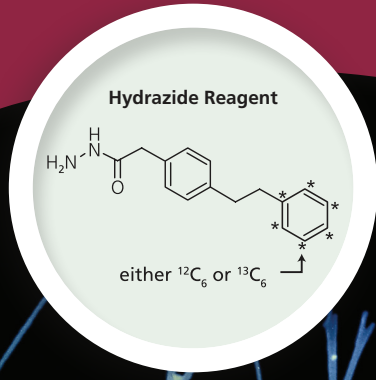
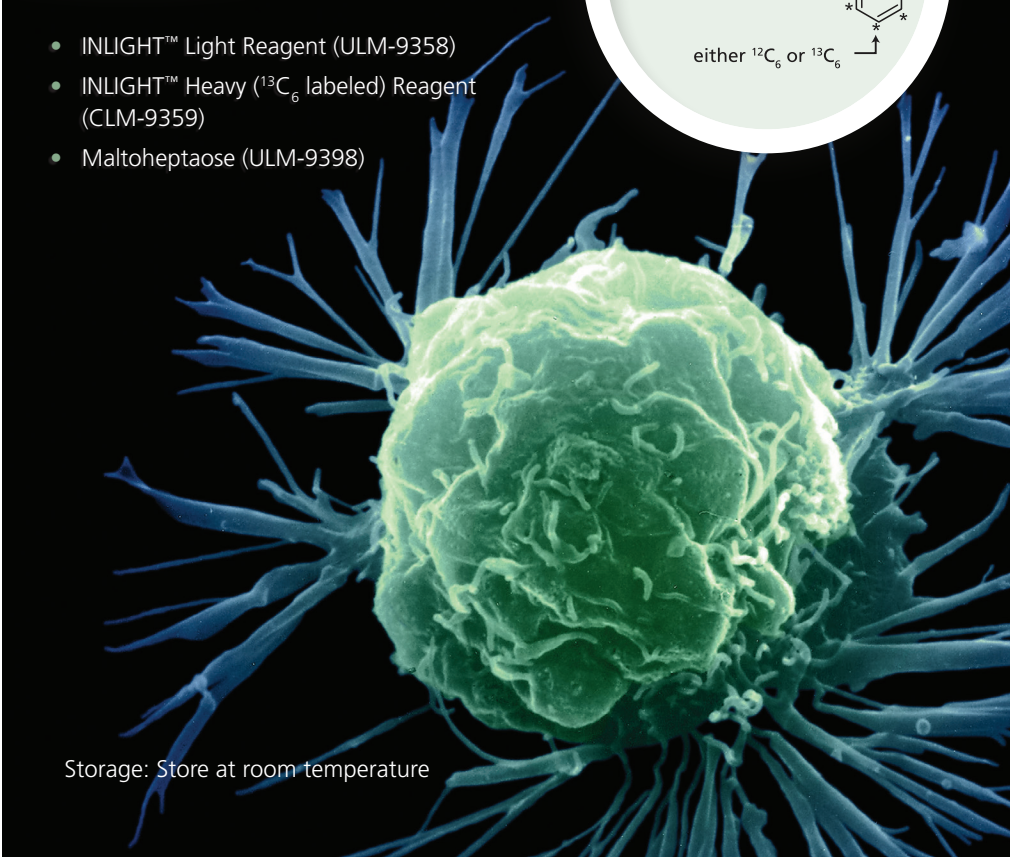
INLIGHT™ Glycan Tagging Kit Protocol

A Glycan Tagging Kit for Comparative Quantification of *N*-linked Glycans

INLIGHT™ Glycan Tagging Kit
Catalog No. GTK-1000



- INLIGHT™ Light Reagent (ULM-9358)
- INLIGHT™ Heavy ($^{13}C_6$ labeled) Reagent (CLM-9359)
- Maltoheptaose (ULM-9398)



Storage: Store at room temperature

INLIGHT™ Strategy

N-Linked Glycan Cleavage and Derivatization via Hydrazone Formation

Cambridge Isotope Laboratories' **INLIGHT™ Glycan Tagging Kit** contains natural (light) and ^{13}C stable isotope-labeled (heavy) 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. Maltoheptaose is provided for use as an internal oligosaccharide (7 hexose units) standard. The **INLIGHT™ Glycan Tagging Kit** (GTK-1000) contains five vials of light reagent and five vials of heavy reagent which in total provide tagging reagent for approximately 25 relative quantification experiments.

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 glycoproteins, Fetuin and RNase B. To ensure appropriate LC/MS conditions, the analysis of glycoproteins fetuin and/or RNase B is highly recommended as a first step in using the **INLIGHT™** strategy of glycan analysis. This will allow proper strategy optimization and troubleshooting for each step, paying particular attention to:

a) time and resource constraints.

b) LC/MS platform verification/optimization.

After the INLIGHT™ strategy has been successfully applied to these well-characterized glycoproteins fetuin and RNase B (see sample data at: isotope.com/chemical-tagging), the strategy may be applied with confidence to complex mixtures.

KIT CONTENTS

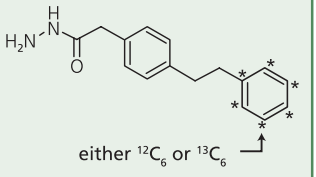
INLIGHT™ Strategy

N-Linked Glycan Cleavage and Derivatization via Hydrazone Formation

INLIGHT Glycan Tagging Kit
GTK-1000

- INLIGHT™ Light Reagent, 5 × 0.25 mg (ULM-9358, provided)
- INLIGHT™ Heavy ($^{13}C_6$ labeled) Reagent, 5 × 0.25 mg (CLM-9359, provided)
- [1]Maltoheptaose, 5 × 10 µg (ULM-9398, provided)

Hydrazide Reagent



2-(4-phenethylphenyl)acetohydrazide: *N*-glycan tagging reagent (see ref. 1)

REQUIRED REAGENTS

Solvents	Manufacturer	Part No./Cat. No.
Acetonitrile – HPLC grade (ACN)	Burdick & Jackson	AH015-4
Methanol – HPLC grade	Burdick & Jackson	AH230-4
Water – HPLC grade	Burdick & Jackson	AH365-4
Formic acid	Sigma-Aldrich	56302
Acetic acid (50%) solution for HPLC	Sigma-Aldrich	45754
Trifluoroacetic acid, Reagent Plus® 9 (TFA)	Sigma-Aldrich	T6508
Ethanol (200 proof) HPLC grade	Sigma-Aldrich	459828

Reagents & Other Supplies	Manufacturer	Part No./Cat. No.
SPE graphitized carbograph columns [300 mg]	Grace Discovery Sciences	210101
Ammonium bicarbonate	Sigma-Aldrich	A6141
Dithiothreitol 1 M aqueous solution	Sigma-Aldrich	646563
[2]Horseradish peroxidase (HRP) Type VI	Sigma-Aldrich	P8375
PNGase F – glycerol free [75,000 units]	New England Biolabs	P0705L
[3]Fetuin	New England Biolabs	P6042S
[4]RNase B	New England Biolabs	P7817S

INSTRUCTIONS FOR USE

A. Solution and Buffer Preparation

1. SPE Wash Solution (solvent #1) 1 Liter – 1 mL TFA in water (HPLC grade).
2. SPE Condition Solution (solvent #2) 1 Liter – 500 µL TFA, 800 mL ACN, 200 mL water (HPLC grade).
3. SPE Elution Solution (solvent #3) 1 Liter – 1 mL TFA, 250 mL ACN, 750 mL water (HPLC grade).

(continued)

4. Digest Buffer – 250 mL – 100 mM ammonium bicarbonate – weigh 1970 mg, dissolve in water (HPLC grade).
5. Derivatization Solution – 100 mL – 75:25 (v/v) methanol:50% acetic acid.

**Note: HPLC-grade solvents should be used if at all possible. However, depending on the number of samples being prepared in parallel, large volumes of wash buffer may be necessary for solid-phase extraction purification of N-glycans, in which case Milli-Q water or equivalent may be substituted for HPLC-grade water in SPE wash buffer.*

[1] Maltoheptaose is a glucose heptamer that can be used as a free oligosaccharide standard. (See references 2 and 3)
[2] HRP (plant glycoprotein) can be used as an internal standard, contains a xylosylated glycan. (See reference 2)
[3] Fetuin (mammalian glycoprotein) contains sialylated *N*-linked and *O*-linked glycans. (See references 4 and 5)
[4] RNase B (mammalian glycoprotein) contains high-mannose-type *N*-linked glycans. (See references 6 and 7)

Standard Glycoprotein Samples: Bovine Glycoproteins Fetuin A and RNase B

Sample Name/Tube Label	Sample Description	Tagging Reagent
F1	50 µL Bovine Fetuin (2 mg/mL) = 100 µg	Light
F2	50 µL Bovine Fetuin (2 mg/mL) = 100 µg	Heavy
R1	50 µL Bovine RNase B (1 mg/mL) = 50 µg	Light
R2	50 µL Bovine RNase B (1 mg/mL) = 50 µg	Heavy

B. Glycoprotein Denaturation

1. Add **50 µL** glycoprotein to **146 µL** 100 mM ammonium bicarbonate (digest buffer).
2. Pipet up and down then briefly vortex to mix.
3. Add **2 µL** of 1 M dithiothreitol (DTT) to each sample, giving a final concentration of 10 mM DTT.
4. Heat denature by alternating between 100°C (boiling water/heat block) and 25°C (wet chamber/room temperature water in wells of a microcentrifuge tube rack) for 15 seconds each. Do this four times (~2 minutes total time).
5. Briefly vortex to mix and spin down in a centrifuge.

C. PNGase F Enzymatic Digestion

1. Add **2 µL** (1000 NEB units; 15.3 IUB mUnits) PNGase F to each sample.
2. Briefly vortex to mix and spin down in a centrifuge. **Final digest volume: 200 µL**.
3. Incubate for 12-18 hours at 37°C.

Note: 12 hours incubation is appropriate for a standard glycoprotein mix. Longer incubation times may be necessary for complex biological samples.

D. Ethanol Precipitation

1. Quench by adding 800 µL chilled ethanol‡ to digest solution.
2. Briefly vortex to mix and spin down in a centrifuge. **Final volume: 1 mL**.
3. Incubate at -80°C for 1 hour.
4. Centrifuge at **13,200 rpm (16,110 × *g*)** for 30 minutes at coldest setting possible.
5. Pipet supernatant into a clean 1.5 mL microcentrifuge tube.
6. Evaporate to dryness in **centrifugal evaporator at 45°C**.
7. Concentrated sample can be stored overnight at -20°C until SPE purification.

‡ 200 Proof ethanol stored at -80°C.

E. Glycan Purification by Solid-Phase Extraction (SPE)

Solvent #1 – SPE wash solution: 0.1% TFA in water
Solvent #2 – SPE conditioning solution: 0.05/80/20% (v/v/v) TFA/ACN/water
Solvent #3 – SPE elution solution: 0.1/25/75% (v/v/v) TFA/ACN/water

1. Condition SPE graphitized carbograph columns in the following order:
   a) Two column volumes of solvent #1.
   b) One column volume of solvent #2.
   c) Two column volumes of solvent #1.
2. Re-suspend samples in 1 mL of solvent #1.
3. Briefly vortex to mix and spin down in a centrifuge.
4. When the last of the wash solution elutes, pour the reconstituted sample into the SPE column.
5. Rinse sample vial with 1 mL of solvent #1, vortex, spin down in centrifuge, and pour into the same cartridge as the initial reconstitution. Repeat rinse once more.
6. Allow meniscus to reach top of filter.
7. Wash loaded column (containing glycome solution) with 40 mL of solvent #1.
8. Elute the glycans with 1 mL of solvent #3 and collect fraction (**Fraction A**).
9. Repeat 3 times (**Fractions B, C & D**).
10. Freeze collected fractions (4) at -80°C.

F. Combine SPE Fractions

1. Concentrate SPE fractions to dryness using **centrifugal evaporator at 45°C** (~5-6 hours).
2. Re-suspend **Fractions A, C & D** with 75 µL HPLC water; briefly vortex to mix and spin down in a centrifuge.
3. Combine suspended fractions into **Fraction B**.
4. Freeze at -80°C.
5. Concentrate to dryness at **45°C** (~2-3 hours).
6. After sample is completely dry, store at -20°C until day of LC/MS analysis.

G. Glycan Derivatization

Note: Each vial contains 0.25 mg of INLIGHT™ reagent [2-(4-phenethylphenyl)acetohydrazide], enough for up to five reactions, depending on the starting concentration of glycan. The following quantity of tag has proven sufficient for derivatization of: 50 µg maltodextrin (linear polysaccharide standard); 50 µg RNase B glycoprotein (1 glycosite; high mannose-type glycans); 100 µg fetuin glycoprotein (3 glycosites; complex type glycans), and 50 µL aliquots of human plasma (approx. 30 mg/mL total protein based on Bradford assay). It is recommended that laboratories optimize INLIGHT™ tag : glyco-substrate ratio to minimize excess tag and maximize reaction efficiency (> 95%).

1. Make a 0.25 mg/mL solution of each: **INLIGHT™ Light Reagent** and **INLIGHT™ Heavy Reagent** by dissolving each reagent in derivatization solution; 75/25 (v/v) methanol: 50% acetic acid.
2. Re-suspend purified glycan samples in either:
   a) 200 µL Light Reagent solution (natural 2-(4-phenethylphenyl)acetohydrazide)
   b) 200 µL Heavy Reagent solution ($^{13}C_6$ labeled 2-(4-phenethylphenyl)acetohydrazide)
3. Pipet up and down, then vortex to further mix, and spin down by centrifuge.
4. Optional: Add 10 µg maltoheptaose (polysaccharide standard) to each reaction, prepared fresh in derivatization buffer.

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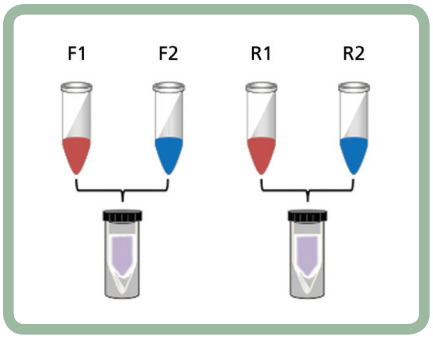

Our process: Re-suspend derivatized glycans in **200 µL** and combine **40 µL** from one "Light"-labeled glycome and **40 µL** from one "Heavy"-labeled glycome for relative *quantification of derivatized N-glycans by LC/MS using INLIGHT™.*[3]

Note: Any remaining derivatized samples can be dried down and stored at -20°C. Be aware that sample integrity may degrade over time. Studies have not been conducted to ensure stability of derivatized glycans during long-term storage.

5. Incubate samples at 56°C for 3 hours.
6. Evaporate to dryness in **centrifugal evaporator at 55°C**. Important: to completely quench reaction, all solvent must be evaporated before LC/MS analysis.

H. LC/MS Preparation

**Use only HPLC-grade solvents*

1. Re-suspend derivatized glycan samples in 25-200 µL of:
   Reversed Phase HPLC: 0.2% formic acid in 98:2 (v/v) water: acetonitrile (Mobile Phase A)
   HILIC: 50 mM ammonium acetate in water (pH 4.5) (Mobile Phase A)
2. Pipet up and down, then vortex for 30 seconds.
   For samples tagged with ≥20µg tag:
   a. Centrifuge at 14,000 × *g* for 5 minutes.
   b. Carefully pipet supernatant (containing derivatized glycans), leaving pellet (containing excess reagent)
5. Combine "Light" and "Heavy"-labeled glycan samples in a 1:1 (v/v) ratio for LC/MS analysis.

I. LC/MS Analysis

There are many LC/MS methods that may be used for analysis of tagged glycans. The following examples have proven successful.[8] *These methods are described for a* ***40 µL Light: 40 µL Heavy combined glycome sample****. An appreciable increase in glycan signal intensity is observed when using a mass spectrometer equipped with a high-pass filter and AGC. Alternative mass analyzers are viable, but may require in-house optimization. Likewise, variability between LC systems may require in-house optimization for efficient glycan separation.*

*Use only HPLC-grade solvents

nano-flow Reversed Phase HPLC:

- Stationary phase: Magic C_{18}AQ packing, 5 µm particle size, 200 Å pore size; Michrom BioResources (Auburn, CA)
- Injection volume: 10 µL
- Flow rate: 500 nL/min
- Mobile Phase A: 0.2% formic acid in 98:2 (v/v) water: acetonitrile.
- Mobile Phase B: 0.2% formic acid in 2:98 (v/v) water: acetonitrile.

Table 1. RP-LC gradient elution profile

Time (min)	%A	%B
0	98	2
5	98	2
6	80	20
39	65	35
40	2	98
45	2	98
46	98	2
56	98	2

nano-flow Hydrophilic Interaction (HILIC) HPLC:

- Stationary phase: TSK-Gel Amide-80 packing, 5 µm particle size, 80 Å pore size; TOSOH Bioscience (King of Prussia, PA)
- Injection volume: 10 µL
- Flow rate: 500 nL/min
- Mobile Phase A: 50 mM ammonium acetate in water (pH 4.5).
- Mobile Phase B: 100% acetonitrile.

Table 2. HILIC gradient elution profile

Time (min)	%A	%B
0	15	85
3	15	85
4	20	80
24	20	80
59	55	45
60	20	80
70	20	80

**For details about data processing and analysis options, please see reference #3 for INLIGHT Strategy, or reference #9 for GlycReSoft Software Method.*

Literature Cited

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Protocol_GTK
6/14 Supersedes all previously published literature