



# **GRable Version 1.0**

## User Manual

Last update: October 30, 2023

# Table of Contents

1. Outline of GRable.....	1
1.1. Introduction.....	1
1.2. Principle of the Glyco-RIDGE method.....	1
1.3. Contact information .....	1
2. System overview .....	2
2.1. Overview of data processing .....	2
2.2. Requirements for input data .....	2
2.3. GUI and functionality .....	3
3. Operation.....	4
3.1. Login .....	4
3.2. Language selection .....	4
3.3. Data registration (Step 1) .....	4
3.3.1. Functional overview .....	4
3.3.2. Operation procedure .....	5
3.4. Range setting (Step 3).....	5
3.4.1. Functional overview .....	5
3.4.2. Operation procedure .....	5
3.5. Monoisotopic peak picking (Step 4) .....	6
3.5.1. Functional overview .....	6
3.5.2. Operation procedure .....	7
3.6. Clustering (Step 5).....	9
3.6.1. Functional overview .....	9
3.6.2. Operation procedure .....	9
3.7. Matching (Step 6) .....	10
3.7.1. Functional overview .....	10
3.7.2. Operation procedure .....	11
3.8. Selection (Step 7).....	13
3.8.1. Functional overview .....	13
3.8.2. Operation procedure .....	14
3.9. Data tree .....	17
3.10. Viewer .....	18
3.11. Monoisotopic peak list .....	19
3.11.1. Functional overview .....	19
3.11.2. Details of the correction function.....	21
4. Preparation details of import files.....	22
4.1. Core peptide list (.xlsx).....	22
4.2. Glycan point list (.xlsx).....	23
5. Details of export files .....	23

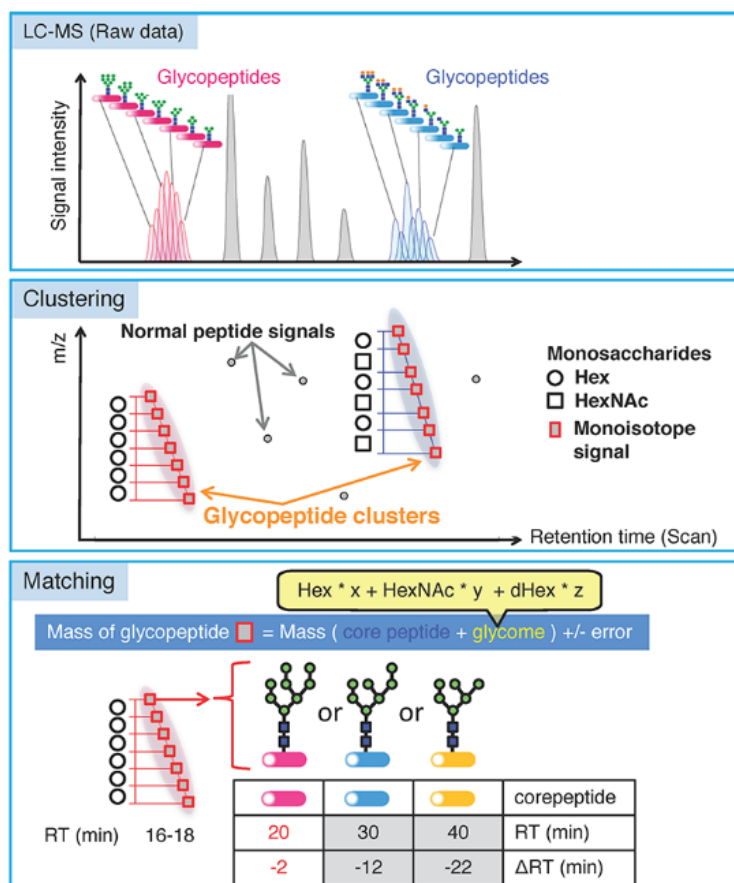
## 1. Outline of GRable

### 1.1. Introduction

GRable (<https://glycosmos.org/grable>) is a freely available online tool for identifying the site-specific glycoforms of glycopeptides. This tool is unique because it utilizes an MS1-based glycoproteomic method named “Glyco-RIDGE” (Glycan heterogeneity-based Relational Identification of Glycopeptide signals on Elution profile).

### 1.2. Principle of the Glyco-RIDGE method

First, this method identifies glycopeptide signals based on the chromatographic properties of the glycopeptides and mass differences owing to glycan heterogeneity. That is, glycopeptides with the same core peptide but different glycans elute within a narrow range of retention time (RT); therefore, glycopeptide signals with similar RT and mass differences corresponding to the masses of glycan units can be a cluster without MS2 spectrum analyses (Figure 1). In parallel, the core peptides present in the glycopeptide sample are identified using IGOT-LC/MS/MS. Considering that the mass value of the glycopeptide is the sum of the core peptide and glycan, the combination of peptide and glycan for each glycopeptide is searched from the mass and RT lists of glycopeptides, peptides, and glycans).



**Figure 1. Overview of the Glyco-RIDGE method.**

(Narimatsu *et al. J Proteome Res*, 2018)

### 1.3. Contact information

If you belong to an academic research institute, you can use the full version without functional limitations by concluding a joint research agreement with AIST. Please contact us: [M-GRable-inquiry-ml@aist.go.jp](mailto:M-GRable-inquiry-ml@aist.go.jp).

## 2. System overview

### 2.1. Overview of data processing

The software proceeds in seven steps (Figure 2-1). In GRable Version 1.0, Step 2 (deconvolution) is currently not applicable. Except for Step 2, each step is systematically executed after uploading the required data and setting the appropriate conditions in the software's main window. The viewers at the main interface can visually confirm the results of Steps 3–5. The detailed steps 4–7 results can be exported as Excel files along with each setting.

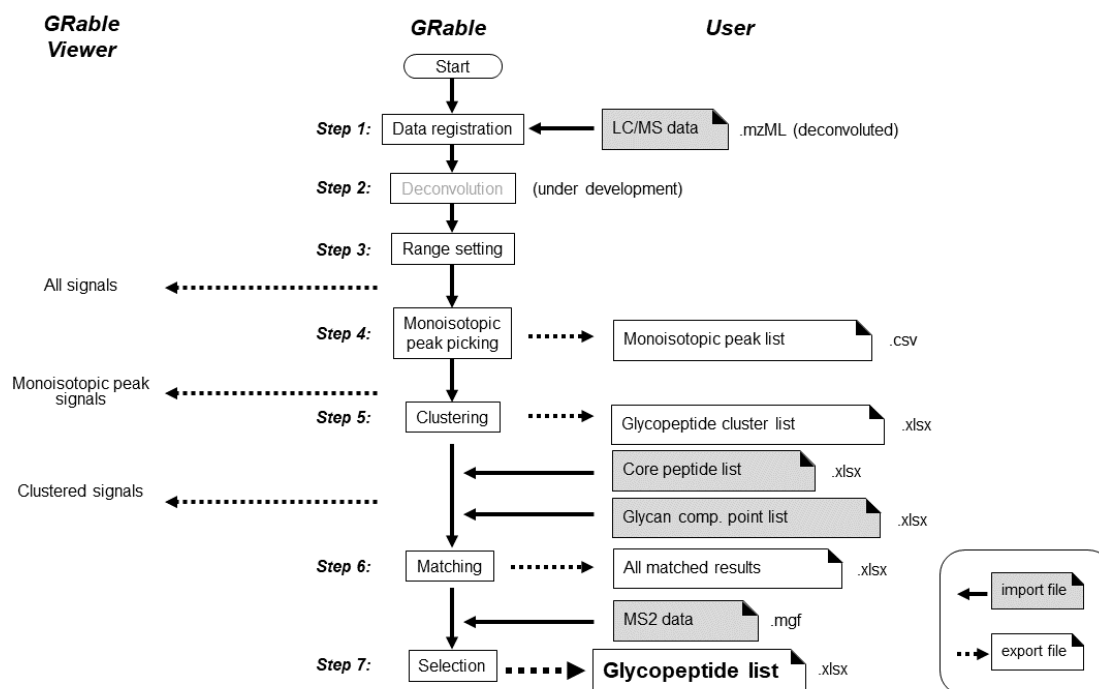


Figure 2-1. Overview of data processing by GRable.

### 2.2. Requirements for input data

The GRable requires the following four files [Note 1]: The details of the input files are described in (Section 4.).

- 1) LC/MS data of glycopeptides (.mzML)** and LC-MS data (. raw) is deconvoluted and exported in mzML format using Proteome Discoverer. ([Note 2, 3])
- 2) Core peptide list (.xlsx):** This list contains information on existing core peptides identified by PNGase-mediated deglycosylation followed by LC/MS analysis. ([Note 4])
- 3) Glycan point list (.xlsx):** This list assigns points to individual members according to the matched glycan compositions. The cluster score is the sum of the points obtained by each cluster member. The score is primarily used to evaluate the matching results for the cluster and assign high points to compositions with a high probability of existence and penalties to biosynthetically impossible compositions.
- 4) LC/MS/MS data of glycopeptides (.mgf):** Peak list of MS2 spectra extracted from the raw data of 1).

#### Notes

1. All data should be less than 300 MB each.
2. The GRable is developed using Thermo Fisher Scientific data. Therefore, data from equipment from other manufacturers are not supported. In addition, liquid chromatography-mass spectrometry (LC-MS) is performed using a C18 column.
3. The analytical data requires a mass accuracy of > 5 ppm. GRable assigns glycopeptide signals using

the accurate mass difference between glycopeptides with the same core peptide and estimates the glycan composition based on the mass difference between the core peptide and glycopeptide. Low mass accuracy increases the likelihood of incorrect matches.

4. This analysis should preferably be performed under the same LC conditions as those used to measure the glycopeptide sample. Presumed peptide sequences can be included in the list of highly purified proteins.
5. The GRable algorithm matches multiple core peptides to a single cluster. It then uses some surrounding information to select the most plausible match among them, for example, the sum of glycan points of cluster members, RT difference with core peptide, and signal intensity of the core. In addition, if cluster members are accompanied by MS2 spectra, the software uses this information.
6. We plan to develop an updated version that can handle data from manufacturers other than Thermo Scientific, allowing for individual user management.

### 2.3. GUI and functionality

Figure 2-2 shows the main window of GRable Version 1.0, and Table 2-1 lists the number of functions. Functions present but not described in this manual are under construction and thus not guaranteed.

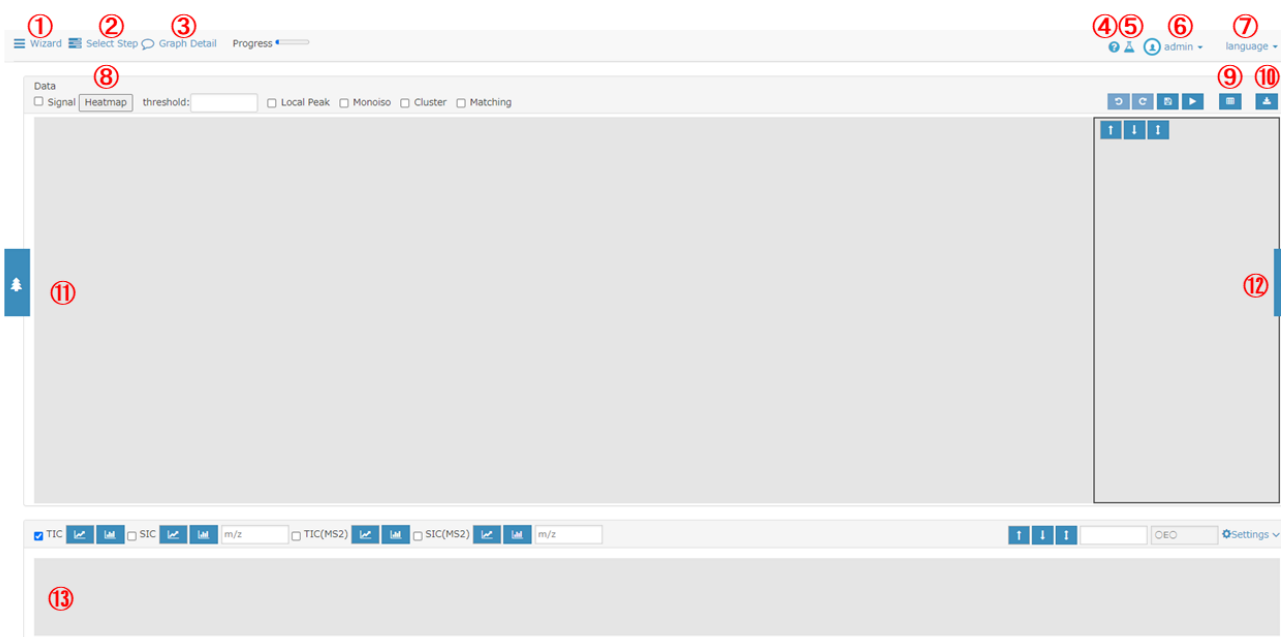


Figure 2-2. Main window.

Table 2-1. List of functions.

Function No. is similar to that in Figure 2-2.

No.	Name of function	Description
1	Wizard	Selects the step you want in the module.
2	Select step	Selects the step you want in the dropdown list.
3	Graph Detail	Displays setting parameters of the displayed graph.
4	System information	Displays system update information.
5	Monoisotopic Mass Table	Displays a Monoisotopic Mass Table.

No.	Name of function	Description
6	User/project management	Manages users and projects and logs out.
7	Language	Selects the display language (Japanese or English).
8	Heatmap	Displays signal intensity as a heatmap.
9	Option	Displays 3D display or monoisotopic peak list.
10	Download	Downloads graph image data, clustering analysis results, and matching results.
11	Tree	Displays data and analysis results in a tree format and displays, analysis, and reanalysis.
12	Settings	Displays the setting panel for heatmap operations (enlargement/reduction, etc.).
13	Chromatograms	Displays the Chromatograms graph.

### **3. Operation**

#### **3.1. Login**

To log into the system, one must enter a login ID and password into the login window ([Figure 3-1](#)).

GRable

ログインIDとパスワードを入力してください。

**Login ID**

**Password**

Login

**Figure 3-1. Login window.**

#### **3.2. Language selection**

The display can be switched between Japanese and English using the language-selection menu ([Figure 2-27](#)) in the upper right of the main window.

#### **3.3. Data registration (Step 1)**

##### **3.3.1. Functional overview**

GRable registers LC/MS data of glycopeptides in the mzML format, ensuring great versatility and ease of data processing. This software is designed to handle the charge-deconvoluted centroid spectra of each scan. This software is tested and optimized using mzML data prepared using Proteome Discoverer (version 2.4 or later; Thermo Fisher Scientific), in which deconvolution is performed with a workflow consisting of Xtract. To

analyze using the Glyco-RIDGE method, high accuracy of mass measurement is required. For example, the mass resolution of MS1 by the Orbitrap analyzer is 120,000 or 240,000 (at  $m/z$  200), and a lock mass at 445.12003 is necessary.

### 3.3.2. Operation procedure

To register the data for analysis, press the Wizard button (Figure 2-2①) on the main window to display the data registration module (Figure 3-2).

- ① Select the type of data to be registered. Select "LC/MS" for uploading mzML format files after deconvolution.
- ② Enter the analysis data set name.
- ③ Select data. After data uploading, the check is added.
- ④ Press the Register button.
- ⑤ The process is complete when "Registration completed" appears. Proceed to the next step.
- ⑥ If you want to cancel this step, press the 'Cancel' button.

Figure 3-2. Data registration module.

## 3.4. Range setting (Step 3)

### 3.4.1. Functional overview

This step (Figure 3-3) is designed to set the RT range, mass range, and minimum signal intensity (threshold) over which the analysis is performed. Analysis of data of long-gradient elution and broad mass ranges is time-consuming. The signals selected in this step (all signals) can be viewed as heat maps in the main viewer window. Because of the large data, the resulting peak list is exported to a CSV (comma-separated value) file, and the set parameters and results in the subsequent steps are exported to Excel (.xlsx) file.

### 3.4.2. Operation procedure

To set the range, press the Wizard button (Figure 2-2①) from the main module to display the range-setting

module (Figure 3-3).

- ① Enter an analysis name.
- ② Set parameters (Table 3-1). Note that "Load settings" is inapplicable in this version.
- ③ Select whether the setting is saved.
- ④ Click the "Run" button to start the analysis.
- ⑤ This process is complete when the range setting is complete. Please proceed to the next steps.
- ⑥ If you want to cancel this step, press the 'Cancel' button.

Figure 3-3. Range setting module.

Table 3-1. Details of range settings.

No.	Parameter	Description
1	rt range	Sets RT range (min) of data used for analysis
2	Intensity threshold	Sets signal intensity threshold of data used for analysis
3	Mass Range	Sets mass range (upper and lower limits) of data used for analysis

### 3.5. Monoisotopic peak picking (Step 4)

#### 3.5.1. Functional overview

This step (Figure 3-4) involves finding and grouping signals of the identical ion as an island based on three parameters: time (scan), mass (MH<sup>+</sup>), and intensity; it involves obtaining the monoisotopic mass of each signal island at the peak time using our unique algorithms. In the previous step, signals without isotopes are removed as noise so that all signals had at least one isotope signal. First, all the signals within the analysis range are surveyed to determine the local peak signal. If a peak signal is found, the same signals are repeatedly searched within the error tolerance (typically set at five ppm) from neighboring scans (toward both sides of the RT) and grouped. The same searches are performed for the other peak isotope signals to obtain a single signal island and set the minimum size of the island to three scans × four isotope signals as the default. Subsequently, three scan spectra centered on each island peak scan are accumulated, and the



resulting spectra are analyzed using a shape-analysis module. This module searches monoisotopic signals from the highest signal to the lowest signal. The monoisotopic signal threshold (lowest intensity) is calculated for the highest signal from the oligomer of carbamidomethylcysteine with the lowest number of carbons per 100 masses (=3.1, mean = 4.3, maximum = 6.1 (Phe)). [Figure 3-5](#) shows the default values in the settings module. If the relative intensity of a candidate monoisotopic signal is less than this value, the signal is not monoisotopic, and the search ends. After this search, a monoisotopic peak list of all the ions is created (exported as a table consisting of the peak RTs, monoisotopic masses, and peak intensities). The selected monoisotopic peak signal can be seen in the viewer overlaid on all the signals.

### 3.5.2. Operation procedure

To perform monoisotopic peak picking, press the Wizard button ([Figure 2-2①](#)) from the main window to display the monoisotopic peak-picking module ([Figure 3-4](#)). The recommended parameters are set as default values.

- ① Select the “Monoisotopic peak picking” tab.
- ② Enter an analysis name.
- ③ Set parameters ([Table 3-2](#)). Note that “Load settings” is inapplicable in this version.
- ④ Select whether the setting is saved.
- ⑤ Click the “Start” button to start the analysis. When “Analysis complete” is displayed, the process is complete.
- ⑥ If you want to cancel this step, press the ‘Cancel’ button.

**Figure 3-4. Monoisotopic peak picking module.**

**Table 3-2. Details of monoisotopic peak picking settings.**

No.	Parameter	Description
1	Max. tolerance	Sets the maximum mass tolerance among a series of scans for treating signals as ones derived from identical ions.

No.	Parameter	Description
2	Max no. data gap	Sets the number of allowable lack of scans for treating signals within a single ion island.
3	Min no. data point/signal	Sets the minimum number of scans of a single island.
4	Min time width	It is unnecessary to set this parameter because it is not utilized for data processing in this version.
5	Min no. isotope signals/ion	Sets the minimum number of isotopes.
6	Max. charge	This parameter should be set as 1 because only deconvoluted data is used for analysis.
7	Local peak search	If "yes" is selected, local peak detection is performed using the Maximum filter, and it is used as the starting point for the monoisotopic peak search. If "NO" is selected, all signals are used as the starting point for monoisotopic peak search in descending order of intensity. It is recommended to select "yes" because this process takes more time without the Maximum filter.
8	Minimum peak intensity	The minimum intensity of signal as a starting point for monoisotopic peak search.
9	Maximum filter window	Specifies the filter size (time width) of the Maximum filter for local peak search.
10	Monoisotopic signal search parameters	Define the parameters for determination of the monoisotopic peak position based on the relative intensities of isotopes in a dialog box (Figure 3-5).

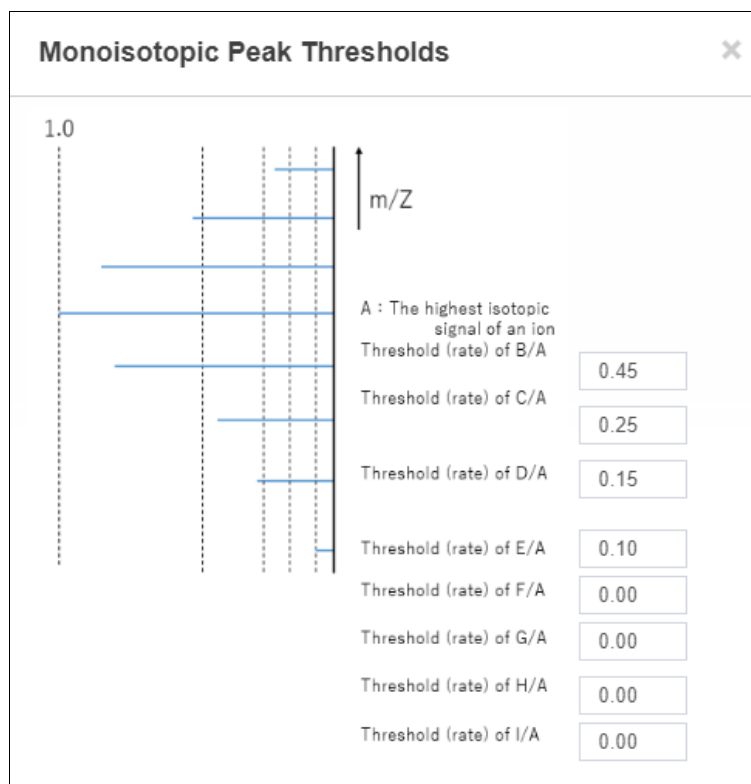


Figure 3-5. Setting window for monoisotopic signal search parameters.

### 3.6. Clustering (Step 5)

#### 3.6.1. Functional overview

This step (Figure 3-6) assigns a series of signals of the glycopeptide group as a cluster based on the elution behavior and mass difference between its members. By setting the maximum RT difference and maximum error of the mass difference for glycan units (such as Hex, HexNAc, and dHex), a pair of putative glycopeptide group signals is searched for all monoisotopic peaks. The pairs are combined to form a cluster if a single signal (node) has multiple relationships (edges). By repeating a similar search, a group with at least a user-defined number of members is considered a single glycopeptide cluster. By default, the minimum number of members in a cluster is set to 4.

#### 3.6.2. Operation procedure

Press the main window's Wizard button (Figure 2-2①) to display the clustering module (Figure 3-6).

- ① Select the "Clustering" tab.
- ② Enter the analysis name.
- ③ Set parameters (Table 3-3). Note that "Load settings" is inapplicable in this version.
- ④ Select whether the setting is saved.
- ⑤ Click the "Start" button to start the analysis. When "Analysis complete" is displayed, the process is complete.
- ⑥ If you want to cancel this step, press the 'Cancel' button.

The screenshot displays the Clustering module interface. At the top, a progress bar shows the current step is 'Clustering'. Below this, a blue bar prompts the user to 'Please select cluster analysis settings'. The settings are organized into several sections:

- Analysis name:** A text input field containing 'test'.
- Monoisotopic peak picking:** A text input field.
- Load Settings:** A 'select' button.
- Input cluster analysis name:** Radio buttons for 'name' (selected, with a red circle 2) and 'auto (use dateTime)'. A red circle 3 is also present near this section.
- Intensity threshold:** A text input field with '0'.
- Error Evaluation:** A dropdown menu set to 'mean'.
- Charge state:** A text input field with '~'.
- Peaks with the same mass are counted as one:** Radio buttons for 'yes' (selected, with a red circle 4), 'Da', 'ppm', and 'no'.
- Summary (within 100 characters):** A text area.
- Save Settings:** A checked 'Save' checkbox (with a red circle 4) and a 'Save' button.
- Step Progress:** A row of buttons for '1st step', '2nd step', '3rd step', '4th step', and '5th step'.
- Minimum number of signals / cluster:** A text input field with '4' and an 'apply this step' checkbox.
- Maximum mass error (ppm):** A text input field with '5'.
- Monosaccharide & RT range to be searched:** A table with columns for 'Monosaccharide', 'ΔM (z1+)', and 'Time range (min)'. The table lists:
 

Monosaccharide	ΔM (z1+)	Time range (min)
<input checked="" type="checkbox"/> Hex	162.052824	from ~ to
<input checked="" type="checkbox"/> HexNAc	203.079373	from ~ to
<input checked="" type="checkbox"/> dHex	146.057909	from ~ to
<input type="checkbox"/> NeuAc	291.095417	from ~ to
<input type="checkbox"/> NeuGc	307.090331	from ~ to
<input type="checkbox"/> Hex+HexNAc	365.132197	from ~ to
<input type="checkbox"/>		from ~ to
- Time range (min):** A section with 'from' and 'to' fields for each monosaccharide.
- Buttons:** 'To Merge Analysis', 'Start' (with a red circle 5), and 'Cancel' (with a red circle 6).

Figure 3-6. Clustering module.

Table 3-3. Details of clustering settings.

No.	Parameter	Description
1	Intensity threshold	Sets the minimum intensity threshold.
2	Error Evaluation	Sets the error evaluation method ("mean" or "sum SQ of SQ"). The default setting is "mean," which is more stringent. GRable calculates the difference between M1 and M2 (masses of two monoisotopic signals). To calculate the difference in ppm, ( $\Delta\Delta M$ obs-calc) between the observed mass difference ( $\Delta M$ obs= $M_2-M_1$ ) and the calculated mass value of any glycan unit (e.g., M(Hex), set by the user as shown below) is divided by either value of: - mean: $(M_1+M_2)/2$ - sum SQ of SQ: $\sqrt{(M_1)^2 + (M_2)^2}$ Suppose the difference (ppm) is smaller than the maximum mass tolerance (set by the user as shown below). In that case, the two monoisotopic signals are considered in the relationship with a difference in the glycan unit.
3	Charge state	This parameter should remain blank because only deconvoluted data are used for analysis.
4	Peaks with the same mass are counted as one.	Selects "yes" or "no". If "yes" is selected (by default), peaks with the same mass but divided into multiple peaks are considered single peaks (composition) when the number of members of a cluster is evaluated.
5	1st step (2nd step...)	Sets the parameters for each step of parallel clustering. By clicking, you can switch tabs and enter settings for each step.
6	Apply this step	Checks each step's checkbox to determine whether they are included in parallel clustering. Users can apply up to five steps in one analysis.
7	Minimum number of signals/cluster	Sets the minimum number of signals for one cluster.
8	Maximum mass error(ppm)	Sets the maximum mass tolerance.
9	Monosaccharide & RT range to be searched	Specifies types of glycan units (mono- and oligo-saccharides) along with their masses and RT ranges to be considered. Hex, HexNAc, dHex, NeuAc, NeuGc, Hex+HexNAc can be entered by default, and the number of free entry fields can be increased to 10.

### **3.7. Matching (Step 6)**

#### **3.7.1. Functional overview**

In this step ([Figure 3-7](#)), GRable searches for a combination of core peptide and glycan compositions matching the mass of the putative glycopeptide within the allowed mass error (user setting), according to the following equation:

$$\text{Observed } M(\text{glycopeptide}) = \text{calculated } M(\text{core peptide identified}) + M(\text{Hex}) \times i + M(\text{HexNAc}) \times j + M(\text{dHex}) \times k + M(\text{NeuAc}) \times l \quad (\text{where } M \text{ is a mass value and } i, j, k, \text{ and } l \text{ are integers})$$

A list of core peptide candidates is required to achieve this. The list can be prepared by PNGase-mediated

deglycosylation followed by LC/MS analysis of the same glycopeptide sample. The identification of deglycosylated peptides by LC/MS is one to two orders of magnitude more sensitive than glycopeptide identification, allowing core peptide listing using only 5–10 % of the sample used for glycopeptide analysis. As the selection step below describes, RT differences between glycopeptides and deglycopeptides are clues for selecting a correct match; therefore, we recommend that LC/MS data be obtained on the same day and in sequential analysis runs. In addition, a list of glycan points is required. Glycan compositions considered for matching are assigned as the number range of each glycan unit (user setting). As a default, the ranges of Hex, HexNAc, dHex, and NeuAc are set to 0-10, 1-10, 0-4, and 0-4, respectively. Then, using a set of three masses of glycopeptides, core peptides, and glycans, matched combinations are searched according to the aforementioned equation: For each glycopeptide, a point is assigned based on the matched glycan composition. The points are set in the glycan point list (provided by the user). Glycan points are classified into three types: 1 = compositions produced by common/familiar biological glycan processing, 0 = possible but not common, and -1 = unusual from the common biological pathway. Users can set this point allocation; for example, higher points are assigned to compositions observed in the glycome analysis, and all matched results can be obtained as an Excel file.

### 3.7.2. Operation procedure

Press the main window's Wizard button (Figure 2-2①) to display the matching module (Figure 3-7).

- ① Select the “Matching” tab.
- ② Enter the analysis name.
- ③ Set parameters (Table 3-4). Note that “Load settings” is inapplicable in this version.
- ④ Check whether the setting is saved and select accordingly.
- ⑤ Click the “Start” button to start the analysis. When “Analysis complete” is displayed, the process is complete.
- ⑥ If you want to cancel this step, press the ‘Cancel’ button.

The screenshot shows the 'Matching' module interface. At the top, a progress bar indicates the current step is 'Matching'. Below this, a blue bar prompts the user to 'Please select matching settings'. The main area contains several configuration options:

- Analysis:** A text input field containing 'test'.
- Monoisotopic peak picking:** A text input field.
- Clustering:** A text input field.
- Load Settings:** A dropdown menu set to 'select'.
- Set Analysis Name:** A radio button for 'name' (selected) and 'auto (use dateTime)'. A red circle ② is next to the 'name' radio button.
- Analysis of relation between clusters:** Radio buttons for 'yes' (selected) and 'no'.
- Select matching mode:** Radio buttons for 'core peptide list-based' (selected) and 'targeted protein'. Below it is a 'select xixx file...' field with a 'Browse' button.
- Select glycan point list(xixx):** A 'select xixx file...' field with a 'Browse' button.
- Maximum mass error:** A numeric input field set to '5', with units 'ppm' and 'Da'.
- Include uncompe negative point:** Radio buttons for 'yes' (selected) and 'no'.
- Input monosaccharide & composition range:** A table with columns for 'Mresidue', 'Min No', and 'Max No'.
 

	Mresidue	Min No	Max No
<input checked="" type="checkbox"/> Hex	162.052824	12	12
<input checked="" type="checkbox"/> HexNAc	203.079373	12	12
<input checked="" type="checkbox"/> dHex	146.057909	4	4
<input type="checkbox"/> NeuAc	291.095417		
<input type="checkbox"/> NeuGc	307.090331		
<input type="checkbox"/>			
<input type="checkbox"/>			
<input type="checkbox"/>			
- Summary:** A text area for a summary (within 100 characters).
- Save Settings:** A 'Save' button (with a red circle ④) and a 'Save' button.
- Bottom:** 'Start' (with a red circle ⑤) and 'Cancel' (with a red circle ⑥) buttons.

Figure 3-7. Matching module.

**Table 3-4. Details of matching settings.**

No.	Parameter	Description
1	Analysis of the relation between clusters	Selects whether inter-cluster analysis is executed. If “yes” is selected, enter the conditions by clicking on the button on the right side to display the setting dialog ( <a href="#">Figure 3-8</a> ). In the search difference setting, specify types of relations and mass tolerance (Da) to be considered. “Delta RT (min)” is an RT range for evaluating other cluster members. “Minimum rate of related members” is a threshold of the parentage of members detected in this search; when the members over the threshold are detected, reference and target clusters are treated as “related,” and the target cluster is indicated in an export file.
2	Select matching mode	Selects “core peptide list-based” mode and upload a core peptide list (in a fixed format). Note that “target protein” mode is not available in this version.
3	Select the glycan point list	Uploads a glycan point list.
4	Maximum mass error	Sets the maximum mass tolerance (ppm or Da).
5	Include uncomp negative point	Selects whether negative points are given for unusual glycan compositions defined in a glycan point list (in a fixed format).
6	Input monosaccharide & composition range	Specifies types of glycan units (mono- or oligo-saccharide) along with their masses and minimum/maximum numbers to be considered. Hex, HexNAc, dHex, NeuAc, and NeuGc can be entered by default, and there are also three free entry fields. Ensure that the glycan units are identical to the ones written in a glycan point list unless an alert appears.

**Search Difference** ✕

<input type="checkbox"/> H > NA	21.981943
<input type="checkbox"/> H > K	37.955882
<input checked="" type="checkbox"/> H > NH4	17.026549
<input checked="" type="checkbox"/> H*3 > Fe	52.911464
<input type="checkbox"/> Sulphation	79.956815
<input type="checkbox"/> Phosphorylation	79.966331
<input type="checkbox"/> deamidation	0.984016
<input type="checkbox"/> oxidation	15.994915
<input type="checkbox"/> ammonia-loss	-17.026549
<input type="checkbox"/> dehydration	-18.010565
<input type="checkbox"/> NeuAc	291.095417
<input type="checkbox"/> NeuGc	307.090331
<input type="checkbox"/> HexA	176.032088
<input type="checkbox"/> HexNH2	161.068808
<input type="checkbox"/> <input style="width: 50px;" type="text"/>	<input style="width: 50px;" type="text"/>
<input type="checkbox"/> <input style="width: 50px;" type="text"/>	<input style="width: 50px;" type="text"/>
<input type="checkbox"/> <input style="width: 50px;" type="text"/>	<input style="width: 50px;" type="text"/>
Mass tolerance	0.05 Da
Delta RT	-30 min ~ 30 min
Minimum rate of related members ≥	50 %

**Figure 3-8. Setting dialog of inter-cluster analysis.**

### **3.8. Selection (Step 7)**

#### **3.8.1. Functional overview**

During matching, multiple combinations are often suggested for each glycopeptide cluster. In this step ([Figure 3-9](#)), GRable collects the following information from the results and additional MS2 information to evaluate their reliability at the cluster and single glycopeptide levels to select the most plausible combination among the candidates.

#### Information

- 1) **Core peptides abundance:** The number of core peptides identified from a small aliquot of the glycopeptide sample using the IGOT-LC/MS/MS method greatly exceeds the number of clusters detected when the Glyco-RIDGE method is employed. This suggests that the detected glycopeptide clusters likely contain core peptides with high abundance or ionization efficiency among the peptides contained in the sample. Accordingly, if multiple core peptides are matched to a single glycopeptide group (cluster), the most abundant core peptide is the most plausible. Thus, the results of the most abundant core are displayed at the top of the list.
- 2) **Glycan abundance:** In preparing a core peptide list using IGOT-LC/MS/MS, the major glycan compositions (glycomes) of the sample can be determined by collecting the released glycans and analyzing them, for example, by MALDI-MS. As with core peptide abundance, the major glycan components will likely attach to major glycopeptides. Thus, GRable utilizes a glycan point list where glycan compositions detected in the glycome of the sample are assigned a point. In the matching module, the sum of the points within the cluster is calculated as the total score used to select a plausible match. In other words, for each cluster, a match with a higher score is considered better among the candidate matches. When an actual glycan composition list is unavailable, a glycan point list can be created by considering the *N*-glycan processing pathways, where common and unusual compositions have positive and negative points, respectively. Users can decide whether negative points should be considered in this selection module.
- 3) **RT difference between glycopeptides and corresponding core peptides:** As the RT of a core peptide is close to that of neutral glycopeptides and is often delayed, core peptides with an RT outside the setting are unlikely to be true. However, when sialic acid is added to a glycopeptide, the RT increases, in contrast to when a neutral glycan is added. Thus, if the matched glycans are sialylated, they should be evaluated using the RT difference.
- 4) **Mass accuracy:** In the clustering and matching steps, mass tolerance is generally set to 5 ppm, slightly more stringent than that used for database searches in proteomic analysis (7 ppm). The delta mass is generally set to 2 ppm in the selection step to guarantee highly reliable results. Therefore, MS1 should be acquired at a higher resolution, as described in [Section 2.2](#).
- 5) **Utilization with MS2 information:** Although this Glyco-RIDGE method is performed only with MS1 information in the clustering and matching steps to list glycopeptide candidates, MS2 information is also utilized for selecting the most plausible matching. In the selection step, we check whether the MS2 information is obtained for each cluster member. In this module, users can select whether the MS2 information is used for analysis. If it is acquired, the following information is retrieved and used for selection:

- i. Presence of diagnostic ions: The presence or absence of diagnostic ions derived from glycans (e.g., HexNAc(204) and Hex+HexNAc(366)) and their signal intensities are obtained automatically for all MS2 scans. This provides supportive information for each matching result, confirming that the ion is derived from a glycopeptide.
- ii. Presence of glycopeptide ions: GRable checks for the presence of peptides and peptide ions remaining in the innermost GlcNAc of N-glycan, called Y0 and Y1, respectively, and ions that frequently occur around them (e.g., Y1+Fuc and Y2). Suppose two of these ions are found, and the mass of the observed or calculated Y0 is identical to that of a matched peptide within a given mass tolerance, the result strongly endorses the matching result.
- iii. DB search results: GRable does not directly identify a glycopeptide based on MS2 spectrum analysis but collects MS2-based search results to evaluate matching results. In the selection result sheet, a peptide sequence corresponding to the Y0 mass in the core peptide list is provided, facilitating easy evaluation of the result.

### 3.8.2. Operation procedure

Press the Wizard button (Figure 2-2①) from the main window to display the selection module (Figure 3-9).

- ① Select the “Selection” tab.
- ② Enter an analysis name.
- ③ Set parameters (Table 3-5). Note that “input Analysis Setting” is inapplicable in this version.
- ④ Select whether the setting is saved.
- ⑤ Click the “Start” button to start the analysis. When “Analysis complete” is displayed, the process is complete.
- ⑥ If you want to cancel this step, press the ‘Cancel’ button.

Figure 3-9. Selection module.



**Table 3-5. Details of Selection setting.**

No.	Parameter	Description
1	Include unmatched cluster	If "yes" is selected, only the cluster information will be included in matching results for clusters that do not match.
2	select by total score	Selects whether a threshold of total score is used for selection. If "yes" is selected, matching results contain only matches whose total score is greater than or equal to the threshold value set by the user. For clusters with no matches above the prescribed score, only the cluster information will be included in the matching results.
3	sort cluster members by	Selects the sorting method for cluster members. - "intensity:" sort in descending order of intensity - "m/z:" sort in descending order of mass
4	allow unusual comp in rank	If "no" is selected, signals with a rank equal to or greater than the user's specified number and with unusual comp will not be present in the selection results.
5	mark match with strict delta mass	If "yes" is selected, matches that have a difference of masses (delta(ppm)) between observed and theoretical glycopeptides within the mass range (specified by user in ppm) will be considered "plausible" and marked with color (specified by user) in an export file. Note that this parameter is used only for marking to allow manual inspection. Thus, all the selected glycopeptide signals (even with out-of-range) are included in the selection results.
6	mark match with strict rt range	If "yes" is selected, matches that have a difference of RTs (delta(RT)) between a glycopeptide and core peptide within the RT range (specified by the user in min) will be considered "plausible" and marked with color (specified by user) in an export file. Note that this parameter is used only for marking to allow manual inspection. Thus, all the selected glycopeptide signals (including out-of-range) are included in the selection results.
7	show peaks having MS2 spectrum	To utilize MS2 information, select "yes" and specify the subsequent parameters. If "yes" is selected, glycopeptide signals with MS2 information will be marked with "1" in the "MS2?" column of a selection result sheet.
8	maximum mass error for MS2	Sets the maximum mass tolerance (ppm or Da) for searching corresponding MS2 spectra for each clustered glycopeptide. If the difference of masses (deltaMH+(Da)) between a clustered glycopeptide (m/z) and the calculated one (MH+calc) based on MS2 information (i.e., charge and precursor mass) is smaller than the threshold, the corresponding MS2 information will be indicated in an "MS2 info for cluster" sheet.
9	maximum time error for MS2	Sets the maximum RT tolerance (sec) for searching corresponding MS2 spectra for each clustered glycopeptide. Suppose the difference of RTs (delta rt) between a clustered glycopeptide (rt) and MS2 signal (rt(min)) is smaller than the

No.	Parameter	Description
		threshold. The corresponding MS2 information will be indicated in a “MS2 info for cluster” sheet.
10	Upload MS2(Mgf/MzML) File	Uploads MS2 data (in mgf or mzML format). When a mzML format file is used, utilize MS2 data before deconvolution.
11	Diagnosis Ions	Specifies glycan fragment ions used as diagnostic ions to confirm that the MS2 spectra are derived from a glycoprotein. The panel that appears with the “set” button allows setting the type of diagnostic ions with their mass (Da) ( <a href="#">Figure 3-10</a> ).
12	tolerance error for diag ion	Sets the mass tolerance (ppm or Da) between theoretical and observed fragment ions for evaluating the presence of diagnostic ions specified by the user as above.
13	tolerance error for Y0/Y1	Sets the mass tolerance (ppm or Da) between theoretical and observed fragment ions for evaluating the presence of Y0 and its related ions.

### Diagnosis Ion

	Diagnosis ion	mass[Da]	
<input type="checkbox"/>	HexNAc(126)	126.055504	Da
<input checked="" type="checkbox"/>	HexNAc(138)	138.055504	Da
<input checked="" type="checkbox"/>	HexNAc(144)	144.066069	Da
<input checked="" type="checkbox"/>	HexNAc(168)	168.066069	Da
<input checked="" type="checkbox"/>	HexNAc(186)	186.076634	Da
<input type="checkbox"/>	NeuAc-H2O(274)	274.092679	Da
<input type="checkbox"/>	NeuGc-H2O(290)	290.087594	Da
<input type="checkbox"/>	Hex(163)	163.060649	Da
<input checked="" type="checkbox"/>	HexNAc(204)	204.087198	Da
<input checked="" type="checkbox"/>	Hex(1)HexNAc(1)(36)	366.140022	Da
<input type="checkbox"/>	NeuAc(292)	292.103242	Da
<input type="checkbox"/>	NeuGc(308)	308.098156	Da
<input type="checkbox"/>	H1 HN1 F1	512.197931	Da
<input type="checkbox"/>	HN2 F1	553.22448	Da
<input type="checkbox"/>	H2 HN1	528.192846	Da
<input type="checkbox"/>	H1 HN2	569.219395	Da
<input type="checkbox"/>	H3 HN1	690.24567	Da
<input type="checkbox"/>	H4 HN1	852.298494	Da
<input type="checkbox"/>	H2 HN2	731.272219	Da
<input type="checkbox"/>	H3 HN2	893.325043	Da
<input type="checkbox"/>	H1 NeuAc1	454.156066	Da
<input type="checkbox"/>	H1 NeuGc1	470.15098	Da
<input type="checkbox"/>	H1 HN1 NeuAc1	657.235439	Da
<input type="checkbox"/>	H1 HN1 NeuGc1	673.230353	Da
<input checked="" type="checkbox"/>	H1 HN1 Fe1	419.051488	Da
<input type="checkbox"/>	<input style="width: 100%;" type="text"/>	<input style="width: 100%;" type="text"/>	Da

**Figure 3-10. Setting dialog of diagnosis ion.**

### 3.9. Data tree

The data tree displays the results of each step for each analysis project in a tree-view format. To display the tree, move the cursor to the tree button on the left of the main window (Figure 2-2①) to display the data tree (Figure 3-11). By right-clicking on the data of interest in the tree, a menu will appear for executing functions (Table 3-6).

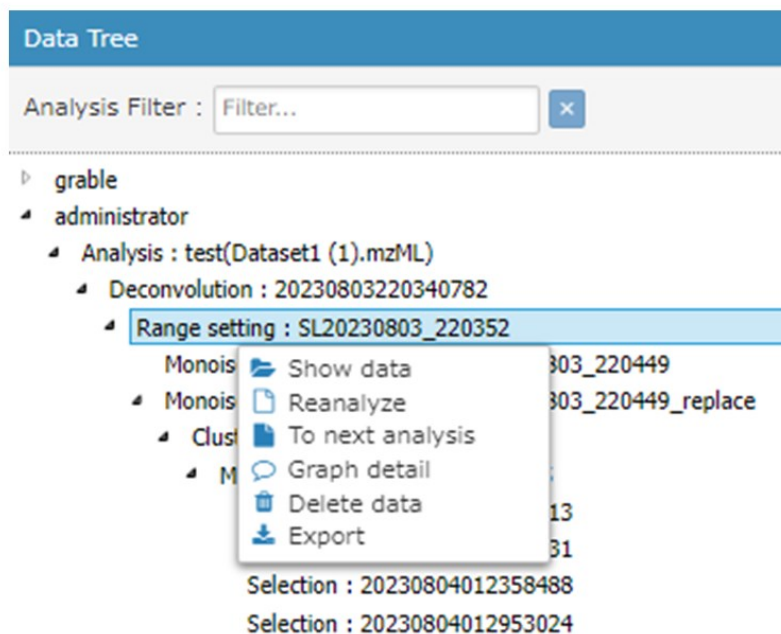


Figure 3-11. Representative image of the data tree.

Table 3-6. Details of data tree functions.

No.	Function	Description
1	Show data	Displays the selected result data on a viewer in the main window. <a href="#">Section 3.10</a> describes the details of the viewer. This operation is valid only for results of “range setting,” “monoisotopic peak picking,” “clustering,” and “matching” steps.”
2	Re-analyze	Re-analyzes the selected data with another different setting in a selected step. The parameters in the setting dialog are the same as the selected data.
3	To next analysis	Proceeds to the next step using the selected result data.
4	Graph detail	Displays the selected analysis settings in a separate dialog.
5	Delete data	Deletes selected data.
6	Export	Exports input data, analysis results, and setting conditions. To export them, check the items to be exported in the download dialog (Figure 3-12) and click on the “download” button. <a href="#">Section 5</a> describes the details of the exported files. The file size of the peak list is large, and thus, it is recommended to uncheck for the peak list unless necessary. The peak list is separately exported as a .csv file. The other selected items are exported in one Excel file.

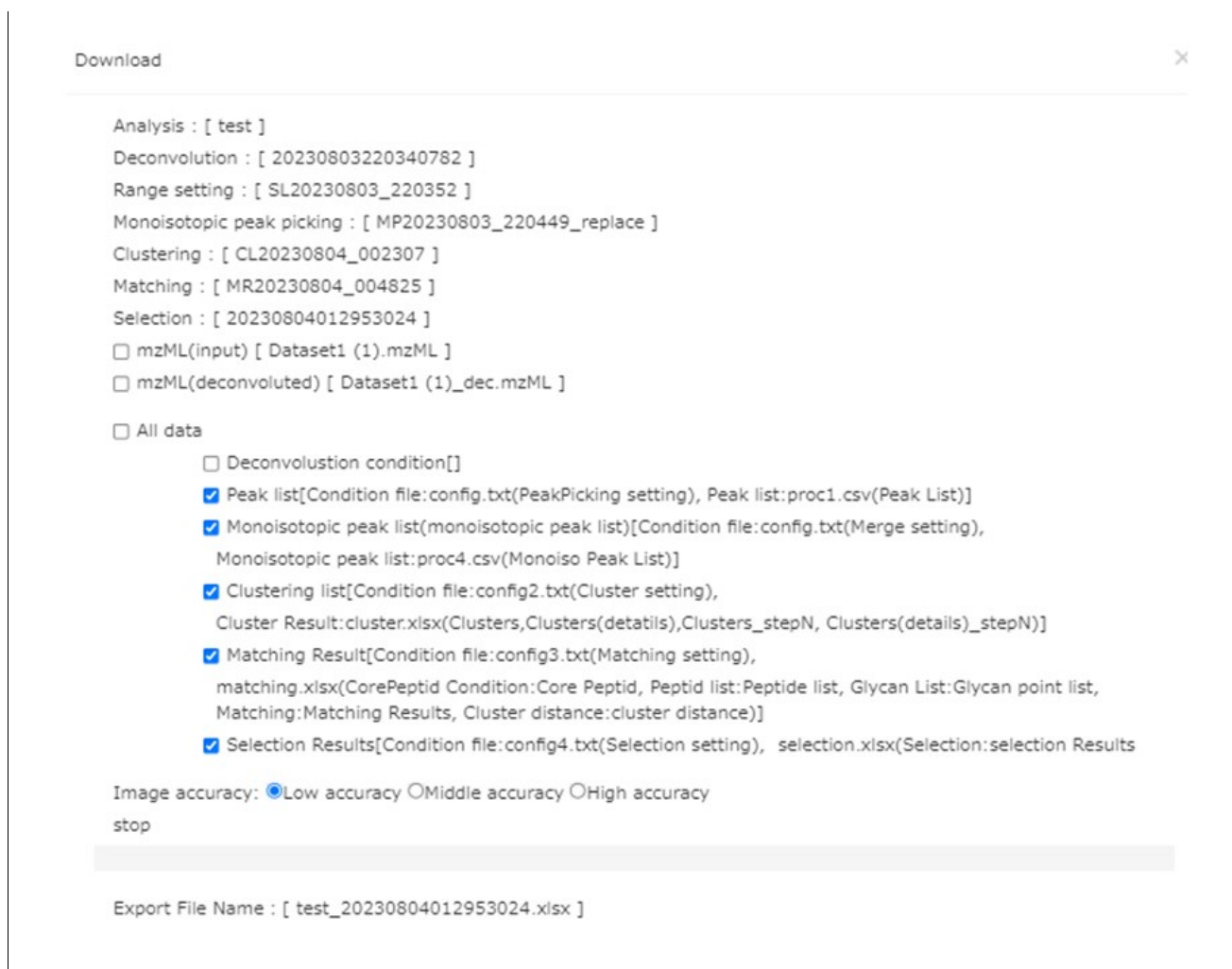


Figure 3-12. Download dialog.

### 3.10. Viewer

The viewer in the main window (Figure 3-13) can graphically visualize the analysis results. The data shown can be selected from a tree view, as documented above (Section 3.9), and its visualization can be optimized using functional buttons (Table 3-7).

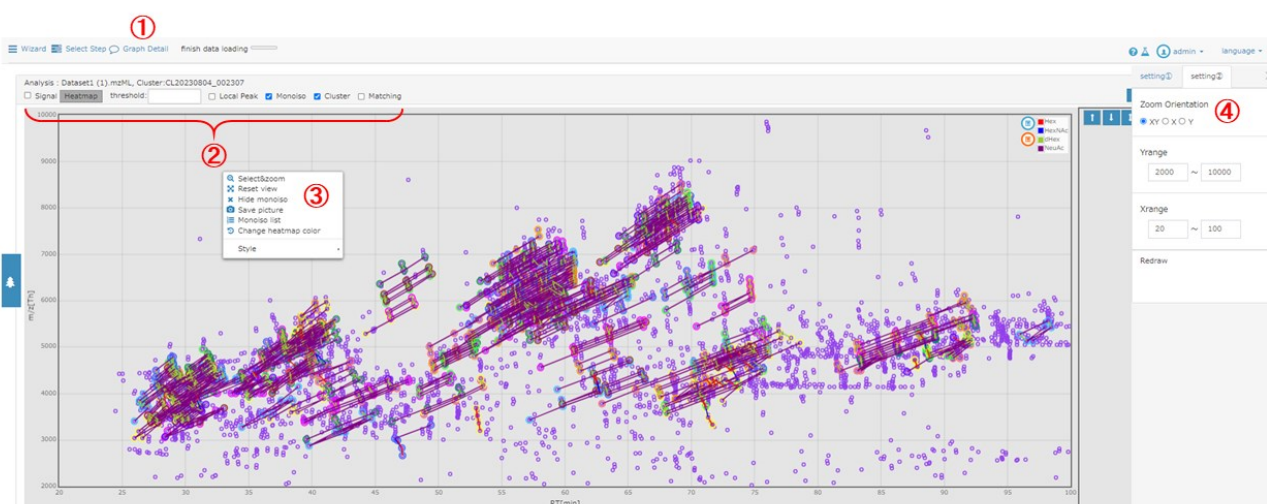


Figure 3-13. Viewer in the main window.

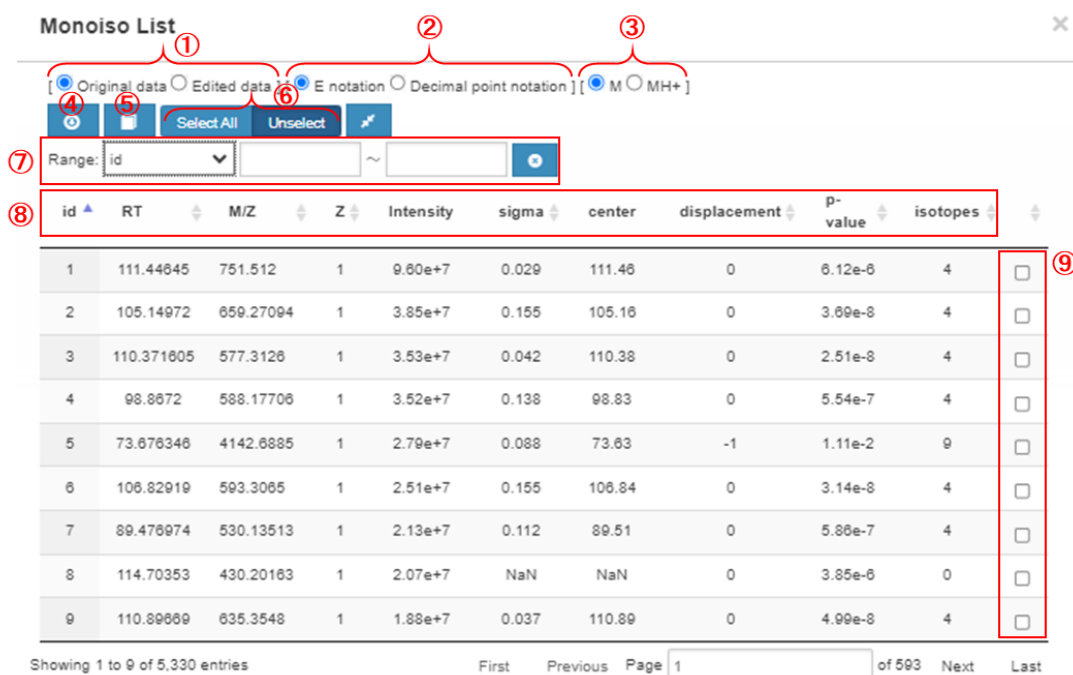
**Table 3-7. Details of viewer functions.**

No.	Function	Description
1	Graph Detail	Displays the selected analysis settings in a separate dialog.
2	Panels	<p>“Analysis” indicates the analysis name and selected result names. A “Heatmap” button and threshold entry form are valid only when the “Signal” is checked. Users can display the mode of data among “Local Peak,” “Monoiso,” “Cluster,” and “Matching.”</p> <ul style="list-style-type: none"> <li>- <b>Local Peak:</b> Displays all the detected local peaks.</li> <li>- <b>Monoiso:</b> Displays all the detected monoisotopic peaks in a black circle.</li> <li>- <b>Cluster:</b> Displays all the detected glycopeptide clusters, where line colors indicate the relationship of glycan units between two glycopeptide signals.</li> <li>- <b>Matching:</b> Displays only the matched glycopeptide clusters when the “Cluster” is unchecked.</li> </ul> <p>The display of “Signal” and “Local Peak” is slow; thus, it is recommended to uncheck these buttons.</p>
3	Select&zoom	Displays the enlarged graph within the range specified using a mouse.
3	Reset view	Returns to default settings.
3	Hide monoiso	Displays only monoisotopic peaks by hiding clusters.
3	Save picture	Saves the viewer's image as a PNG file.
3	Monoiso list	Displays a dialog of a monoisotopic peak list. <a href="#">Section 3.11</a> describes the details of the operation of this list.
3	Change heatmap color	This function is inapplicable in this version.
3	Style	The style of monoisotopic peaks can be changed using this function
4	Zoom Orientation	Specifies the orientation when the viewer is enlarged and reduced using a mouse wheel.
4	Y range / X range	Specifies the range of data (X: RT and Y: m/z) to be visualized. The setting values will be applied when the “Redraw” button is clicked.

### **3.11. Monoisotopic peak list**

#### **3.11.1. Functional overview**

This list ([Figure 3-14](#)) displays the monoisotopic peaks obtained by monoisotopic peak picking (Step 4) in Dialog format. To display this list, show the analysis results after Step 4 according to the instruction mentioned above ([Section 3.10](#)) and then press the “option” button in the main window ([Figure 2-2⑨](#)). This dialog is movable, expandable, and contractable. [Table 3-8](#) summarizes the list of the items.



**Figure 3-14. Monoisotopic peak list.**

**Table 3-8. Details of monoisotopic peak list items.**

No.	Function / Item	Overview
1	Data switching	This function switches between original data and edited data. In an initial state, original data are displayed.
2	Decimal display switching	The “intensity” display can be switched between E format and decimal format. An initial state is the display in the E format.
3	M / MH <sup>+</sup> switching	The “M/Z” display can be switched between M and MH <sup>+</sup> . An initial state is the display in the M format.
4	Data download	The monoisotopic peak list can be downloaded in .csv format.
5	Save edits	The modified list can be saved. This button is used for the data correction function described below.
6	Select All / Unselect	All signals in the list can be selected or unselected together. This button is used for the data correction function described below.
7	Filter	Signals in the list can be filtered for each item with ranges specified by the user. To use this function, select an item in the dropdown list and enter the range you want to display in the textbox. The filtering can be reset by pressing the “x” button. Only half-width numbers can be entered. This button is used for the data correction function described below.
8	Id	ID number of the isotope group
8	RT	RT of the isotope group
8	M/Z	m/z of the monoisotopic peak
8	Z	Charge state of the monoisotopic peak.
8	Intensity	Signal intensity of the monoisotopic peak
8	Sigma	Width of chromatographic distribution (average approximately 0.1)
8	Center	RT of the monoisotopic peak.

No.	Function / Item	Overview
8	Displacement	The degree of monoisotopic shift differs from a multinomial distribution fitting curve in the spectrum. The theoretical spectrum is created based on the abundance ratios of four isotopes: C, N, O, and S.
8	P-value	The <i>P</i> -value for the fitting of an observed spectrum to its theoretical spectrum.
8	Isotopes	The number of isotopes
9	Check box	Each ID can be selected/unselected individually by checking/unchecking this check box. This function is used for the data correction function described below.

### 3.11.2. Details of the correction function

The data correction function is used in the monoisotopic peak list ([Figure 3-15](#)) according to the following procedure:

- ① Select “replace” in the dropdown list.
- ② Enter the range of “displacement” values to be corrected. Once the range is entered, the list is updated.
- ③ Click the “Select All” to check all the selected IDs. Notably, this step is time-consuming.
- ④ When the update is complete, “SelectAll Complete” will appear, and all the checkboxes in the list will be checked.
- ⑤ Click the “Save edits” button, enter an analysis name, and then click the “Create” button to save the edited data as a new one. Notably, this step is time-consuming.
- ⑥ The updated data appear in the data tree of the analysis ([Section 3.9](#)).

**Monoiso List**

[  Original data  Edited data ] [  E notation  Decimal point notation ] [  M  MH+ ]

Range: replace ① -1 ②

id	RT	M/Z	Z	Intensity	sigma	center	displacement	p-value	isotopes	
5	73.676346	4142.6885	1	2.79e+7	0.088	73.63	-1	1.11e-2	9	<input type="checkbox"/> ④
29	76.149994	4798.9175	1	7.42e+6	0.138	76.09	-1	1.77e-2	9	<input type="checkbox"/>
52	31.205032	3637.3892	1	3.81e+6	0.096	31.20	-1	3.35e-2	8	<input type="checkbox"/>
83	44.22881	3455.423	1	2.25e+6	0.074	44.24	-1	2.32e-2	8	<input type="checkbox"/>
94	92.05852	5049.183	1	1.98e+6	0.260	92.19	-1	7.60e-3	8	<input type="checkbox"/>
103	88.81853	4758.088	1	1.84e+6	0.276	88.70	-1	5.07e-3	8	<input type="checkbox"/>
106	73.15075	4507.825	1	1.71e+6	0.124	73.16	-1	1.30e-2	9	<input type="checkbox"/>
109	39.87414	4508.809	1	1.67e+6	0.069	39.87	-1	2.30e-2	9	<input type="checkbox"/>
117	89.28525	5157.3364	1	1.58e+6	0.139	89.33	-1	3.53e-3	9	<input type="checkbox"/>

Showing 1 to 9 of 1,771 entries (filtered from 5,330 total entries)

First Previous Page 1 of 197 Next Last

**Figure 3-15. Procedure of correction function.**

## 4. Preparation details of import files

### 4.1. Core peptide list (.xlsx)

To prepare the core peptide list used for matching (Step 6), an aliquot of glycopeptides was treated with peptide-*N*-glycosidase F (PNGase F) in <sup>18</sup>O-labeled water to remove *N*-glycan and labeled deglycosylated Asn as <sup>18</sup>O-labeled Asp (isotope-coded glycosylation site-specific tagging; IGOT). The IGOT-treated deglycopeptides were analyzed by LC-MS/MS, and the MS2 data were used for a database search using Mascot. Using the Mascot Export file, a core peptide list was created in a fixed format (Figure 4-1), and some items were manually added to the Mascot export file, a few of which are mandatory for analysis (Table 4-1). To ensure correct performance, unknown parameters were as "0" (not to remain blank).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	CP No.	prot_acc	prot_desc	pep_start	pep_end	pep_seq	pep_var_mod_pos	Siteseqpos:Nigot		pep_calc	Mpep	prot_seq	intensity	RT
2	1	P19652	>sp P19652 A1AG2	19	38	QIPLCANLVPVPIATNATLDR	3.0000000000000000;33(NAT)/	1	2190.16	2187.172	MALSWVL	1.13E+08	89.98356	
3	2	P02763	>sp P02763 A1AG1	19	42	QIPLCANLVPVPIATNATLDQITGK	3.0000000000000000;33(NAT)/	1	2561.366	2558.378	MALSWVL	35100836	95.63262	
4	3	P19652	>sp P19652 A1AG2	19	38	QIPLCANLVPVPIATNATLDR	0.0000000000000000;33(NAT)/	1	2207.187	2204.199	MALSWVL	29672064	89.96731	
5	4	P19652	>sp P19652 A1AG2	19	42	QIPLCANLVPVPIATNATLDRTGK	3.0000000000000000;33(NAT)/	1	2589.409	2586.42	MALSWVL	7390537	90.28969	
6	5	P19652	>sp P19652 A1AG2	102	113	ENGTVSRVYEGGR	0.0200000000000000.0	103(NGT)/	1	1326.605	1323.617	MALSWVL	6000682	34.65462
7	6	P00738	>sp P00738 HPT_HI	236	249	VVLHPNYSQVDIGL	0.0000020000000000.241(NYS)/	1	1555.813	1552.825	MSALGAV	4919720	69.47055	
8	7	P02765	>sp P02765 FETUA	145	165	VCQDCPLLAPLNDTRVVAHAAK	0.0000000000002000.156(NDT)/	1	2379.193	2376.204	MKSLVLL	3312263	63.15908	
9	8	P19652	>sp P19652 A1AG2	87	101	QNQCIFYNSSYLVNQR	0.0000002000000000.93(NSS)/	1	1922.847	1919.858	MALSWVL	2250460	60.54092	
10	9	P19652	>sp P19652 A1AG2	19	42	QIPLCANLVPVPIATNATLDRTGK	0.0000000000000000;33(NAT)/	1	2606.435	2603.447	MALSWVL	1832450	90.02454	
11	10	P19652	>sp P19652 A1AG2	26	38	LVPVPIATNATLDR	0.00000002000000.0	33(NAT)/	1	1410.797	1407.809	MALSWVL	1713041	64.11006

Figure 4-1. Representative image of core peptide list.

Table 4-1. Details of core peptide list.

N o.	Manually addition	Mandatory	Item	Description
1	Required		CP No.	Core peptide number manually numbered in descending order of signal intensity
2			prot_acc	Protein accession No.
3			prot_desc	Protein description
4			pep_start	Start position of the peptide within a protein.
5			pep_end	The end position of the peptide within a protein
6		yes	pep_seq	Peptide sequence
7			pep_var_mod_pos	Position of the following variable modifications: 1: ammonia-loss (peptide N-term, carbamidomethyl C) 2: Delta:H(-1)N(-1)18O(1)(N) 3: Gln->pyro-Glu (peptide N-term, Q) 4: oxidation (M)
8	Required		Siteseqpos	Position of IGOT-labeled Asn residue (+2.98822096000004) within a protein along with its consensus sequence
9	Required	yes	Nigot	Number of IGOT-labeled Asn residues
10			pep_calc_mr	Calculated mass of peptide
11		yes	Mpep	Calculated mass of peptide without IGOT labeling. This information is the most important to obtain results.
12			prot_seq	Protein sequence



N o.	Manually addition	Mandatory	Item	Description
13			intensity	Signal intensity of MS2 fragment ion
14	Required	yes	RT	RT (min) of MS2 fragment ion. Note that RT (sec) in mascot export files should be converted into RT (min)

#### 4.2. Glycan point list (.xlsx)

The glycan point list used for matching (Step 6) can be prepared based on the glycans observed in the glycome analysis of the same glycoprotein sample or the biosynthetic pathway of glycans. In the glycan point list ([Figure 4-2](#)), glycan compositions (highlighted in pink) are listed along with a given point in a “point” row. Unusual glycan compositions can also be specified by listing along with a negative point in an “unusual” row. These unusual glycan compositions are used for analysis only when the “uncomp negative point” parameter is “yes” in the matching setting ([Table 3-4](#)). Note that the names of the glycan units (e.g., Hex, HexNAc, dHex, and NeuAc) should be the same as those used in the matching setting ([Figure 3-7](#)).

	A	B	C	D	E	F	G	H
1		point	Hex	HexNAc	dHex	NeuAc		
2	point	1	0	1	0	0		
3	point	1	0	1	1	0		
4	point	1	0	2	0	0		
5	point	1	0	2	1	0		
6	point	1	1	2	0	0		
7	point	1	1	2	1	0		
8	point	1	2	2	0	0		
9	point	1	2	2	1	0		
10	point	1	3	2	0	0		
11	point	1	3	2	1	0		
12	point	1	4	2	0	0		
13	point	1	4	2	1	0		
14	point	1	5	2	0	0		
15	point	1	5	2	1	0		
16	point	1	6	2	0	0		
17	point	1	6	2	1	0		
18								
19	unusual	-1	9	2	1			
20	unusual	-1	9	2	2			
21	unusual	-1	9	2	3			
22	unusual	-1	9	2	4			
23	unusual	-1	8	2	1			
24	unusual	-1	8	2	2			
25	unusual	-1	8	2	3			
26	unusual	-1	8	2	4			
27	unusual	-1	7	2	1			
28	unusual	-1	7	2	2			
29	unusual	-1	7	2	3			

**Figure 4-2. Representative image of glycan point list.**

#### 5. Details of export files

The analysis results are exported using the “Export” function in the data tree ([Section 3.9](#)), the peak list ([Table 5-1](#)) separately as a .csv file, and the selected items ([Table 5-2](#)) to Excel.

**Table 5-1. Details of peak list.**

Item	Description
SCAN	Scan number of MS1 spectra
RT	RT (min) of a local peak
MASS	Observed mass (m/z) of a local peak
INTENSITY	Signal intensity of a local peak

Item	Description
GID	Glycopeptide ID that is the same as the “monoiso no” in the “Monoiso Peak List” sheet
FLAG	This parameter is not used.

**Table 5-2. List of data exported in an Excel file.**

Sheet name	Related step	Description	Details of items
Range setting	Range setting	Parameters specified for this step	
Monoiso Peak List	Monoisotopic peak picking	List of monoisotopic peaks detected in this step	<a href="#">Table 5-3</a>
Monoiso Peak Picking log	Monoisotopic peak picking	Log information for this step	
Monoiso Peak Picking setting	Monoisotopic peak picking	Parameters specified for this step	
Clustering setting	Clustering	Parameters specified for this step	
Clusters	Clustering	List of clusters detected in this step	<a href="#">Table 5-4</a>
Clusters(details)	Clustering	Detailed information on clusters detected in this step	<a href="#">Table 5-5</a>
Clusters_stepX	Clustering	List of clusters detected only in the applied condition (step X)	<a href="#">Table 5-4</a>
Clusters(details)_step X	Clustering	Detailed information of clusters detected only in the applied condition (step X)	<a href="#">Table 5-5</a>
Matching setting	Matching	Parameters specified for this step	
Glycan point list	Matching	Glycan point list identical to an input file	See <a href="#">Section 4.2</a>
Peptide list	Matching	Core peptide list identical to an input file	See <a href="#">Section 4.1</a>
Matching Results	Matching	List of all matches of glycopeptide cluster, core peptide, and glycan compositions	<a href="#">Table 5-6</a>
relation between clusters	Matching	Inter-cluster analysis results (*Included only when inter-cluster analysis is applied)	<a href="#">Table 5-7</a>
Selection setting	Selection	Parameters specified for this step	
Selection Results	Selection	List of matches selected based on the specified parameters	<a href="#">Table 5-6</a>
MS2 info for clusters	Selection	MS2 information for each selected cluster	<a href="#">Table 5-8</a>
All MS2 info	Selection	MS2 information for all scans in input MS2 data	<a href="#">Table 5-8</a>
logging	Selection	Log information for this step	
peptide chart	Selection	2D-map (RT – Mass of predicted Y0 ion) for core peptides observed in the “All MS2 info” sheet	<a href="#">Table 5-8</a>

**Table 5-3. Details of monoisotopic peak list.**

Item	Description
Monoiso no	Monoisotopic peak No. numbered in a descending order of peak intensity
Monoiso retention time	RT (min) of monoisotopic peak
Monoiso mass	Mass (M) of monoisotopic peak
Peak intensity	Signal intensity of a local peak
Monoiso rtid	This parameter is not used.
Monoiso intensity	Signal intensity of a monoisotopic peak
Charge	Charge state
Center of Distribution	RT of the center of chromatographic distribution
Sigma of distribution	Width of chromatographic distribution (average approximately 0.1)
Valid groups	Number of isotopes
Multinomial p-value	The P-value for the fitting of an observed spectrum to its theoretical spectrum.
Composition	This parameter is a part of the algorithm for fitting an observed spectrum to its theoretical spectrum.
Suggest monoiso displacement	The degree of monoisotopic shift is expressed as a difference from a multinomial distribution fitting curve in the spectrum.
Suggested monoiso mass	Mass of a monoisotopic peak selected after check using the correction function.
P-value suggested monoiso	The P-value for a suggested monoisotopic peak
Suggested monoiso signal found	This parameter is not used. (Always "0")
Original Mz	Mass of a monoisotopic peak selected before the correction function
Update flag	If there is an update using the correction function, "1" is indicated.

**Table 5-4. Details of Clustering result sheets.**

Item	Description
Cluster_no	Cluster No. is in the descending order of the maximum intensity within the cluster.
Peak_no	Monoisotopic peak No. the same as "monoiso no" in the monoisotopic peak list.
Charge	Charge state of the monoisotopic peak
m/z	m/z of the monoisotopic peak (*When "Peaks with the same mass are counted as one" is "yes" in the setting, the masses having the same mass within the cluster are highlighted in yellow.)
RT	RT (min) of the monoisotopic peak
Intensity	Signal intensity of the monoisotopic peak
No_member	No. of members in the cluster

Item	Description
	(*When “Peaks with the same mass are counted as one” is “yes” in the setting, the masses having the same mass within the cluster are counted as one.)
Step	Step No. where the monoisotopic peak is found

**Table 5-5. Details of Clustering result (details) sheet.**

Item*	Description
cluster_no	Cluster No.
charge	Charge state of the monoisotopic peak
peak_no	Monoisotopic peak No.
rt	RT (min)
scan_no	Scan No.
m/z	Mass (MH <sup>+</sup> )
mass	Mass (M)
intensity	Signal intensity
target_peak_no	Monoisotopic peak No.
rt	RT (min)
scan_no	Scan No.
m/z	Mass (MH <sup>+</sup> )
mass	Mass (M)
intensity	Signal intensity
residue	A glycan unit equivalent to the difference between reference and target peaks
diff_mass	Difference in observed masses between reference and target peaks
diff_theor	Theoretical mass difference between reference and target peaks, corresponding to the theoretical mass of the glycan unit
Delta	Delta value (Da) between “diff_mass” and “diff_theor.”
diff(ppm)	Delta value (ppm) converted from “delta” by the method selected for “Error Evaluation” of the setting.
diff_rt	Difference in RTs between reference and target peaks
step	Step No. that found the monoisotopic peak

\*Red: Information on reference peaks. Blue: information on target peaks examined for relationships with reference peaks.

**Table 5-6. Details of matching and selection result sheets.**

Item *1	Description
cluster_no	Cluster No.
member	No. of the members within the cluster
peak_no	Monoisotopic peak no.

Item *1	Description
charge	Charge state
scan_no	Scan No. of the monoisotopic peak
m/z	Mass (MH <sup>+</sup> )
rt	RT (min)
intensity	Signal intensity
M(gpep, obs)	Observed mass (M)
step	Step No. that found the monoisotopic peak
CP No.	Core peptide No.
prot_acc	Protein accession No.
prot_desc	Protein description
pep_start	Start position of the peptide within a protein.
pep_end	End position of the peptide within a protein
pep_seq	Peptide sequence
pep_var_mod_pos	Position of the following variable modifications: 1: ammonia-loss (peptide N-term, carbamidomethyl C) 2: Delta:H(-1)N(-1)18O(1)(N) 3: Gln->pyro-Glu (peptide N-term, Q) 4: oxidation (M)
Siteseqpos	Position of IGOT-labeled Asn residue (+2.98822096000004) within a protein along with its consensus sequence
Nigot	Number of IGOT-labeled Asn residues
pep_calc_mr	Calculated mass of peptide
Mpep	Calculated mass of peptide without IGOT labeling
prot_seq	Protein sequence
intensity	Signal intensity of MS2 fragment ion
RT	RT (min) of MS2 fragment ion. Note that RT (sec) in mascot export files should be converted into RT (min)
rank	Ion score rank in the mascot search results
Hex	No. of Hex residues in the matched glycan composition
HexNAc	No. of HexNAc residues in the matched glycan composition
dHex	No. of dHex residues in the matched glycan composition
NeuAc	No. of NeuAc residues in the matched glycan composition
Hex[-core]	No. of Hex residues after subtracting by the trimannosyl core of N-glycan (i.e., 3) in the matched glycan composition
HexNAc[-core]	No. of HexNAc residues after subtracting by the trimannosyl core of N-glycan (i.e., 2) in the matched glycan composition
dHex	No. of dHex residues in the matched glycan composition
NeuAc	No. of NeuAc residues in the matched glycan composition
unusual comp	If the glycan composition is defined as "unusual" in the glycan point list, "1"

Item <sup>*1</sup>	Description
	will be indicated.
point	A point assigned for the glycan composition
factor	A coefficient value for weighting a glycan composition of interest. The factor is “1” unless other values are specified in the glycan point list. This function is not guaranteed in this version.
score	A score for the glycan composition calculated by multiplying “point” and “factor.”
total score	The sum of scores for all the members of the cluster
mass	Calculated mass (M) of theoretical glycopeptide in a combination of the core peptide and glycan composition
delta(Da)	Delta value (Da) between observed and calculated masses for the glycopeptide
delta(ppm)	Delta value (ppm) converted from delta(Da)
delta(RT)	Delta value (min) between RTs of the glycopeptide and core peptide
MS2? <sup>*2</sup>	If the MS1 spectra have any MS2 information, “1” will be indicated with a link to the corresponding data in the “MS2 info for clusters” sheet.
predict peptide <sup>*2</sup>	This is identical to the “peptide[seq]” information in the “MS2 info for clusters” sheet.
Y0 (z=1) <sup>*2</sup>	Y0 mass (MH <sup>+</sup> ; z=1) calculated using the mass of the core peptide
Y0 (z=2) <sup>*2</sup>	Y0 mass (MH <sup>+</sup> ; z=2) calculated using the mass of the core peptide
Y0 (z=3) <sup>*2</sup>	Y0 mass (MH <sup>+</sup> ; z=3) calculated using the mass of the core peptide
Y1 (z=1) <sup>*2</sup>	Y1 mass (MH <sup>+</sup> ; z=1) calculated using the mass of the core peptide
Y1 (z=2) <sup>*2</sup>	Y1 mass (MH <sup>+</sup> ; z=2) calculated using the mass of the core peptide
Y1 (z=3) <sup>*2</sup>	Y1 mass (MH <sup>+</sup> ; z=3) calculated using the mass of the core peptide

<sup>\*1</sup> **Red**: glycopeptide clusters. These items are identical to those in the “Cluster(details)” sheet ([Table 5-5](#)).  
**Blue**: Core peptides and glycan composition. These items are identical to those in the “Peptide list” sheet ([Table 4-1](#)).

<sup>\*2</sup> These items will only be indicated when the “show peaks having MS2 spectrum” is “yes” in the selection setting ([Table 3-5](#)).

**Table 5-7. Details of inter-cluster analysis result sheet.**

Item	Description
cluster_no <sup>*</sup>	Cluster No.
#member <sup>*</sup>	No. of members in the cluster
group_no <sup>*</sup>	Group No. for annotating each glycopeptide (C, cluster; G, group) (*The peaks with the same mass are counted as one.)
peak_no <sup>*</sup>	Monoisotopic peak No.
charge <sup>*</sup>	Charge state
m/z <sup>*</sup>	m/z of the monoisotopic peak
rt <sup>*</sup>	RT (min) of the monoisotopic peak

Item	Description
intensity*	Signal intensity of the monoisotopic peak
#members	Non-redundant No. of the combination of reference and target peaks with a relationship specified by the user. When the ratio of members is over the threshold specified in the setting ( <a href="#">Figure 3-8</a> ), these peaks are considered “related.”
Delta m/z	Difference in masses (m/z) between reference and target peaks
Relation	The relationship between reference and target peaks is identified by comparing the “Delta m/z” with the value entered in the setting.
Direction	Direction of relationship indicating which peak is a naked or adduct
Delta Delta m/z	Delta value (Da) between the “Delta m/z” and the value entered in the setting. When this value is lower than the threshold specified in the setting, these peaks are considered “related.”
Delta rt	Delta value (min) of RTs between reference and target peaks. When this value is within the range specified in the setting, these peaks are considered “related.”

\* Columns A-H indicate items for the reference cluster, whereas columns I-P indicate target clusters to examine whether they have a relationship specified by the user.

**Table 5-8. Details of MS2 information sheets.**

Item *1	Description
cluster_no	Cluster No.
peak_no	Monoisotopic peak No.
charge	Charge state
m/z	Observed mass (MH <sup>+</sup> ) of the monoisotopic peak
rt	RT (min) of the monoisotopic peak
intensity	Signal intensity of the monoisotopic peak
no_member	Ascending numbering for monoisotopic peaks in the cluster
precursors	Ascending numbering for precursor ions in one monoisotopic peak
MH+calc	Calculated mass (MH <sup>+</sup> ) of the glycopeptide based on the mass of precursor ion and its charge state.
deltaMH+(Da)	Delta value (Da) between observed and calculated masses (MH <sup>+</sup> ) of the monoisotopic peak
delta rt	Delta value (min) between RTs of a monoisotopic peak (MS1) and its corresponding MS2 spectra
scan	Scan No. of MS2 spectra annotated in the uploaded mgf file. The link is inactive in this version.
rt(min)	RT (min) of MS2 spectra
charge	Charge state of the precursor ion
precursor mass	Mass (MH <sup>+</sup> ) of the precursor ion in the indicated charge state
Intensity	Signal intensity of the precursor ion

Item <sup>*1</sup>	Description
z[Y0/Y1]	Charge state of Y0 and Y1 ions
matches[Y0/Y1]	No. of matches for glycopeptide-derived ions
Y0 mass predict	Predicted mass (M) of Y0 ion
peptide[seq]	CP No. and sequence of the core peptide that has the mass (M) corresponding to the “Y0 mass predict”. (*Present only if a corresponding core peptide is in the list.)
peptide[mass]	Observed mass (M) of the core peptide
peptide-Y0 mass	Delta value (Da) between the “peptide[mass]” and “Y0 mass predict”
Y0 mass obs <sup>*2</sup>	Observed mass of the Y0 ion
Y0 int <sup>*2</sup>	Signal intensity of the Y0 ion
Y1-Y0 dif <sup>*2</sup>	Delta value (Da) between masses of Y1 and Y0 ions
isotope count	Ascending numbering of isotopes
HexNAc(138) obs <sup>*3</sup>	Observed mass of a diagnostic ion (specified by user)
HexNAc(138) dif <sup>*3</sup>	Delta value (Da) between observed and calculated masses of a diagnostic ion (specified by user). When the delta value is lower than the “tolerance error for diag ion” value entered in the setting (Table 3-5), the observed ion will be considered a diagnostic ion.
HexNAc(138) int <sup>*3</sup>	Signal intensity of a diagnostic ion (specified by user)
used in cluster <sup>*4</sup>	Cluster No. that includes a monoisotopic peak corresponding to the MS2 scan.
precursor intensity <sup>*4</sup>	Signal intensity of the precursor ion

<sup>\*1</sup> **Red**: information on assigned clusters shown in the “Selection Results” sheet.

<sup>\*2</sup> Similar items are also indicated for other glycopeptide-related ions (Y0x, Y1, Y1F, and Y2).

<sup>\*3</sup> Similar items are also indicated for other diagnostic ions specified by the user.

<sup>\*4</sup> Indicated only in the “MS2 info for clusters” sheet.