

# MIRAGE protocol

## Filling in the MIRAGE web-form

This step is to generate Excel spreadsheet(s) compatible with MIRAGE guideline.

1. Go to website: <http://unicarb-dr.biomedicine.gu.se/generate>
2. The Mirage spreadsheet contains three parts. The users can choose individual or all spreadsheets fitting the experimental setting. Then click **NEXT**.

- MIRAGE-Sample Preparation
- LC as part of Mass Spectrometry
- MIRAGE-MS

**NOTE:** Required fields are marked with an \* (If the field is not relevant for the experiment, it can be filled in with a "NA"-Not applicable).

After filling in the selected part(s), an Excel file can be created after clicking Generate. The user can modify the content during the filling-in (website) or after downloaded Excel spreadsheet(s).

### 3. MIRAGE-Sample Preparation section

The user needs to fill in basic information especially those marked with \*. If the cursor stays in column for a few second, a guideline will appears which will help the user to fill in the information. Or the user can select items from drop list if provided.

General features — (a) Global descriptors

Date stamp	<input style="width: 150px; border: 1px solid #ccc;" type="text" value="07 / 05 / 2018"/>	*	①
Responsible person. Provide name,	<input style="width: 150px; border: 1px solid #ccc;" type="text" value="Chunsheng Jin"/>	*	②
Affiliation	<input style="width: 150px; border: 1px solid #ccc;" type="text" value="edicine, Gothenburg University"/>	The (stable) primary contact person for this data set; this could be the experimenter, lab head, line manager etc. Where responsibility rests with an institutional role (e.g., one of a number of duty officers) rather than a person, give the official name of the role rather than any one person.	
Stable contact information	<input style="width: 150px; border: 1px solid #ccc;" type="text" value=":hunsheng.jin@medkem.gu.se"/>		

#### 1. Sample origin

Here "sample" is defined as any carbohydrate, polysaccharide, oligosaccharide or glycoconjugate that originates from any given starting material. The starting material may be a compound, mixture or cell product used to produce the oligosaccharide sample of interest. The source and/or methods used to produce the starting sample material can vary considerably but minimum information that describes its origin is outlined.

General information

Membrane protein samples from adult and neonatal healthy rats (Sprague Dawley) heart	*	③
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- (1) The date on which the work described was completed; given in the standard 'YYYY-MM-DD' format (with hyphens).
- (2) The (stable) primary contact person for this data set; this could be the experimenter, lab head, line manager etc. Where responsibility rests with an institutional role (e.g., one of a number of duty officers) rather than a person, give the official name of the role rather than any one person. In all cases give affiliation and stable contact information. This information can be made available as part of an authors' list or in an acknowledgment section
- (3) Describe how original starting sample material was generated or where it was obtained. Starting material descriptions are further delineated by biologically or chemically derived material.

## 1.1 Biologically derived material

## Biologically derived material - Recombinantly produced material

Cell type 4 

Growth/harvest conditions. Other modifications.

 5

## Biologically derived material - Biological origin of Material

Origin (biological fluids, tissue, etc) Species 

Describe treatments and/or storage conditions

Glycoprotein (Uniprot ID) 6 

## Biologically derived material - Purchased from commercial manufacturer

Vendor and applicable item information

## 1.2 Chemically derived material

Synthesis steps or specify where the equivalent reaction protocol is available.

 7Description of starting material 8 

## 2. Sample Processing

## 2.1 Sample Processing - Isolation

## Enzymatic treatments

Enzymes used	Vendor or enzyme production	Reaction conditions
Glycosidase-PNGASE-F <span style="border: 1px solid black; border-radius: 50%; padding: 2px;">9</span>	Prozyme	In-solution digestion

- (4) Name of cell line (e.g., CHO, HEK, NS0 etc.)
- (5) Growth/harvest conditions should be specified. Any modifications to cells that influence the characteristics of the starting material (e.g. genetic manipulations) should also be stated.
- (6) Uniprot ID (e.g., PQ2771)
- (7) If samples were synthetically derived, provide information.
- (8) Define the type of starting material used or produced that contains the oligosaccharide to be used/analysed in subsequent experiments. These may include glycoprotein(s), proteoglycan, glycolipid, GPI-anchored, free-oligosaccharides, sugar-nucleotides or synthetically derived material but are not limited to these definitions.
- (9) Processing may include methods to remove the oligosaccharides from the starting material prior to downstream experiments or conversely the starting material may also be altered so the oligosaccharide remains conjugated to non-carbohydrate material such as chemical (e.g. linker) or biological (e.g. peptides) components.

For enzymatic treatments, (i) describe any enzymes used to for the purpose of oligosaccharide removal (e.g. PNGase F) or for modification of the starting material (e.g. trypsin protease); (ii) specify where it was obtained (vender) or for enzymes produced in-house, describe expression and purification procedure; (iii) state if sample material was treated in-solution or immobilized (SDS-PAGE, PVDF etc.) as well as temperature, duration, volume, enzyme concentration.

For chemical treatments, it refers to the technique for oligosaccharide release or other chemical modifications (e.g., hydrazinolysis,  $\beta$ -elimination etc.). The reaction condition should contain temperature, duration, volume and chemical concentrations.

The screenshot displays a web form for the MIRAGE protocol, divided into several sections:

- Section 9: Chemical treatments**
  - Sub-section: Chemical methods
  - Field: Release method-REDUCTIV (dropdown)
  - Field: Reaction conditions (50 mM NaOH and 0.5 M NaBH<sub>4</sub>)
- Section 10: 2.2 Sample Processing - Modification**
  - Sub-section: Enzymatic modifications
  - Fields: Enzymes used (dropdown), Reaction conditions, Origin of novel enzyme
- Section 11: 2.3 Sample Processing - Purification**
  - Field: Purification steps (empty text area)
- Section 12: 3. Defined sample**
  - Field: Sample name (Oligosaccharides)
- Section 13: Chemical treatments**
  - Fields: Chemical methods (dropdown), Type of modification, Reaction conditions

## (10) Sample processing-Modification

For enzymatic modifications, (i) describe any treatments made to the isolated material; (ii) enzyme concentration, supplier, biological source, incubation time and temperature, (iii) if novel glycosidase was used, provide information indicating the origin (i.e. species) of the enzyme.

For chemical modifications, (i) describe any treatment made to the isolated material; (2) explain the type of modification employed (e.g., hydrolysis, sample tagging including fluorescent labels, isotopic labelling, permethylation/peracetylation, etc.); (3) source of materials, description of kits used, reaction conditions and detailed workflow.

## (11) Sample processing-Purification

Specify all steps used to purify starting material after isolation/modification steps. Examples of procedures include solid phase extraction (SPE), liquid-liquid extraction or other chromatographic methods. For each method describe the all experimental materials (e.g., stationary phase) and methods (e.g., flow rates, fractionation etc.).

## (12) Defined sample

Name or specify the type of sample material to be analysed or used in other experiments. These may include but are not limited to glycoconjugates, glycosaminoglycans, N- or O-glycans, glycopeptides, glycolipids, monosaccharides, poly- and oligosaccharides.

## 4. LC settings

This applies for both online and offline liquid chromatography (LC) separation.

### LC-MS Settings

For uploading of data to UniCarb-DB

#### General features — (a) Global descriptors

Date stamp  \*

Responsible person. Provide name,  \*

Affiliation  \*

Stable contact information  \*

#### 1. HPLC

HPLC manufacturer  ▼

HPLC brand  ▼

Injector

Injector settings

#### 2. Method run

Temperature (°C)

Solvent a  \*

Solvent b  \*

Other solvent

Flow rate  \*

Gradient

Run time

Phase  \*

#### 3. Column

Manufacturer  ▼

Model  ▼

Type of chromatography

Type of material

Column diameter (mm)

Column length (mm)

Particle size (µm)

## 5. MS part 1

## MIRAGE MS Guidelines

Guidelines for reporting mass spectrometric analysis data of glycans

## 1. General features

## General features — (a) Global descriptors

Date stamp	<input type="text" value="07 / 05 / 2018"/>	*
Responsible person. Provide name,	<input type="text" value="Chunsheng Jin"/>	*
Affiliation	<input type="text" value="Institute of Biomedicine, Gothe"/>	*
Stable contact information	<input type="text" value="chunsheng.jin@medkem.gu.se"/>	*
Instrument manufacturer	<input type="text" value="Thermo Fisher"/>	1
Instrument model	<input type="text" value="LTQ Linear Ion Trap"/>	2
Customizations	<input type="text"/>	3
Ion mode	<input type="text" value="Negative"/>	

## General features — (b) Control and analysis software

Software name	Version	Upgrades	Switching criteria	Isolation width	Location of 'parameters' file
Xcalibur	2.2				<input type="text" value=""/> + 4

- (1) The manufacturing company name for the mass spectrometer.
- (2) The model name for the mass spectrometer.
- (3) Any significant (i.e., affecting behavior) deviation from the manufacture's specification for the mass spectrometer.
- (4) Control and analysis software

The instrument management and data analysis package name, and version; where there are several pieces of software involved, give name, version and role for each one. Also mention upgrades not reflected in the version number.

For switching criteria, it is for tandem MS only. The list of conditions that cause the switch from survey or zoom mode ( $MS^1$ ) to or tandem mode ( $MS^n$  where  $n > 1$ ); e.g., 'precursor ion' mass lists, neutral loss criteria and so on.

For isolation width, it refers to global or by MS level. For tandem instruments (i.e., multi-stage instruments such as triple quads and TOF-TOFs, plus ion traps and equivalents), the total width (i.e., not half for plus-or-minus) of the gate applied around a selected precursor ion  $m/z$ , provided for all levels or by MS level.

The location and name under which the mass spectrometer's parameter settings file for the run is stored, if available. Ideally this should be a URI including filename, or most preferably an LSID, where feasible. Location of file should be mentioned.

## 2. Ion Sources

Ion Sources — (a) Electrospray Ionisation (ESI) Supply type (static, or fed)  \*Interface name 

Catalog number, vendor, and any modifications made to the standard specification.

Sprayer name  \*

Sprayer type, coating, manufacturer, model and catalog number (where available)

Relevant voltages where appropriate (tip, cone, acceleration)

Degree of prompt fragmentation evaluated Yes Whether in-source dissociation performed Yes 

Other parameters if discriminant for the experiment (such as nebulizing gas and pressure)

Ion sources — (b) MALDI Plate composition (or type) NA  \*Matrix composition (if applicable) NA  \*Deposition technique NA  \*Relevant voltages where appropriate NA  \*Degree of prompt fragmentation evaluated NA PSD (or LID/ISD) summary, if performed NA Operation with or without delayed extraction NA  \*Laser (e.g., nitrogen) and wavelength (nm) NA  \*

Other laser related parameters, if discriminating for the experiment

The ion sources includes (a) electrospray ionization (ESI) or (b) MALDI

For ESI,

- (5) Whether the sprayer is fed (by, for example, chromatography or CE) or is loaded with sample once (before spraying).
- (6) Where the interface was bought from, plus its name and catalog number; list any modifications made to the standard specification. If the interface is entirely custom-built, describe it or provide a reference if available.
- (7) Where the sprayer was bought from, plus its name and catalog number; list any modifications made to the standard specification. If the sprayer is entirely custom-built, describe it briefly or provide a reference if available.
- (8) Voltages that are considered as discriminating from an understood standard measurement mode, or important for the interpretation of the data. These might include the voltage applied to the sprayer tip, the voltage applied to the sampling cone, the voltage used to accelerate the ions into the rest of the mass spectrometer (mass analysis + detection) by MS level.
- (9) Yes/No. If yes, provide data showing results.
- (10) State whether in-source dissociation was performed (increased voltage between sample orifice and first skimmer).

- (11) Where appropriate, and if considered as discriminating elements of the source parameters, describe these values.

For MALDI,

- (12) The material of which the target plate is made (usually stainless steel, or coated glass); if the plate has a special construction.
- (13) The material in which the sample is embedded on the target (e.g., 2,5-dihydroxybenzoic acid (DHB)).
- (14) The method of laying down (matrix and) sample on the target plate (including matrix concentration and solvents applied); for example, matrix+sample in single deposition; or matrix, then matrix+sample (if several matrix substances are used, name each), Recrystallization using volatile solvent; where chromatographic eluent is directly applied to the plate by apparatus, or for other approaches, describe the process and instrumentation involved very briefly and cross-reference.
- (15) Voltages considered as relevant for the interpretation of the data. This might include the grid voltage (applied to the grid that sits just in front of the target), the acceleration voltage (used to accelerate the ions into the analyzer part of the mass spectrometer (mass analysis + detection), etc.
- (16) Yes/No. If yes, provide data showing results.
- (17) Confirm whether post-source decay, laser-induced decomposition, or in-source dissociation was performed; if so provide a brief description of the process (for example, summarize the stepwise reduction of reflector voltage).
- (18) State whether a delay between laser shot and ion acceleration is employed.
- (19) The type of laser and the wavelength of the generated pulse (in nanometers).
- (20) Other details of the laser used to irradiate the matrix-embedded sample if considered as important for the interpretation of data; this might include the pulse energy in microJoules, focus diameter in microns, attenuation details, pulse duration in nanoseconds at full-width half maximum, frequency of shots in Hertz and average number of shots fired to generate each combined mass spectrum.

## 3. Ion transfer optics

Hardware options <b>(21)</b>	<input type="text"/>	Post-source componentry — (b) TOF drift tube <b>(25)</b>	<input type="checkbox"/>	<input type="text" value="NA"/>
		Reflectron status (on, off, none)		<input type="text" value="NA"/>
Post-source componentry — (a) Collision cell		Post-source componentry — (c) Ion trap <b>(26)</b>	<input type="checkbox"/>	<input type="text" value="NA"/>
Collision-Induced Dissociation (CID) <b>(22)</b>	<input type="checkbox"/>	Final MS stage achieved		<input type="text" value="NA"/>
Gas composition	<input type="text" value="NA"/>	Post-source componentry — (d) Ion mobility <b>(27)</b>	<input type="checkbox"/>	<input type="text" value="NA"/>
Gas pressure	<input type="text" value="NA"/>	Gas		<input type="text" value="NA"/>
Collision energy CID/function	<input type="text" value="NA"/>	Pressure		<input type="text" value="NA"/>
		Instrument-specific parameters		<input type="text" value="NA"/>
Electron Transfer Dissociation (ETD) <b>(23)</b>	<input type="checkbox"/>	Post-source componentry — (e) FT-ICR <b>(28)</b>	<input type="checkbox"/>	<input type="text" value="NA"/>
Reagent gas	<input type="text" value="NA"/>	Peak selection		<input type="text" value="NA"/>
Pressure	<input type="text" value="NA"/>	Pulse		<input type="text" value="NA"/>
Reaction time	<input type="text" value="NA"/>	Width		<input type="text" value="NA"/>
Number of reagent atoms	<input type="text" value="NA"/>	Voltage		<input type="text" value="NA"/>
Electron Capture Dissociation (ECD) <b>(24)</b>	<input type="checkbox"/>	Decay time		<input type="text" value="NA"/>
Emitter type	<input type="text" value="NA"/>	IR		<input type="text" value="NA"/>
Voltage	<input type="text" value="NA"/>	Other parameters		<input type="text" value="NA"/>
Current	<input type="text" value="NA"/>	Post-source componentry — (f) Detectors <b>(29)</b>	<input type="checkbox"/>	<input type="text" value="NA"/>
		Detector type		<input type="text" value="NA"/>

(21) Hardware options refer to e.g. “simple” quadrupoles, hexapoles, stacked ring electrodes, TOF, ...

(22) For collision-induced dissociation (CID)

The composition and pressure of the gas used to fragment ions in the collision cell (TOF-TOF, linear trap, Paul trap, or FT- ICR cell) should be indicated.

Collision energy CID/function refers to the specifics for the process of imparting a particular impetus to ions with a given  $m/z$  value, as they travel into the collision cell for fragmentation. This could be a global figure (e.g., for tandem TOFs), or a complex function; for example a gradient (stepped or continuous) of  $m/z$  values (for quads) or activation frequencies (for traps) with associated collision energies (given in eV).

(23) For electron transfer dissociation (ETD)

Reagent gas, pressure, reaction time, and number of reagent ions should be filled in.

(24) Electron capture dissociation (ECD)

Emitter type, voltage, and current should be filled in.

(25) TOF drift tube

Whether a Reflectron is present, and if so, whether it is used. Depending on the type of instrument provide exact details on the reflectron mode (e.g. V or W mode).

(26) Ion trap

The final MS level achieved in generating this data set with an ion trap or equivalent (e.g., MS<sup>10</sup>).

(27) Ion mobility

The gas, pressure, and instrument-specific parameters (e.g. wave velocity/height depending on the particular vendor's options for tuning this component) should be filled in.

(28) FT-ICR

Peak selection, pulse width, voltage, decay time, IR and other important experiment parameters should be filled in.

(29) Detectors

Need to define detector type if non OEM detector were used (e.g. microchannel plate, channeltron etc.).

6. MS part 2

## 4. Spectrum and peak list generation and annotation

For this section, if software other than that listed in 1b (Control and analysis software) is used to perform a task, it must be supplied in each case.

Software name	Version
Xcalibur	2.2

## Spectrum and peak list generation and annotation — (a) Spectrum description

Location of source ('raw') files

Software	Name	Format	Link to the target area	URL
Xcalibur				

## Peak list generation and annotation — (b) Peak list generation

Since several different applications may be used for the data acquisition, data post processing and spectrum annotation each used software should be recorded separately together with the information what modification has been done to the data.

Software Name	Version	Customizations	Software settings

Data file(s)

Software	Name	Format	URL

Acquisition number for all acquisitions.	<input type="text"/>
Generation of peak lists from raw data	<input type="text"/>
Raw data scoring	<input type="text"/>
Smoothing; whether applied, parameters	<input type="text"/>
Background threshold, or algorithm used	<input type="text"/>
Signal-to-noise estimation and method	<input type="text"/>
Percentage peak height for centroiding	<input type="text"/>
Retention times for all acquisitions	<input type="text"/>
m/z and intensity values	<input type="text"/>

- (1) For this section, if software other than that list in **Control and analysis software** is used to perform a task, the producer, name and version of that software must be supplied in each case.
- (2) The location and filename under which the original raw data file from the mass spectrometer is stored, if available.

Give the type of the file where appropriate, or else a description of the software or reference resource used to generate it. Due to the nature of the raw files (proprietary formats, no open source software, licensing, etc), the validation of raw data can only be possible if the information is provided in an open XML format (mzXML, mzData, mzML). Input either a spot number or some other form of coordinates if more appropriate, that link the spectrum to the

analyzed area of the sample (2D imaging). Ideally this should be a URL or filename, or most preferably an LSID, where feasible.

(3) For peak list generating software,

This includes the name of the software, the version number, any changes made to the original program code that may affect the results and any settings made in the software that may affect the results (e.g. thresholds).

(4) Provide information about the produced data file. This includes the name of the software, the name of each file, the file format, the availability of the file and if applicable the URL to access the file.

(5) Where available, the reference numbers of all the scans (as numbered in the raw file) that were combined to produce a peak list, the total number of acquisitions combined to produce the peak list, and whether the peak list was produced by summing or averaging the scans that are listed.

(6) The total ion count or S/N threshold for a spectrum and the minimum number of ions detected in that scan, for it to be a candidate for grouping in a peak list; plus the mass tolerance (Da) on the precursor ion masses for MS/MS spectra.

(7) Describe method and software for selection of peaks for inclusion in the peaklist.

(8) Any peak smoothing should be described, along with the parameters supplied to the algorithm.

(9) The ion abundance or S/N cut-off used to filter background noise; or a description of the algorithm used to gate the noise, if complex.

(10) The ratio of signal to noise for each significant peak in a peak list; significance is defined as being above a given ion abundance(which should be supplied) or being otherwise of interest; the method of calculation should also be named (if available).

(11) The percentage peak height at which centroids are calculated; if a more complex algorithm is used to perform the process, it should be named here.

(12) The times relative to the start of the MS run for all acquisitions that were combined in the peak list so that those acquisitions may later be correlated to a chromatogram (continuously-fed electrospray sources only).

(13) The actual data (m/z versus ion abundance); as described in the preceding sections.

## Peak list generation and annotation — (c) Annotation and scoring

Since several different applications may be used for the data acquisition, data post processing and spectrum annotation each used software should be recorded separately together with the information what modification has been done to the data.

Software name	Version	Software type	Customizations	Software settings
<input type="text"/>				

(14)

Data file(s) generated by the software

Software	Name	Format	URL
<input type="text"/>	<input type="text"/>	<input type="text"/>	<input type="text"/>

(15)

Database settings and matching

Database queried	<input type="text"/>	(16)
Taxonomical restrictions	<input type="text"/>	(17)
Other restrictions	<input type="text"/>	(18)
Allowed cleavages	<input type="text"/>	(19)
Parent error	<input type="text"/>	(20)
Fragment error	<input type="text"/>	
Scoring method	<input type="text"/>	(22)
Scoring value format	<input type="text"/>	
Scoring algorithm	<input type="text"/>	
Scoring result	<input type="text"/>	
Validation status	<input type="text"/>	(23)
Validation value format	<input type="text"/>	(25)
Validation result	<input type="text"/>	

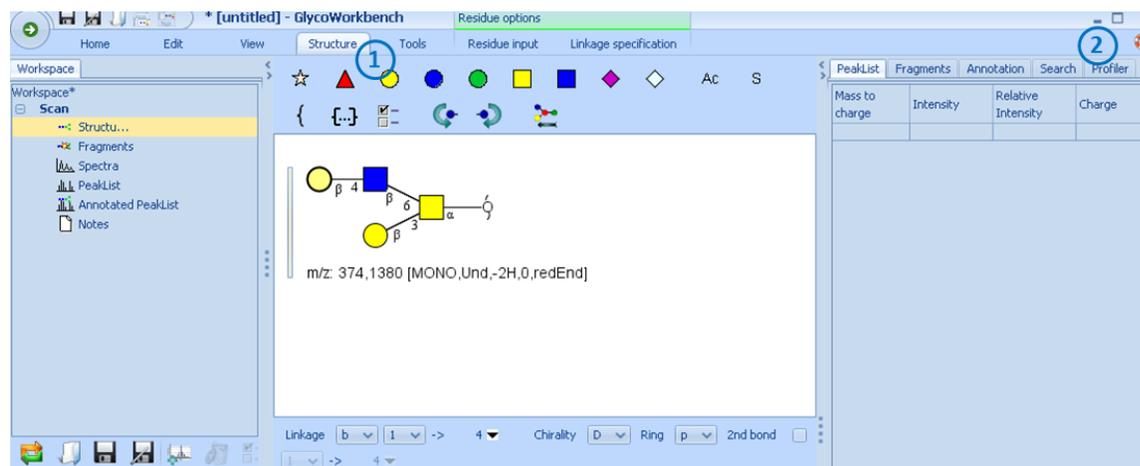
- (14) This includes the name of the software, the version number and type of data processing that was performed with the software. Any changes made to the original program code that may affect the results. Any settings made in the software that may affect the results (e.g. thresholds).
- (15) Information about the annotation data file. This includes the file format, the availability of the file and if applicable the URI to access the file.
- (16) List of databases used for the annotation of the data. Also specify databases version, annotation date and number of entries.
- (17) List of species the search was limited to.
- (18) Other settings to the software that filtered out certain sequences from the database (e.g. allow only certain glycan types (N-Glycan) or restriction by composition). This also includes the usage of threshold for scoring values.
- (19) List of allowed cleavages for the annotation run (A, B, C, X, Y, and Z). This includes also the number of allowed cross-ring cleavages and glycosidic cleavages.
- (20) Mass accuracy settings of both precursor and fragment for the annotation run.
- (21) Used scoring function with references to the algorithm and of software.
- (22) Validation status for all glycan structures, specify if accepted without post-processing of database/de-novo interpretation or if manually accepted or rejected.

- (23) Confirmation of preliminary assignment with tandem MS data. In the case of glycan tandem/multistage MS profiling (MSn) describe the number of fragmentation stages and m/z values associated to the identified glycan.

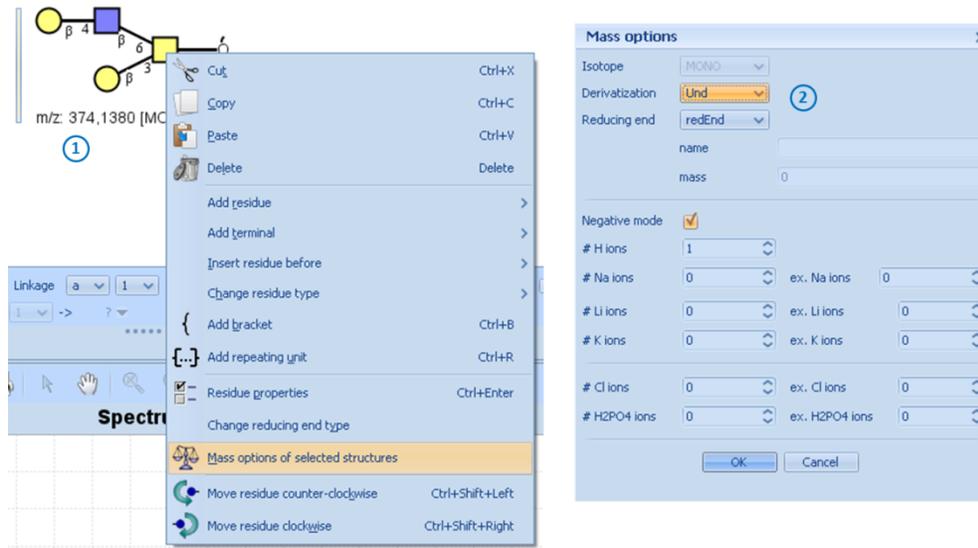
## Glycoworkbench file

This protocol is used to deposit annotated glycan structures, peak list, and other related mass spectral information (e.g., annotation) into an integrated file (glycoworkbench workspace file, .gwp format). The GWP file contains all content that needed to present in Unicarb-DR (<http://unicarb-dr.biomedicine.gu.se/>).

1. Download and install the software (<https://code.google.com/archive/p/glycoworkbench/>).
2. Download the sample Glycoworkbench file from Unicarb-DR (<http://unicarb-dr.biomedicine.gu.se/generate>).
3. Open downloaded sample Glycoworkbench file and draw the first structure in Glycoworkbench.

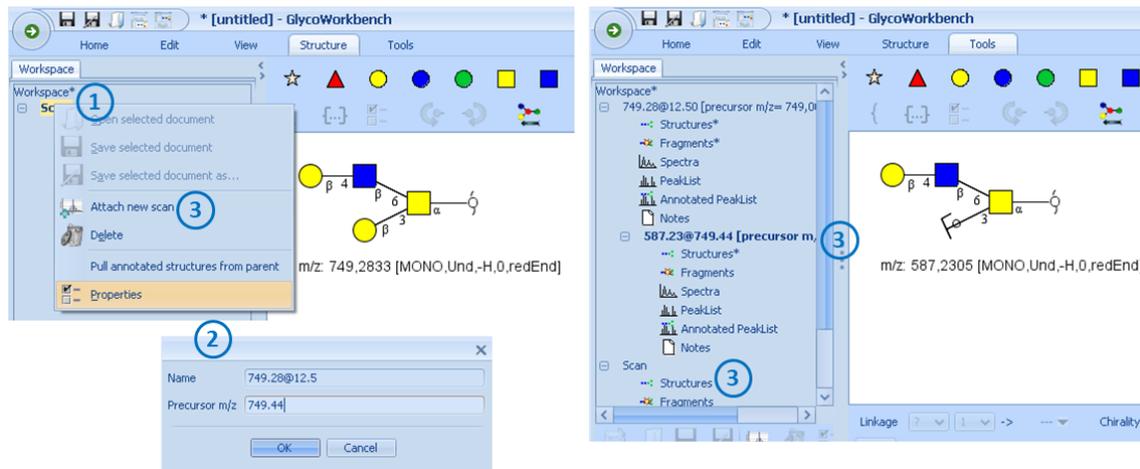


- (1) Using Structure function to draw the structures. For uncertain residue(s), just put e.g. hexose (blank circle). For uncertain linkage, put question mark.
- (2) The detailed manual can found here.
4. Set the calculated mass close to observed mass



- (1) Select the structure and right click. Choose **Mass options of selected structures**.
- (2) Choose the settings for observed mass. Click **OK**. In case of positive mode such as  $[M-H+2Na]^+$ , it can be expressed as one H ion with two ex Na ions. For the negative ions,  $[M-2H+Na]^-$ , it can be expressed as two H ion with one ex Na ion.

5. Change the scan name to observed mass.



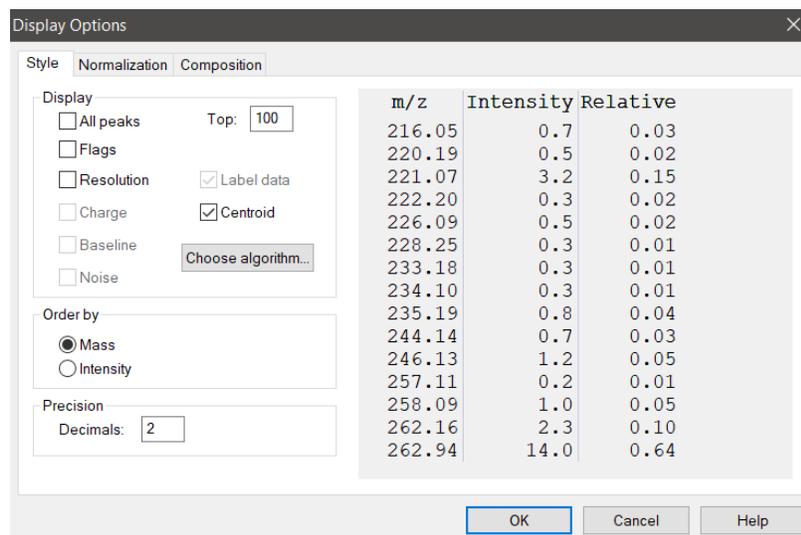
- (1) Right click **Scan** and choose **Properties**.
- (2) In the column **Name**, add calculated mass with either retention time or G.U. value separating with @ (e.g., 749.28@12.50 or 749.28@gu5.2). In the column **Precursor m/z**, add observed mass. Click **OK**. **NOTE**: use period as decimal point rather than comma.
- (3) If MS3 is available, a new scan should be added by choosing **Attach new scan**. Then fragmental structure can be drawn in the new scan window.

If one wants to add second structure, right click **Workspace** and click **Attach new scan**.

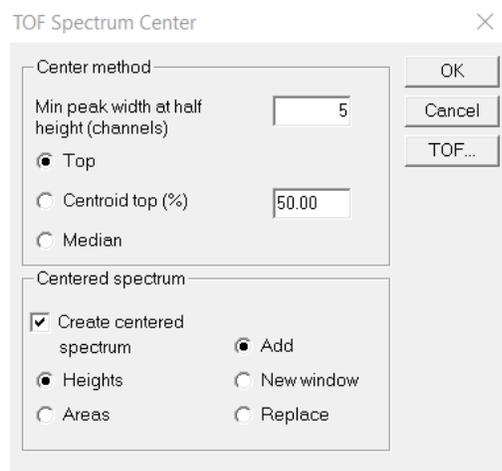
6. Input peak list.

The MS/MS peak list is a two-column matrix containing peaks (m/z values) and ion abundance of each peak. The peak list refers to any kind of centroid data, which are not in profile mode, i.e. do not have continuous m/z data. Preferably, the list should contain 100-200 mass peaks.

For Thermo raw data, open raw file in **Qual Browser** which contains the target MS/MS spectra. In Spectrum window, right click and choose **View>Spectrum List**. Right click and choose **Display Options**. Select **Centroid** and input 100 or tick "All peaks". Click OK. To export peak list in Spectrum window, right click and choose **Export>Clipboard (Exact Mass)**.



For Waters raw data, open raw file in MassLynx and display MS/MS spectra in spectrum window. In the spectrum window, go to **Process>Center....** In the **Min peak width at half height (channels)**, input 5 or higher values so that the peak list would contain 100-200 top peaks. In order to export peak list, click **Edit** and choose **Copy Spectrum List**.



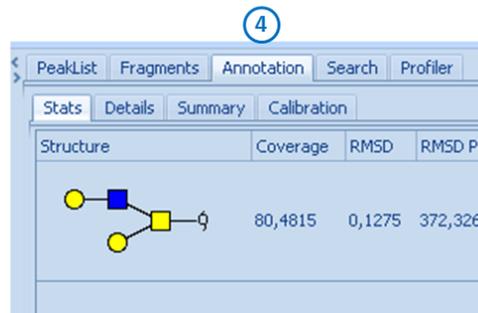
To input the peak list to Glycoworkbench,

(1) Click **PeakList** and select the first cell under **Mass to charge**. Right click and select **Paste**.

(2) Click **PeakList**, the corresponding MS/MS spectrum will appear in Spectrum window.

## 7. Annotation

To annotate the peaks, select the structure and go to **Tools**. Select **Annotation>Annotate peaks with fragments from selected structures**. The **Fragment options** window appears.



- (1) For MS/MS spectra obtained from positive-ion mode, no cross ring fragments should be selected in general. For MS/MS spectra obtained from negative-ion mode, only A fragments should be selected for non-sialylated oligosaccharides; both A and X fragments should be selected for sialylated oligosaccharides.
- (2) Depending on molecular fragmentation technology, specify the maximal number of cleavages. For CID, it allows two maximal glycosidic cleavages and one cross-ring cleave.
- (3) If the precursor ions are doubly charged (e.g.,  $[M-2H]^{2-}$ ) when drawing the structure, both singly and doubly charged fragment ions will be annotated. If the precursor ions are singly charged (e.g.,  $[M-H]^{-}$ ), only singly charged fragment ions will be assigned.
- (4) Annotation results will appear in right panel of Glycoworkbench after click **OK** in **Fragment options**.

## 8. Validation of annotation

The result of annotation needs validation before uploading to UniCarb-DR to remove ambiguous assignments.

The screenshot shows the GlycoWorkbench software interface. At the top, there are tabs for PeakList, Fragments, Annotation, Search, and Profiler. Below these are sub-tabs for Stats, Details, Summary, and Calibration. The main area displays a list of mass spectrometry annotations. A detailed view of a selected annotation is shown at the top, with a circled '1' indicating the 'Annotation' tab. The detailed view shows a chemical structure and the text 'm/z: 749,2833 [MONO,Und.-H,O,redEnd]'. The main table lists annotations with columns for Mass to charge, Intensity, Relative Intensity, Ion, Type, and Score. A row with m/z 235,1900 and Type 3,5A<sub>GlcNAc</sub> is highlighted in yellow, with a circled '2' indicating a right-click context menu. The context menu includes options like Cut, Copy, Paste, Delete, Copy fragments into canvas, and Show isotopic distributions.

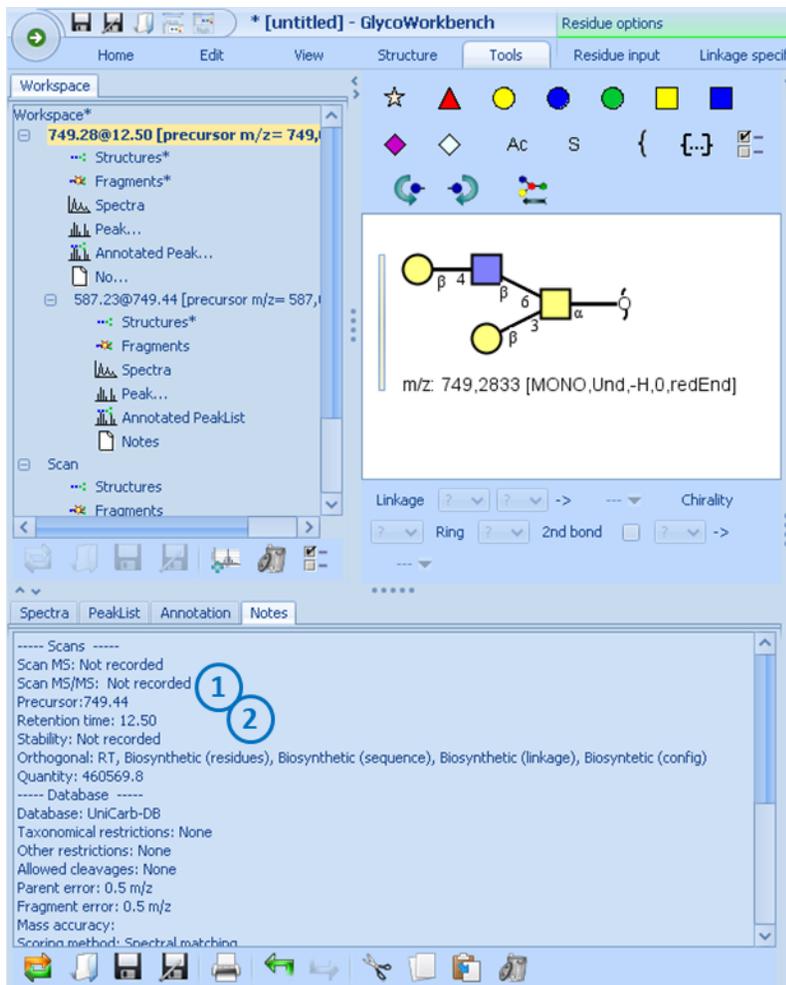
Mass to charge	Intensity	Relative Intensity	Ion	Type	Score
221,0700	3,2000	0,1462		2,4A <sub>GlcNAc</sub>	0,0000
222,2000	0,3000	0,0137		YY	0,0000
226,0900	0,5000	0,0228			0,0000
228,2500	0,3000	0,0137			0,0000
233,1800	0,3000	0,0137			0,0000
234,1000	0,3000	0,0137			0,0000
235,1900	0,8000	0,0365		3,5A <sub>GlcNAc</sub>	0,0000
244,1400	0,7000	0,0320		0,4A <sub>GalH</sub>	
246,1300	1,2000	0,0548			
257,1100	0,2000	0,0091			
258,0900	1,0000	0,0457		3,5A <sub>GalH</sub>	

(1) Select **Annotation>Details**, where detailed annotation can be found.

(2) To remove ambiguous annotation (mainly cross-ring fragments), click the structure that will be removed and right click. Select **Delete**, which only remove type of fragment (e.g., <sup>3,5</sup>A<sub>GlcNAc</sub>) rather than fragment ions from list. Usually, <sup>0,2</sup>A, <sup>0,4</sup>A and <sup>2,4</sup>A cleavages are kept. For N-glycans, <sup>0,3</sup>A of βMan and <sup>1,3</sup>A of αMan residues are also kept. For sialylated structure, <sup>0,2</sup>Xsialic acid ions are considered if present.

## 9. Note of annotated structure

In sample Glycoworkbench file downed from Unicarb-DR (<http://unicarb-dr.biomedicine.gu.se/generate>), there is **Note** section to record all information of selected structure. The content of Note section can be copied and pasted.



(1) At least, the m/z value of precursor ions should be recorded.

(2) At least, the retention time or g.u. values should be recorded.

10. Save the file as Glycoworkbench workspace file (.gwp) if no more structure is added.

## Uploading to Unicarb-DR

This step is to upload MIRAGE File and Glycoworkbench files (.gwp).

1. Sing up or log in Unicarb-DR.
2. Click the user name>**Submit data**.

3. Upload MIRAGE file and Glycoworkbench file.

Manuscript  ①

PubMed ID ②

30104209

30104209

Authors:  
Hykollari A, Malzi D, Eckmair B, Vanbeselaere J, Scheidl P, Jin C, Karlsson NG, Wilson IBH and Paschinger K

Title:  
Isomeric Separation and Recognition of Anionic and Zwitterionic N-glycans from Royal Jelly Glycoproteins.

Journal:  
Molecular & cellular proteomics : MCP

Year:  
2018

Mirage File (.xlsx) ③

2018-11-15 ...IRAGE.xlsx

GlycoWorkbench Files (.gwp) ④

Royaljelly.gwp

- (1) Tick it if it is a manuscript.
  - (2) Put PMID if the manuscript was published and click **Fetch details from PubMed**. The brief description will appear.
  - (3) Upload MIRAGE file.
  - (4) Upload Glycoworkbench file. Click **Next**.
4. A window for the user's inspection will appear. If no mistake is detected, click **Submit**.