Ludger Standard N-glycan Release, 2AB labelling, Clean-up and HILIC-(U)HPLC Methods

Ludger 标准的N-多糖释放, 2AB标记，纯化和HILIC-(U)HPLC方法

This document provides the details of one standard set of methods used at Ludger for glycan release and profiling of 2AB labelled N-glycans. This is the typical set of methods that we would use to analyse N-glycans from mammalian derived glycoproteins. 本文提供了标准的Ludger多糖释放和2AB标记的 N-多糖分析方法的细节。这是我们分析哺乳动物衍生的糖蛋白中的N-多糖的典型方法。

This document should be used in conjunction with the individual product guides that can be located under the Product Categories at: http://www.ludger.com/products/该文件应该与相应的产品使用指南联合使用,可以在产品类别中下载:http://www.ludger.com/products/

Overview for workflow
实验流程概述

Stage 1 第 1 阶段: Release N-glycans 释放N-多糖

Stage 2 (optional) 第 2 阶段(可选的): Conversion of released Nglycans to aldoses 转换释放的N-多糖成醛糖

Stage 3第 3 阶段: Clean-up纯化

Stage 4第 4 阶段: 2-AB labeling 2-AB标记

Stage 5第 5 阶段: Post-labelling clean-up标记后纯化

Stage 6第 6阶段: HILIC-HPLC or HILIC-UPLC analysis. HILIC-HPLC or HILIC-UPLC分析

Method Details
方法细节

Stage 1第 1 阶段: Release N-glycans释放N-多糖

Reagents: E-PNG-01; Pure water (resistivity above 18 MΩ-cm, particle free (>0.22 µm), TOC <10 ppb) is used throughout. 试剂:E-PNG-01;纯水(电阻率高于18 MΩ-cm，不含粒子(> 0.22µm), TOC < 10 ppb) 通篇使用。

1. Add up to 200µg of glycoprotein to an Eppendorf tube. Adjust to 35 µl final volume with de-ionized water. 加200µg糖蛋白到一个微量离心管。用去离子水调整到最后体积为35µl。

Add 10 µl 5x Reaction Buffer pH 7 and 2.5 µl of Denaturation Solution. Heat at 100 ℃ for 5 minutes.
加10µl pH 7的5 倍反应缓冲液和2.5µl变性液。加热5分钟至100° C。

2. Cool. Add 2.5 µl of Triton X-100 and mix. 冷却：加2.5µl曲通X-100然后混合。

3. Add 2.0 µl of PNGase F to the reaction. Incubate 3 to 24 hours at 37°C (recommend overnight if a time course has not been performed). 添加2.0 µl 糖苷酶到反应样本。在37° C温度下培养3到24小时 (如果未曾进行时间进程反应,建议培养一夜)。

TIME: 3.5 to 24 hours
时间:3.5 - 24小时

Stage 2 (optional): Conversion of released N-glycans to aldoses
第 2 阶段(可不做): 转换释放的N-多糖成醛糖

This stage is optional, but will ensure that all of the released N-glycans are converted to aldoses, enabling maximum labelling efficiency. 这个阶段是可选的,但这一步可以确保所有释放的N-多糖转换为醛糖,使提高标记效率。

Reagents: 1% Formic acid.
试剂:1%甲酸。

1. Dry the glycans: Dry the glycans in a vacuum centrifuge. We do not recommend applying heat at this stage as extended drying times or heat can cause desialylation of the glycans. 1。干燥聚糖:在真空离心机里干燥聚糖。我们不建议在此阶段加热干燥，因为延长干燥时间或加热会导致聚糖中唾液酸的去掉。

2. Add 20µL of 1% formic acid to each sample, vortex thoroughly making sure that all of the sample is re-dissolved, then briefly centrifuge. 添加20µl浓度为1%的甲酸到每个样本,漩涡彻底确保所有样品重新溶解,然后短暂地离心。

3. Incubate at room temperature for 40 to 50 min. *Following this incubation do not leave the samples in acid, take the samples through the next stage straight away*. 在室温下培养40到50分钟。培养之后不要将样品留在酸里,直接拿样品进行下一阶段的实验。

TIME: 1 hour plus drying time (2 to 5 hours)
时间:1小时+干燥时间(2到5个小时)

Stage 3: Clean-up
阶段3:纯化

Reagents: LC-PBM-96 kit; Vacuum manifold suitable for 96 well format, SPE plates with Vacuum trap (e.g. **LC-VAC-MANIFOLD Kit** & **LC-VACUUM-TRAP-KIT**) with vacuum pump; 2 mL collection plate for collecting glycans **[LP-COLLPLATE-2ML-96]**; Collection plate lid (optional) **[LP-COLLPLATE-LID-96].**
试剂: LC-PBM-96试剂;真空歧管适合96孔格式, SPE平板与真空聚集槽(例如LC-VAC-MANIFOLD装置& LC-VACUUM-TRAP-KIT装置) 与真空泵;2毫升收集板收集聚糖[LP-COLLPLATE-2ML-96]收集板盖子(可选) [LP-COLLPLATE-LID-96]。

1. Wash the protein binding plate: Pipette 100 µL of methanol into the plate wells [LC-PBM-96] that are to be used (this is to wet the membrane). Apply a vacuum and adjust to between -0.1 and -0.2 bar until

the methanol has passed through the wells. Pipette 300 µL of water into each well to wash away the methanol. Apply a vacuum and adjust to between -0.1 and -0.2 bar until the water has passed through the wells. The membrane will remain wet. 清洗蛋白结合板: 将100 µL的甲醇滴入要使用的孔板 [LC-PBM-96](这是为了打湿薄膜)。施加真空然后将刻度调到-0.1和-0.2之间,直到甲醇通过孔板。吸取300µL的水到每个孔板冲走甲醇。施加真空然后将刻度调到-0.1和-0.2之间,直到水通过孔板。薄膜将保持湿润。

2. Apply the sample: Pipette the samples into the plate wells washing out each tube with 100 µL of water and adding this to the well. Apply a vacuum and adjust to between -0.05 and -0.1 bar until the liquid has completely gone through the wells. Pipette 100µL of water into each well. Apply a vacuum and adjust to between -0.05 and -0.1 bar until the liquid has completely gone through the wells. 添加样品: 吸取样品滴入孔板然后用100µL的水洗净每个试管，施加真空然后将刻度调到-0.05和-0.1之间,直到液体已经完全通过孔板。滴入100µl的水到每个孔板。施加真空然后将刻度调到-0.05和-0.1之间,直到液体已经完全通过孔板。

3. Samples can be stored at ~ 4°C for a day or two, but should be stored long term at ~ -20°C. 样品可以存储在~ 4°C一两天,但长时间应该存储在 -20° C

TIME: 0.5 to 1 hour
时间:0.5 ~ 1小时

Stage 4: 2AB Labelling
阶段4: 2AB标记

Reagents: LT-KAB-VP24 2AB labelling kit
试剂:LT-KAB-VP24 2AB标记试剂盒

1. Dry the glycans: Dry the glycans in a vacuum centrifuge. We do not recommend applying heat at this stage as extended drying times or heat can cause desialylation of the glycans.干燥聚糖:在真空离心机里干燥聚糖。我们不建议在此阶段加热干燥，因为延长干燥时间或加热会导致聚糖去唾液酸。

2. Prepare the labelling and reductant solution: Add 150 µL of kit component LT-ACETIC-DMSO-01 (30% acetic acid in DMSO) to a vial of dye (LT-2AB-03) and mix by pipette action until the dye is dissolved. Sometimes heat (30-65°C) is required to help dissolve the dye. Transfer the 150 µL of dissolved dye solution to a vial of reductant (LT-PB-01) and mix by pipette action until the reductant is dissolved. Sometimes heat (30-65°C) is required to help dissolve the reductant. 准备标记和还原剂溶液: 加150µL LT-ACETIC-DMSO-01(在DMSO中30%的乙酸)的试剂成分到染料瓶(LT-2AB-03)中然后用吸管混合直到染料溶解。有时需要加热(30 - 65° C)来帮助染料溶解。转移150µL溶解的染料到还原剂溶液瓶(LT-PB-01)中，然后用吸管混合直到还原剂溶解。有时需要加热(30 - 65° C)来帮助还原剂溶解。

3. Label the samples: Add 10 µL water to the dried samples and mix. Add 10 µL of labelling reagent and mix. Incubate at 65°C for 1 hour (remixing at ~15 min). Cool. 1。标记样品: 添加10µL水到干的样品中然后混合。加10µL的标记试剂然后混合。在65° C中培养1个小时(~ 15分钟后再混合)。冷却。

TIME: Drying time (2 to 5 hours) plus 1.5 hours
时间:干燥时间(2到5个小时)+ 1.5小时

Stage 5: Post-Labelling Clean-up
第五阶段:标记后纯化

Reagents: LC-T1-A6 LudgerClean T1 cartridges; Vacuum manifold suitable for 96 well format, SPE plates with Vacuum trap (e.g. **LC-VAC-MANIFOLD Kit** & **LC-VACUUM-TRAP-KIT**) with vacuum pump; 2 mL collection plate for collecting glycans **[LP-COLLPLATE-2ML-96]**; Collection plate lid (optional) **[LP-COLLPLATE-LID-96]**; Acetonitrile; 96 % acetonitrile in water (v/v). Base plate for array wells **[LP-HOLDER-96]**; Base plate plugs to fill unused spaces in base plate **[LP-PLUG-96]**

试剂:LC-T1-A6 LudgerClean T1纯化柱; 真空歧管适合96孔格式,SPE平板与真空聚集槽(例如LC-VAC-MANIFOLD装置& LC-VACUUM-TRAP – 装置)和真空泵;2毫升收集板收集聚糖[LP-COLLPLATE - 2ML - 96];收集板盖子(可选) [LP-COLLPLATE-LID-96];乙腈;96%的乙腈水(v / v)。排孔的底板[LP-HOLDER-96];底板塞用来填补在底板上未使用的空间[LP-PLUG-96]

1. Wash and prime the cartridge: Pipette 1mL of water into each cartridge; apply a vacuum of between -0.05 and -0.3 bar until the liquid has completely gone through the wells. Pipette 1mL of 96% acetonitrile into each cartridge; apply a vacuum of between -0.05 and -0.3 bar until the liquid has completely gone through the wells.
   清洗和预备纯化柱:吸取1毫升的水到每个纯化柱; 施加真空然后将刻度调到-0.05和-0.3之间,直到液体已经完全通过孔板。吸取1毫升96%乙腈到每个纯化柱; 施加真空然后将刻度调到-0.05和-0.3之间,直到液体已经完全通过孔板。

2. Apply the samples: Pipette 200 µL of 96% acetonitrile into the sample tube, mix by pipette action and transfer the whole of the solution to the top of a cartridge. Allow to drain under gravity. It should take between 30 seconds and 5 minutes to drain. If after 5 min the sample has not drained, then apply a minimum vacuum to pull some of the sample into the cartridge, and then switch off the vacuum and allow to drain under gravity (repeat as necessary).
   添加样品:吸取200µL 96%的乙腈滴入样品管,用吸管混合然后转移所有溶液到一个纯化柱。使其在重力下排水。它需要30秒至5分钟排干净。如果5分钟后样品还没有干, 就用最低限度的抽真空来抽一些样品到柱子里边,然后关掉真空使其在重力下排水(必要时重复)。

3. Wash the cartridge: Add 1 mL of 96% acetonitrile to each cartridge and allow to drain under gravity. It

should take between 30 seconds and 2 minutes (or more) to drain. If after 5 min the 96% acetonitrile has not drained, then apply a minimum vacuum to elute slowly. Repeat with 2 additional washes of 1ml 96% acetonitrile.
清洗纯化柱:加1毫升96%乙腈到每个柱子,使其在重力下排水。需要30秒到两分钟(或更长时间)排干净。如果5分钟后96%乙腈没有排净, 就用最低限度的抽真空慢慢洗脱。用2个额外的1毫升96%乙腈重复冲洗。

4. Elute the glycans: Insert a collection plate **[LP-COLLPLATE-2ML-96]** into the manifold. Make sure the vacuum is on the lowest setting, remove one of the plugs so that when the vacuum is switched on it does not surge. It is important that the glycans are eluted slowly. Add 1 mL water to each cartridge. Leave to drain under gravity for 5 min. Apply the minimum vacuum to the samples by opening the tap. Once the vacuum has settled, put the plug back in. Apply low vacuum for one second or so to allow the liquid to enter the cartridge (~100 µL). Stop the vacuum and allow the sample to drain under gravity for a further 10 min. Apply a minimum vacuum to complete the elution slowly. After the liquid has drained through, increase the pressure to maximum to remove the final drops from the end of the cartridge.
洗脱聚糖: 把一个收集板**[LP-COLLPLATE-2ML-96]** 放进歧管。确保抽真空装置设置为最低值,拿掉一个塞子,这样当真空装置开启时它不会涌出。聚糖要慢慢地洗脱，这点很重要。每个柱子加1毫升水。使其在重力下排出5分钟。打开最小量的真空。一旦真空稳定了，把塞子放回去。使用低真空一秒钟左右让液体进入柱子(~100 µL)。停止真空, 让样品在重力下再流干10分钟。使用最小真空缓慢地完成洗脱。在液体排通后,增加真空到最大限度把最后一滴液体从柱子底部排出来。

5. Concentrate the eluted glycans (optional): Samples can be dried in a vacuum centrifuge. We do not recommend applying heat at this stage as extended drying times or heat can cause desialylation of the glycans. Reconstitute in water (e.g. 100 µL).
富集洗脱的聚糖(可不做):样品可以在真空离心机里烘干。我们不建议在此阶段加热干燥，因为延长干燥时间或加热会导致聚糖去唾液酸。在水中复原(如100µL)。

6. Samples can be stored at ~ 4°C for a day or two, but should be stored long term at ~ -20°C
样品可以存储在~ 4°C一两天,但长时间应该存储在~ -20°C

TIME: 45 min plus optional drying
时间:45分钟+干燥（可不做）

Stage 6: HILIC-HPLC *or* HILIC-UPLC Analysis
第六阶段:HILIC-HPLC或HILIC-UPLC分析

Reagents: LS-N2-4.6x150 LudgerSep N2 HPLC Column 4.6 x 150mm, 3 µm, or a Waters ACQUITY UPLC® BEH-Glycan 1.7 µm, 2.1 x 150 mm column; **LS-N-BUFFX40** LudgerSep

Ammonium Formate Buffer, pH 4.4, 50 mL; **CAB-GHP-30** 2-AB Glucose Homopolymer Ladder for system suitability standard and for calibration of GU; **CAB-IGG-01** IgG N glycan library or other 2AB-labelled Reference standards **[CAB-XXX-01]**.

试剂: **LS-N2-4.6x150** LudgerSep N2高效液相色谱柱4.6×150毫米, 3 µm或Waters的ACQUITY UPLC® BEH –多糖 1.7 µm, 2.1 x 150毫米柱;LS-N-BUFFX40 LudgerSep甲酸铵缓冲液,pH值4.4,50毫升;CAB-GHP-30 2-AB葡萄糖均聚物梯系统适用性标准品和校准峰值;CAB-IGG-01免疫球蛋白g的N多糖文库标准品，或其他2AB-标记的参照标准品[CAB-XXX-01]。

Solvent溶剂A: 50 mM ammonium formate pH 4.4, made from 50 ml of Ludger product # LS-N-BUFFX40 diluted to 2 litres with water.
50 mM甲酸铵pH值4.4, Ludger的产品# LS-N- BUFFX40用水稀释到2升配出来的。

Solvent溶剂 B : Acetonitrile乙腈

Column Temp柱子温度: 40°C

Sample样品: prepared in 35% aqueous/65% acetonitrile for HPLC or 28% aqueous/72% acetonitrile for UPLC; injection volume 25 µL. 样品准备在35%水溶液/ 65%乙腈为HPLC法或28%水溶液/ 72%乙腈UPLC法;注入体积为25 µL。

Detector检测器: λex = 330 nm, λem = 420 nm for HPLC; λex = 250 nm, λem = 428 nm for UPLC (or whichever gives the highest signal for your detector或者任何一个能给你最高信号的检测器)

Gradient # LSN2-10m-Start-35%: N-Glycan HPLC Analysis Start-up
梯度# LSN2-10m -开始- 35%:N-多糖高效液相色谱分析开始。

Time (min)	Flow mL/min	%A	%B
0	0.0	35	65
2	1.0	35	65
10	1.0	35	65

Gradient # LSN2-30m-Nlink-35%: N-Glycan HPLC Analysis Gradient
梯度# LSN2-30m-Nlink-35%:N-多糖高效液相色谱分析梯度

Use as a gradient for analysis of N-glycans with GU between 4 and 12; the gradient can be extended for larger glycans. 用作N-多糖GU值在4和12之间的分析梯度; 梯度可以扩展为较大的聚糖作分析。

Time (min)	Flow mL/min	%A	%B
0	1.0	35	65
22	1.0	46	57
22.5	1.0	100	0

24.5	1.0	100	0
26	1.0	35	65
30	1.0	35	65

Gradient # BEH-10m-Start-28%: N-Glycan UPLC Analysis Start-up

梯度# BEH-10m -开始- 28%:N-多糖 UPLC分析开始

Time (min)	Flow mL/min	%A	%B
0	0.0	28	72
2	0.4	28	72
10	0.4	28	72

Gradient # BEH-35m-Nlink-28%: N-Glycan UPLC Analysis Gradient

梯度# BEH-35m-Nlink-28%:N-多糖 UPLC分析梯度

Use as a gradient for analysis of N-glycans with GU between 4 and 12; the gradient can be extended for larger glycans. 用作N-多糖GU值在4和12之间的分析梯度; 梯度可以扩展为较大的聚糖作分析。

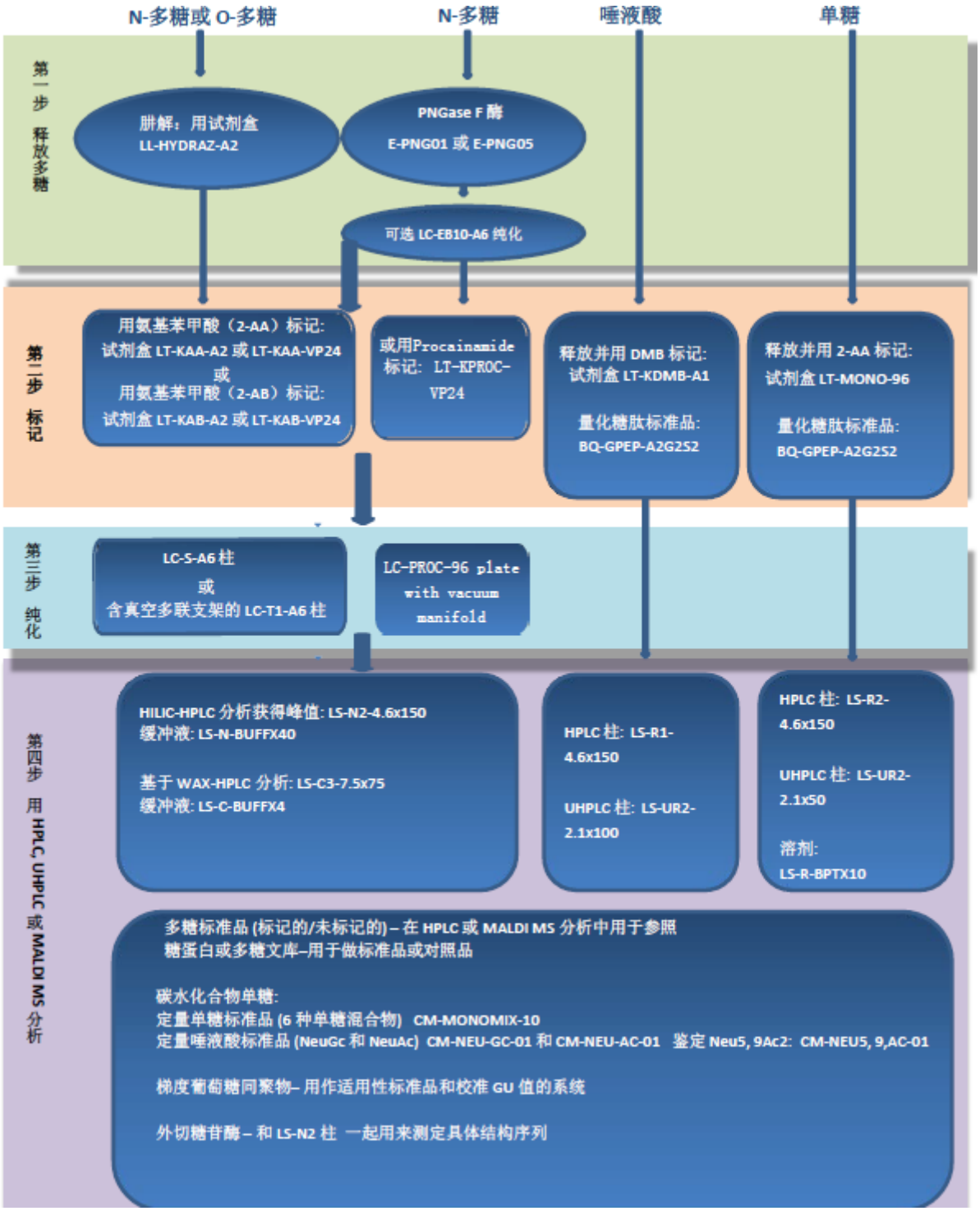
Time (min)	Flow mL/min	%A	%B
0	0.4	28	72
27	0.4	38	62
30	0.4	100	0
31	0.4	100	0
32	0.4	28	72
35	0.4	28	72

1. Start the gradient by running the Start-up method. 通过开启方法,开始梯度。
2. Precondition the column: running a complete analysis gradient cycle without any sample injected. 准备柱子: 在不注入任何样品的情况下运行一个完整的分析梯度循环。
3. Check the column performance: run the labelled glucose homopolymer [CAB-GHP-30, reconstituted in 300 µL water, diluted with acetonitrile to the gradient starting conditions] as a system suitability standard using the analytical gradient. Run a minimum of 2 GHP standards to ensure that the profiles overlap, that the peak shape is symmetrical, and that the resolution is optimum (monitor the peak width at half height over column lifetime).
   检查柱子性能: 使用分析梯度运行标记的葡萄糖均聚物标准品(CAB-GHP-30,用300µL的水复原，用乙腈稀释达到梯度起始条件)于作为系统适用性标准品。运行至少2次GHP的标准来确定 图谱是重叠的,峰的形状是对称的,分辨率是最佳的(在柱子的使用期监控峰在一半高度的宽度超过)。
4. Run samples using the analysis gradient. 使用分析梯度运行样品。
5. Integrate the peaks and assign GU values to peaks by comparison to the GHP standard. Use software such as GCP software in Waters Empower or within Chromeleon for Dionex choosing a cubic spline fit. 用GHP标准品作对比于指定各个峰然后分配GU值到各个峰上。。使用有立方样条拟合的软件如Waters授权的GCP软件或Dionex的Chromeleon软件。

System Suitability Standards and Controls
系统适用性标准和对照

It is good practice to take the appropriate positive and negative controls through the whole analytical procedure to ensure that the procedure is performing correctly. Ludger's standard procedure is to analyse samples in triplicate; with triplicate positive controls (we use the standard glycoproteins **GCP-IGG-100U** (human IgG) with MAbs, or Fetuin **GCP-FET-50U** with samples that have more complex glycosylation); plus buffer and water negative controls. A released unlabelled glycan library (e.g. **CLIBN-IGG-01** IgG N-glycan library) or individual glycans **[CN-XX-01]** can be used as labelling positive controls. 2AB labelled glucose homopolymer **[CAB-GHP-30]** is run as a (U)HPLC system suitability control. 2AB labelled standard glycans **[CAB-xx-01]** and glycan libraries [**CAB-IGG-01** 2AB-labelled IgG N-glycan library] are run for comparison of GU values to aid in identification of peaks. Exoglycosidase sequencing and other complementary methods such as MALDI and WAX-HPLC are used for cross correlation of data and identification of structures.

在整个分析过程中采取适当的阳性和阴性对照,以确保过程中的正确执行是一种很好的做法,。Ludger的标准程序是分析样品时一式三份,用一式三份的阳性对照(我们使用标准的单克隆抗体的糖蛋白**GCP-IGG-100U** (人类免疫球蛋白),或具有更复杂糖基化的胎球蛋白样品**GCP-FET-50U**);加缓冲液和水阴性对照。一个已释放未标记的多糖文库(例如**CLIBN-IGG-01**免疫球蛋白N-多糖文库)或个别的聚糖**[CN-XX-01]**可以用作标记的阳性对照。2AB标记的葡萄糖均聚物**[CAB- GHP-30]**作为(U)HPLC系统适用性对照。2AB标记的标准聚糖**[CAB-xx-01]**和多糖文库[**CAB-IGG-01** 2AB标记的免疫球蛋白N-多糖文库)作为GU值的比较来帮助峰的识别。外切糖苷酶测序和其他互补的方法,如MALDI和WAX-HPLC用于交互相关的数据和结构的识别。


N-多糖或 O-多糖
N-多糖
唾液酸
单糖
第一步 释放多糖
肼解：用试剂盒
LL-HYDRAZ-A2
PNGase F 酶
E-PNG01 或 E-PNG05
可选 LC-EB10-A6 纯化
第二步 标记
用氨基苯甲酸（2-AA）标记:
试剂盒 LT-KAA-A2 或 LT-KAA-VP24
或
用氨基苯甲酸（2-AB）标记:
试剂盒 LT-KAB-A2 或 LT-KAB-VP24
或用Procainamide 标记: LT-KPROC-VP24
释放并用 DMB 标记:
试剂盒 LT-KDMB-A1
量化糖肽标准品:
BQ-GPEP-A2G2S2
释放并用 2-AA 标记:
试剂盒 LT-MONO-96
量化糖肽标准品:
BQ-GPEP-A2G2S2
第三步 纯化
LC-S-A6 柱
或
含真空多联支架的 LC-T1-A6 柱
LC-PROC-96 plate with vacuum manifold
第四步 用 HPLC, UHPLC 或 MALDI MS 分析
HILIC-HPLC 分析获得峰值: LS-N2-4.6x150
缓冲液: LS-N-BUFFX40
基于 WAX-HPLC 分析: LS-C3-7.5x75
缓冲液: LS-C-BUFFX4
HPLC 柱: LS-R1-4.6x150
UHPLC 柱: LS-UR2-2.1x100
HPLC 柱: LS-R2-4.6x150
UHPLC 柱: LS-UR2-2.1x50
溶剂:
LS-R-BPTX10
多糖标准品 (标记的/未标记的) – 在 HPLC 或 MALDI MS 分析中用于参照
糖蛋白或多糖文库–用于做标准品或对照品
碳水化合物单糖:
定量单糖标准品 (6 种单糖混合物) CM-MONOMIX-10
定量唾液酸标准品 (NeuGc 和 NeuAc) CM-NEU-GC-01 和 CM-NEU-AC-01 鉴定 Neu5, 9Ac2: CM-NEU5, 9,AC-01
梯度葡萄糖同聚物– 用作适用性标准品和校准 GU 值的系统
外切糖苷酶 – 和 LS-N2 柱 一起用来测定具体结构序列