Product Guide for LudgerLiberate™

Orela

Glycan Release Kit

(Ludger Product Code: LL-ORELA-A2)

Ludger Document # LL-ORELA-A2-Guide-v3.0

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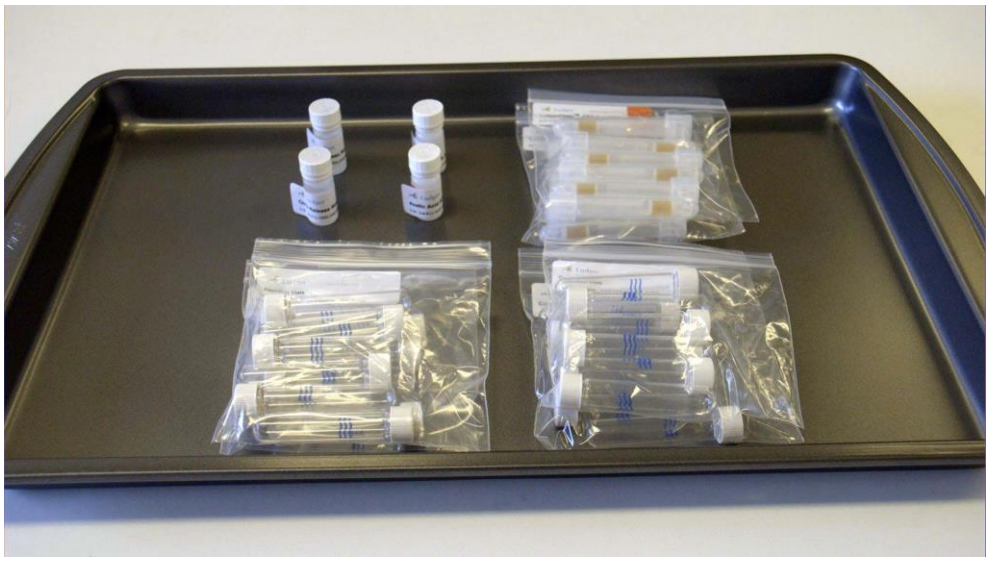
Contents

Specifications for LL-ORELA-A2

Application	For release of O-linked glycans from glycoprotein therapeutics.
Description	The kit contains reagents for the release of O-linked glycans from glycoprotein biopharmaceuticals. Released glycans have free-reducing termini to allow fluorescent tagging by reductive amination.
Number of Samples	The kit contains reagents and materials for up to 12 glycoprotein samples analysed in parallel or two sets of 6 samples.
Amount of Sample	Typically, up to 1 mg of glycoprotein per sample.
Suitable Samples	Biopharmaceutical glycoproteins.
Storage:	Store refrigerated at 4 to 10°C in the dark. If you have limited cold storage space, store the LudgerClean™ CEX-H cartridges (Cat # LC-CEX-H-01) at 4°C and the rest of the kit at room temperature. Protect from sources of heat, light, and moisture. The reagents are stable for at least 18 months from the date of manufacture.
Shipping:	The product should be shipped at ambient temperature.

For research use only. Not for human or drug use

Kit Contents



Each kit contains the following materials and reagents:

Cat. #	Item	Quantity
LL-ORELAREAGENT-01	Orela Release Reagent	2 x 3 mL
LL-ACETIC-50P-01	Acetic Acid Solution	2 x 3 mL
LC-CEX-H-01	LudgerClean™ CEX-H cartridges	2 x 6 cartridges
LL-REACT-01	Glass reaction vials with PTFE lined caps	2 x 6 vials
LL-COLLV-01	Glass collection vials with PTFE lined caps	2 x 6 vials

Additional Reagents and Equipment Required

- Pure water: resistivity 18 MΩ-cm, particle free (>0.22 μm), TOC <10 ppb
- Dialysis membranes, PD10 columns or similar for removal of salts and detergents from your glycoprotein samples*
- Syringe (glass or PTFE) to transfer Orela reagent – e.g. 1 ml Hamilton or SGE glass syringe for liquids with Teflon tipped plunger and stainless steel or PTFE needle. Do not use plastic syringes.
- Heating block, oven, or similar dry heater (a water bath cannot be used) that can be set between 40 and 100°C
- Centrifugal evaporator (e.g.ThermoSavant SpeedVac® or GeneVac®). If using the ThermoSavant SpeedVac® we recommend the use of the Thermo Savant RH32-13 Rotor.
- Pipettes
- Optional - depending on your sample

Safety and Handling

- Please read the Material Safety Data Sheets (MSDSs) for all chemicals used (see Appendix 2).
- All processes involving the kit reagents should be performed using appropriate personal safety protection - eyeglasses, good quality chemically resistant gloves (e.g. nitrile), etc. - and where appropriate in a laboratory fume cupboard.
- Ensure that any glass, plasticware or solvents used are free of glycosidases and environmental carbohydrates. Use powder-free gloves for all sample handling procedures and avoid contamination with environmental carbohydrates.
- Once individual vials of reagents are opened, their contents should be used immediately, and excess then be discarded according to local safety rules.

The Orela Reaction

The Orela reaction involves the following step:

Liberation of the glycans as the hydroxyl derivatives

The Orela reagent reacts as a base on the terminal monosaccharide, attached to the serine or threonine, to cleave the covalent bond between the glycan and peptide backbone to liberate glycans with a terminal cyclic monosaccharide with a hydroxyl functional group.

Timeline for Orela

Procedure	Time
Start with pure glycoprotein samples	
Transfer samples to reaction vials and dry completely	24 hours
Add Orela release reagent	15 min
Incubate samples*	5 - 16 hour
Remove release reagent	2 hours
Acidification	overnight
Purification of released glycans	2 hours

* The incubation time will vary depending on your particular glycoprotein samples. We recommend that at the start of a project you conduct a pilot study with an incubation time course to optimize the release conditions.

The following are typical incubation regimes:

O-Mode Orela (Fast): Incubate 5 hours at 60˚C
O-Mode Orela (Normal): Incubate 16 hours at 50˚C

Outline of the Orela Protocol

- ## Prepare the glycoconjugate

  Prepare the glycoprotein or glycopeptide samples by removing contaminants such as salts, detergents and dyes that could interfere with the labelling procedure.

- ## Dry the glycans

  Place the samples in reaction vials and dry them down.

- ## Add release reagent to glycoconjugates

  Add Orela release reagent to each sample.

- ## Incubate

  Incubate the samples to allow the release reaction to progress.

- ## Acidification

  Add acetic acid solution and incubate at 4°C to neutralise any remaining base and protonate the glycans.

- ## Post-release cleanup

  Remove the peptide or protein using a cation exchange column

- ## Store or analyse released glycans

  The released glycans are now ready for analysis

Sample Preparation

1 Purify the Glycoprotein

The glycopeptide or glycoprotein samples must be free of contaminants that can interfere with the release reaction. These include the following:

- Non-volatile solvents
- Non-volatile salts, in particular, transition metal ions
- Detergents
- Dyes and stains such as Coomassie Blue

Methods that are generally good for the removal of such contaminants include the following:

- Dialysis against water or 0.1% trifluoroacetic acid (TFA) as some glycoproteins tend to precipitate in water
- Size exclusion chromatography using a small desalting column (e.g. PD10) with water or 0.1% TFA as eluant

2 Transfer Samples to Reaction Vials

The amount of sample for each reaction vial (cat # LL-REACT-01) should be in the range of 50 µg to 1 mg.

The reaction vials (5 mL glass vials with Teflon PTFE lined screw caps) included in the kit are pre-cleaned.

3 Dry the Samples

Dry the samples using a centrifugal evaporator or a freeze-dryer.

If freeze-drying, be careful to ensure that the sample dries to a small, compact mass at the very bottom of the vial.
Do not subject samples to high temperatures (>28°C) or extremes of pH as these conditions can result in acid-catalyzed loss of sialic acids (high temperatures, low pH) or uncontrolled glycan release (at high pH).

Release Reaction


4 Add Orela Reagent

Using a clean, dry glass or PTFE syringe with a PTFE tipped plunger and Teflon or stainless steel needle transfer 200 µL of Orela release reagent (vial LL-ORELAREAGENT-01) to each dried sample.
Cap the reaction vials and mix by vortexing.

This step must be performed in a chemical fume hood.
Ensure that the reaction vial caps are tightly screwed on. For extra security and to minimize you can seal the caps onto the vials using Parafilm, PTFE tape or similar.

5 Orela Incubation

Place the reaction vials in a heating block, sand tray, or dry oven and incubate according to the type of glycan release you require:

O-Mode Orela (Fast):	Incubate 5 hours at 60°C
O-Mode Orela (Normal):	Incubate 16 hours at 50°C

Use an oven or dry block - do not use a water bath.
The samples must be completely dissolved in the Orela reagent for efficient glycan release. To encourage complete dissolution the samples can be re-vortexed 15 and 30 minutes after the start of the incubation then the incubation continued.
During this step, the O-glycans are liberated from the glycoprotein as hydroxyl derivatives.
The kinetics of glycan release depends on the type of sample and the glycans. The incubation regimes above give good release for most samples. However, in some cases, it may be useful to perform a time-course to optimize the release conditions. When performing a time-course, there are two factors to consider; (a) the yield and (b) side reactions (particularly peeling). The shorter the incubation time, the lower the peeling and the lower the yield. Increasing the incubation time increases yield but can result in higher levels of peeling.

6 Cool the Samples

After the incubation period, remove the samples from the incubation apparatus and allow them to cool completely to room temperature.

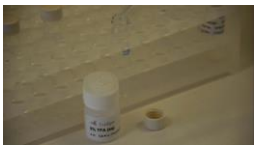
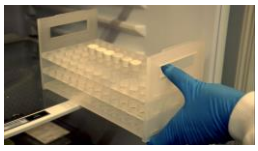
Orela Reagent Removal


7 Evaporate off Unreacted Orela Reagent

Remove unreacted Orela reagent by evaporation in a centrifugal evaporator.

Use an evaporation chamber temperature of 30 to 40°C.
N.B. Make sure that your centrifugal evaporator is rated to handle strong ammonia-like bases. Your evaporator should be serviced and cleaned with good seals. Use an efficient cold trap with a temperature of 40°C or lower between the evaporation chamber and the pump. Dispose of the cold trap waste according to hazardous waste regulations. You can contact your local waste management service for advice.

Acidification

8 Add 50% Acetic Acid (aq)

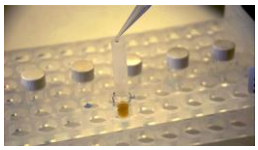
Add 200 µL of 50% acetic acid solution (vial LL-ACETIC-50P-01) to each sample, cap the reaction tube, and vortex to mix.

9 Incubate for Acidification

Incubate in a refrigerator at 4°C overnight.

This step allows the neutralisation of any remaining base and protonation of the free unreduced glycans. The reaction is performed at 4°C to minimize acid-catalyzed desialylation.

Glycan Purification

10 Prime the LudgerClean™ CEX Cartridges

For each sample, prepare a LudgerClean™ CEX cartridge (cat # LC-CEX-H-01) by washing with 10 x 1 mL water

If the flow is restricted, e.g. by an air gap, then apply a slight pressure to the top of the cartridge (e.g. using a pipette) to resume normal flow.
Do not allow the resin to dry out.
Allow each aliquot to flow through the resin bed before the next solution is applied.

11 Apply the Sample and Elute Glycans

a. Place the cartridges over a collection vial (cat # LL-COLLV-01)
b. Apply each sample to a prepared LudgerClean™ CEX cartridge (cat # LC-CEX-H-01) and allow the solution to flow through the resin bed slowly under gravity.
c. Wash out each vial with 200 µL water and add to the top of each column.
d. Further elute with 3 x 0.5 mL water.

The eluted fluid will contain the purified, released glycans.
If the flow through the column is restricted, e.g. by an air gap, then apply a slight pressure to the top of the cartridge (e.g. using a pipette) to resume normal flow.
Note that at this stage glycans will be in slightly acidic water after elution from the CEX cartridge.

Sample Storage

12 Dry the Glycan Solutions

If required, the samples should be dried by centrifugal evaporation

Keep the sample temperature <28°C to minimize desialylation.
This step is optional and can be omitted if you are analyzing aliquots by any method that first involves drying (e.g. addition to a MALDI-MS plate or fluorescence labelling).

13 Store the Glycans Frozen

For long-term storage, store the glycans at -20°C or lower temperature.

The released glycans can be stored frozen either dried or after reconstitution with water.

Analysis of Released Glycans

The released glycans can be analyzed by a variety of techniques including the following:

- Fluorescence labelling with LudgerTag™ fluorophores followed by HPLC, CE or MS.
  The following table lists the current LudgerTag™ fluorophores and rates them according to their suitability for various analysis methods.

Fluorophore	HPLC	MS	CE
2-AB (2-aminobenzamide)	* * * * *	* * *	
2-AA (2-aminobenzoic acid)	* * * * *	* * * * *	* *
AA-Ac (3-(Acetylamino)-6-aminoacridine)	* * * * *	* * * * *	* * * *
APTS (1-aminopyrene-3,6,8-trisulfonate)			* * * * *
2-AP (2-aminopyridine)	* * *	* *	

Key:
5 stars = excellent, 4 stars = good, 3 stars = fair, 1 - 2 stars = poor, no stars = not applicable

- Mass spectrometry
- HPAE-PAD (high pH anion exchange chromatography with pulsed amperometric detection)

Example Data: Repeatability using Bovine Fetuin

To obtain statistical data on the repeatability of the Orela glycan release kit, triplicate samples of bovine fetuin glycoprotein (GCP-FET-250) were subjected to Orela for release of the O-linked glycans. This method was repeated on three separate occasions. Released glycans were 2-aminobenzamide (2-AB) labelled and separated on a LudgerSep™N2 column (cat. No. LS-N2-4.6x150). The areas of each glycan peak were compared replicate to replicate and day to day.

Orela was performed using two separate conditions: 50°C for 16 hours or 60°C for 6 hours.

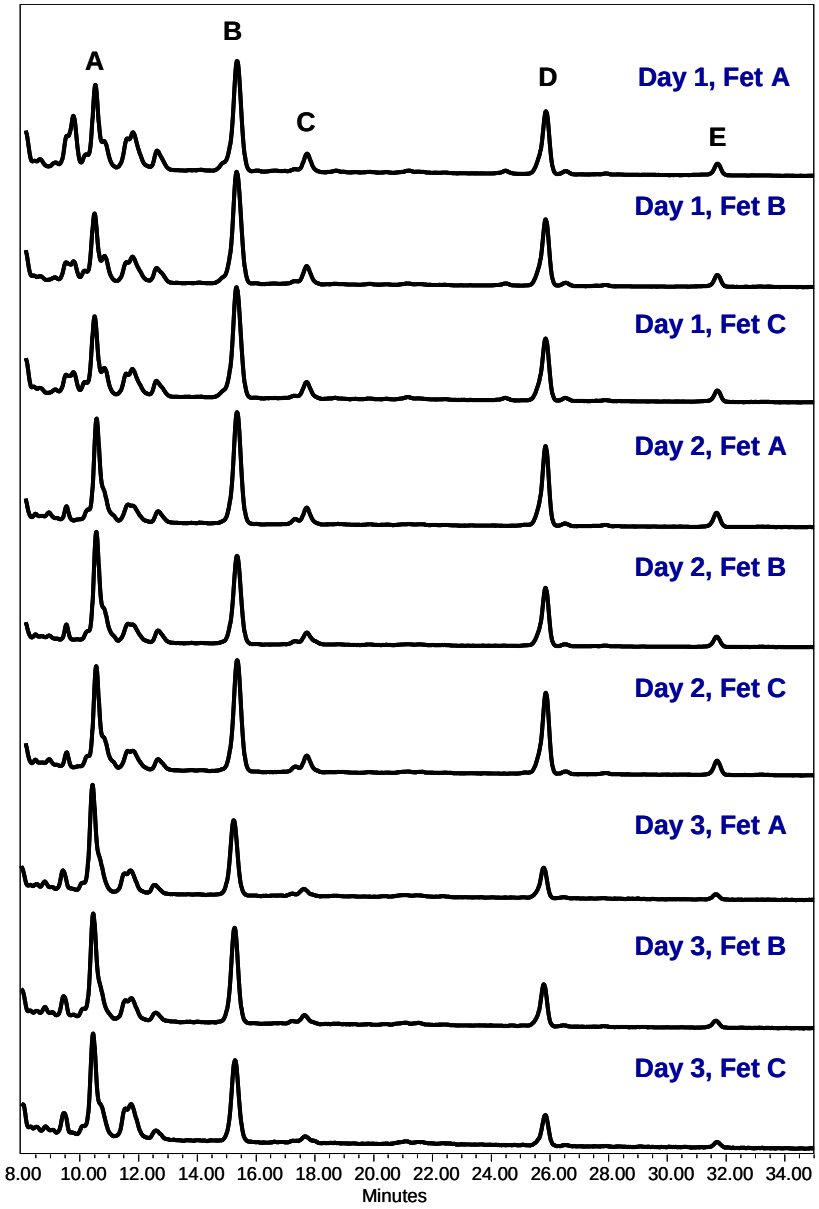


Figure 1: LudgerSep™N2 chromatograms showing 2-AB labelled glycans released from fetuin at 50°C for 16 hours.

Peak, GU	Peak A GU 2.29	Peak B GU 2.95	Peak C GU 3.26	Peak D GU 4.54	Peak E GU 5.59
	% area				
Average	36.52	36.39	5.49	18.79	2.81
SD	8.43	3.85	1.08	4.30	0.76
CV	**23.09**	**10.57**	**19.68**	**22.87**	**26.87**

Table 1: % peak area data including the peeled peak: A, GU 2.29, for fetuin glycans released at 50°C for 16 hours.

Peak, GU	Peak B GU 2.95	Peak C GU 3.26	Peak D GU 4.54	Peak E GU 5.59
	% area			
Average	57.74	8.59	29.30	4.38
SD	5.44	0.72	3.95	0.82
CV	**9.42**	**8.40**	**13.47**	**18.84**

Table 2: % peak area data not including the peeled peak: A, GU 2.29, for fetuin glycans released at 50°C for 16 hours.

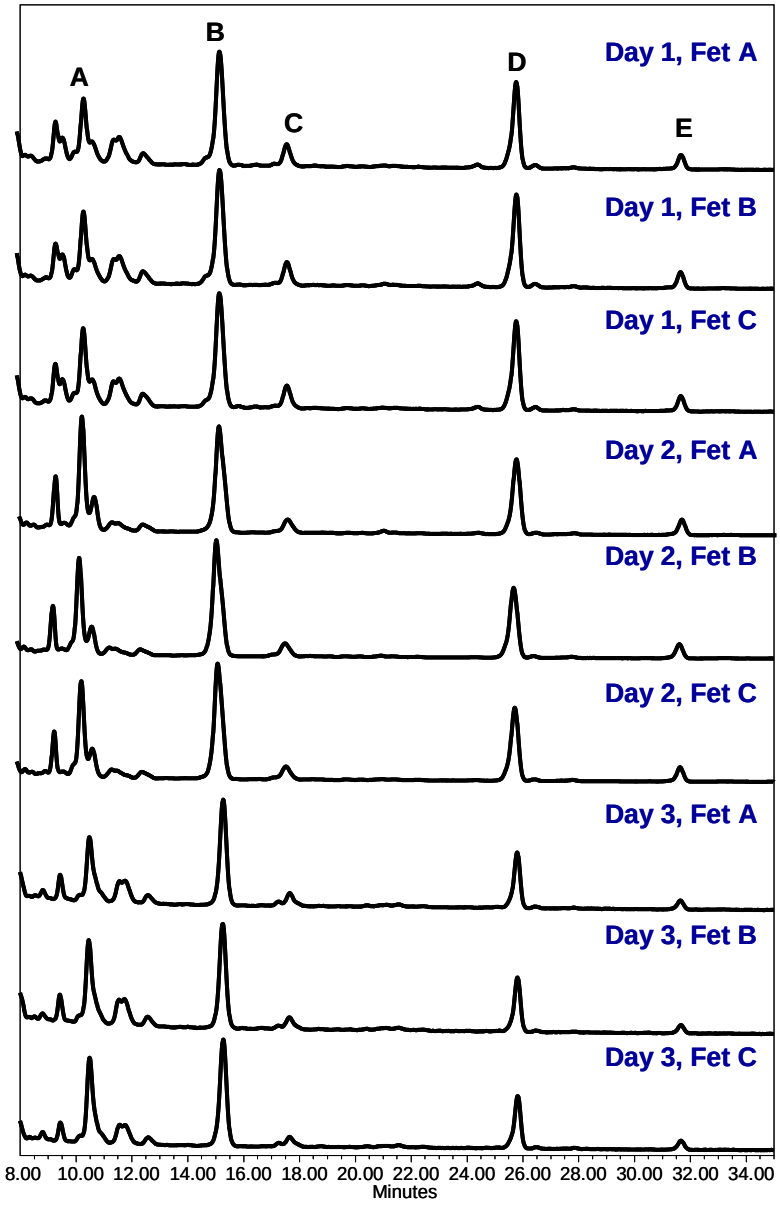


Figure 2: LudgerSep™ N2 chromatograms showing 2-AB labelled glycans released from fetuin at 60°C for 6 hours.

Peak, GU	Peak A GU 2.29	Peak B GU 2.95	Peak C GU 3.26	Peak D GU 4.54	Peak E GU 5.59
	% area				
Average	29.28	38.50	5.92	22.75	3.54
SD	3.89	1.69	1.03	3.02	0.52
CV	**13.29**	**4.39**	**17.34**	**13.28**	**14.80**

Table 3: % peak area data including the peeled peak: A, GU 2.29, for fetuin glycans released at 60°C for 6 hours.

Peak, GU	Peak B GU 2.95	Peak C GU 3.26	Peak D GU 4.54	Peak E GU 5.59
	% area			
Average	54.58	8.34	32.08	5.00
SD	3.69	1.09	2.85	0.67
CV	**6.77**	**13.04**	**8.88**	**13.46**

Table 4: % peak area data not including the peeled peak: A, GU 2.29, for fetuin glycans released at 60°C for 6 hours.

Warranties and Liabilities

Ludger warrants that the above product conforms to the attached analytical documents. Should the product fail for reasons other than through misuse Ludger will, at its option, replace free of charge or refund the purchase price. This warranty is exclusive and Ludger makes no other warrants, expressed or implied, including any implied conditions or warranties of merchantability or fitness for any particular purpose.
Ludger shall not be liable for any incidental, consequential or contingent damages.

This product is intended for *in vitro* research only.

Document Revision Number

Document # LL-ORELA-A2-Guide-v3.0

Appendix 1: Troubleshooting Guide

The Orela protocol is an efficient, robust method. If problems do arise they can normally be corrected without difficulty. The following is a guide to the most likely problems, possible causes, and solutions.

Low Yield

The temperature for Orela incubation was incorrect.

Please ensure that the oven or heating block is equilibrated to the incubation temperature and that the reaction tube is subjected to this temperature for the entire release period.

The sample was incompletely solubilized.

The glycoconjugate sample must be completely dissolved in the Orela reagent for maximum release efficiency. Please ensure that the sample is thoroughly mixed with the Orela reagent before the incubation and, as a precaution, re-mix the samples 15 and 30 minutes after the start of the incubation.

The sample contained contaminants that interfered with the release

Ensure that all samples are adequately purified before Orela release (see protocol step 1).

There was less starting glycoprotein or glycopeptide than was originally estimated.

The glycans were lost during the sample workup

Please ensure that the acidification and glycan purification steps are performed as in the protocol.

Peeling of Glycans

The peeling reaction is a degradation of the released glycans characterized by the loss of monosaccharide residues from the reducing terminus. O-glycans are generally more susceptible to peeling than N-glycans.

The temperature-time profile for the Orela reaction was too harsh for the glycans

Use the temperature-time profiles given in this protocol as a starting point. If you see peeling then for subsequent experiments reduce the temperature or time for the Orela reagent incubation.

Desialylation of the Glycans

The sample was subjected to acidic conditions in aqueous solutions at elevated temperatures

Avoid prolonged periods of exposure to sialylated glycan or glycoprotein samples in aqueous solutions at low pH and elevated temperatures.

In general, try to keep samples in solutions in the pH range of 5 – 8.5 and avoid exposure to temperatures above 28°C. Samples in pH-buffered aqueous solutions (with pH between 5 and 8.5) tend to be resistant to acid-catalyzed de-sialylation even at temperatures higher than 28°C. However, even then it is wise to err on the side of caution and keep the samples cool whenever possible.

Cannot Assign Peaks on Samples Analyzed by HPLC, MS or CE

Use glycoprotein and glycan standards appropriate for your project

Select reference glycoprotein standards to use as positive controls for hydrazinolysis and use relevant glycan standards in subsequent analyses. Ludger is developing a range of matched glycoproteins and released glycans as certified reference standards for use in glycoprofiling studies. Please contact us for advice on what standards to use for your particular application.