
**HOWTO, example workflow and data files.**

**(Version 5.9.2018)**

# Introduction:

SugarQb (www.imba.oeaw.ac.at/sugarqb) is a freely available collection of computational tools for the automated identification of intact glycopeptides from high-resolution HCD MS/MS data-sets in the Proteome Discoverer environment. SugarQb has now been migrated to the latest and free version of Proteome Discoverer 2.1

For further information on the algorithm, please refer to the corresponding publication Stadlmann. J., Taubenschmid J. Mechtler K., Penninger JM., et al. Comparative gycoproteomics of stem cells identifies new players in ricin toxicity, Nature (2017).

This document is intended to provide you with a quick guide on who to download, install and test SugarQb, analyzing example data of tryptic glycopeptides derived from human plasma. All relevant .dll files, additional parameter files and a Glycan mass data-base are available at:

www.imba.oeaw.ac.at/SugarQb.

**Contact: SugarQb@imp.oeaw.ac.at**

## Download and Installation:

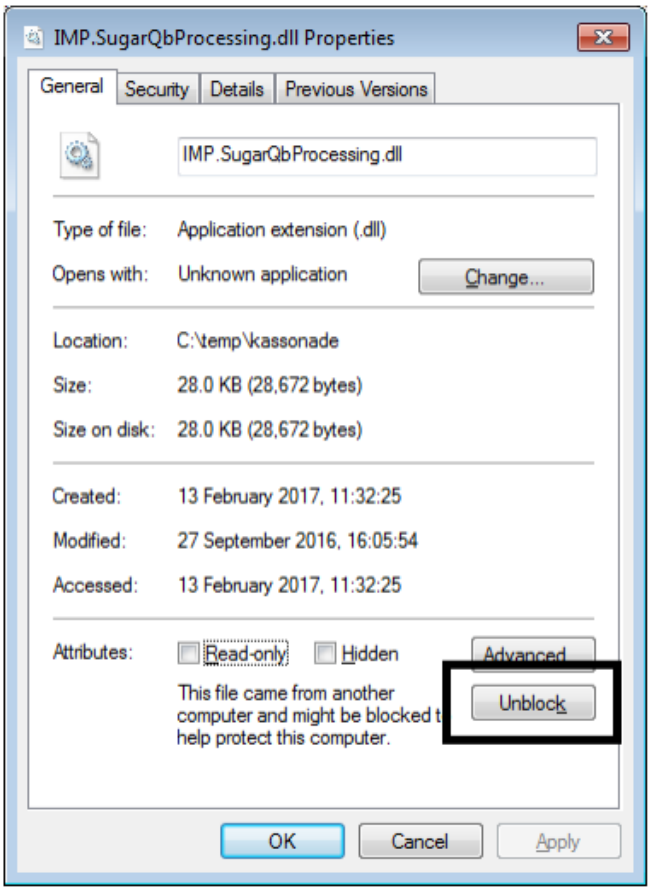
- Download SugarQb for Thermo Scientific Proteome Discoverer 2.1 using the following URL: www.imba.oeaw.ac.at/SugarQb

- Save all your files and shutdown Thermo Scientific Proteome Discoverer

- Navigate to the folder where you have installed Thermo Scientific Proteome Discoverer (Tip: You can easily find out the path by right-clicking the Thermo Scientific Proteome Discoverer desktop icon and open the Properties window. The older path is written in the field Target.)

- Copy the .dll files into the Thermo Scientific Proteome Discoverer folder.

- Unblock the .dll files if required by right-clicking each .dll file, opening its properties window and clicking the Unblock button if available.




- Restart the Thermo Scientific Proteome Discoverer, navigate to the licencing page and click on Scan for missing features. Subsequently, restart the program once more.

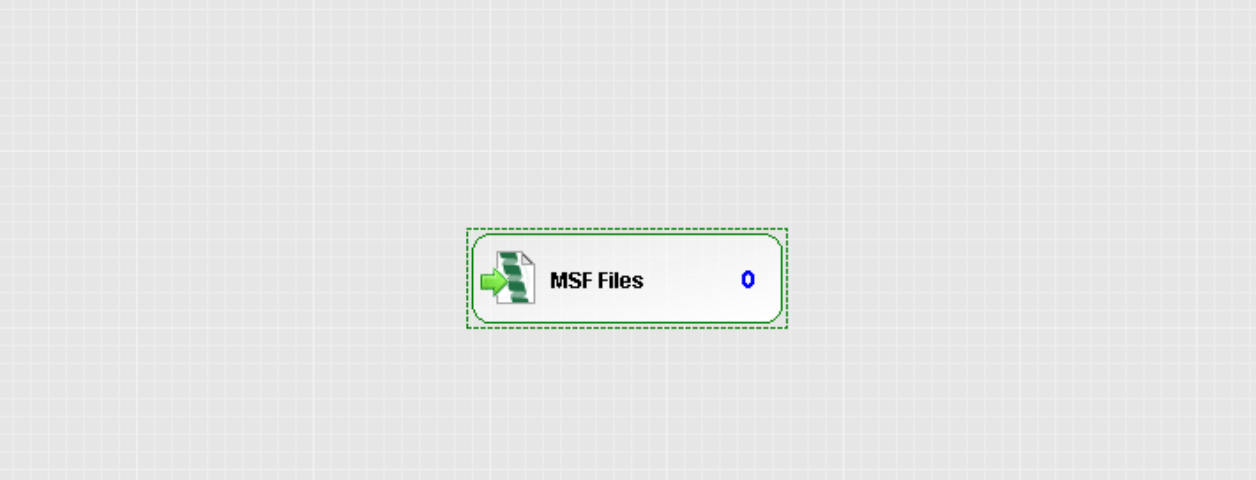
## Example Workflow:

In Proteome Discoverer there are two different type of Workflows the Consensus Workflow and the Processing Workflow.

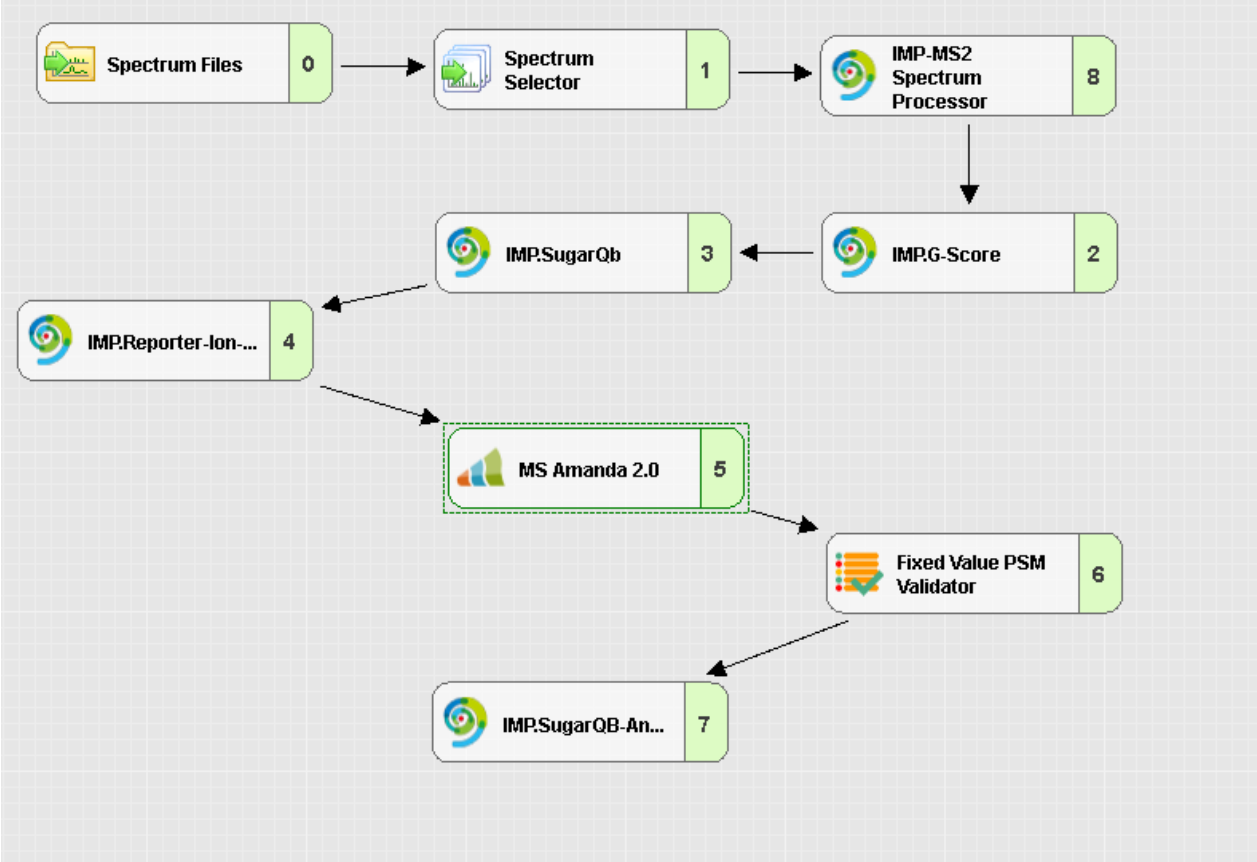
- Download the test data file “LUMOS_SugarQb_Test_humanPlasma_HCDonly.raw” from: www.imba.oeaw.ac.at/SugarQb . This data has been generated by analyzing IP-HILIC-enriched, tryptic glycopeptides derived from a chemically de-sialylated human plasma, using HCD on an OrbiTrap Fusion LUMOS instrument.

- After Installation of the SugarQb Nodes, in Thermo Scientific Proteome Discoverer 2.1., create the following Workflow. Parameter settings of the respective Nodes are detailed below.

## Consensus Workflow:



## Processing Workflow:



## Recommended Settings & Parameters:

### Spectrum Selector:

N:B.: The default settings of the Spectrum Selector Node were modified, to also allow “higher” mass precursor ions(i.e. up to 10.000 Da) to be analyzed.

Parameters

Show Advanced Parameters

| | |
|---|---|
| **1. General Settings** | |
| Precursor Selection | Use MS1 Precursor |
| **2. Spectrum Properties Filter** | |
| Lower RT Limit | 0 |
| Upper RT Limit | 0 |
| First Scan | 0 |
| Last Scan | 0 |
| Ignore Specified Scans | |
| Lowest Charge State | 0 |
| Highest Charge State | 0 |
| Min. Precursor Mass | 350 Da |
| Max. Precursor Mass | 10000 Da |
| Total Intensity Threshold | 0 |
| Minimum Peak Count | 1 |
| **3. Scan Event Filters** | |
| Mass Analyzer | (Not specified) |
| MS Order | Is MS2 |
| Activation Type | (Not specified) |
| Min. Collision Energy | 0 |
| Max. Collision Energy | 1000 |
| Scan Type | Is Full |
| Polarity Mode | Any |
| **4. Peak Filters** | |
| S/N Threshold (FT-only) | 1.5 |
| **5. Replacements for Unrecognized Properties** | |
| Unrecognized Charge Replacer | Automatic |
| Unrecognized Mass Analyzer R | ITMS |
| Unrecognized MS Order Replac | MS2 |
| Unrecognized Activation Type F | CID |
| Unrecognized Polarity Replacer | + |
| Unrecognized MS Resolution@ | 60000 |
| Unrecognized MSn Resolution@ | 30000 |

## MS2 – Spectrum Processor

This Node provides two MS2-spectrum preprocessing steps: Deisotoping of isotopic clusters and charge-deconvolution. For this, spectra are searched for isotopic clusters by determining the distances in m/z values between pairs of peaks. For every cluster detected, only the monoisotopic peaks remain in the spectrum, other peaks are removed.

Subsequently, the spectra are deconvolved to charge state 1. Every peak with a charge state greater than 1 will be removed from the spectrum and replaced by a peak at the corresponding singly-charged m/z-position with the same intensity. Note that the algorithm only works on peaks having charge state information available. For a more detailed description of the algorithm, please refer to: http://ms.imp.ac.at/?goto=pd-nodes

In this exemplary workflow, the following parameter settings are recommended:

Parameters

Hide Advanced Parameters

| | |
|---|---|
| **1. General Settings** | |
| Perform De-Isotoping | True |
| Select DeIsotoping Method | Standard |
| Isotope Distance Deviation Tolerance | 25 mmu |
| Minimal Isotope Ratio | 0.3 |
| Use Adaptive Isotope Distance Deviation Toler | True |
| Deisotope Reporter Region | True |
| Perform Charge De-Convolution | True |
| Select Charge-Deconvolution method | Standard |
| **2. Averagine Modelling Settings** | |
| Modelling Tolerance | 0.5 |
| Use Relative Intensity Threshold | False |
| Intensity Threshold | 0 |
| Apply Adaptive Modelling | False |
| Use Pattern Scoring (Best - Fit Isotope Pattern S | False |
| **3. MS1 Preprocessing Settings** | |
| Recalculate Precursor mass from MS1 | False |
| Use 3d Peaks | True |
| 3d peak-picking tolerance | 5 ppm |
| Minimum profile points for 2d peak | 5 |
| Detect 3d split-peak | True |
| Regression window | 4 |
| Number of Skip-Scans | 1 |
| Use Isotopes | True |
| Isotope Distance Tolerance | 5 mmu |
| Use Averagine Modeling | True |

## G-Score (optional):

The G-Score Node filters MS2 spectra based on the occurrence and intensity of various glycan-derived oxonium ions (for more details see Stadlmann J., Taubenschmid J., et al. Nature (2017)), and thus allows for a more efficient analysis of glycopeptides. Optimal threshold settings need to be empirically established for each instrument acquisition method. In this example, a G-Score threshold of 0.4 was used. N.B. the use of this Node is optional.

Parameters

Hide Advanced Parameters

| 1. Scoring Parameters | |
|---|---|
| Mass Tolerance | 5 ppm |
| G-Score Threshold | 0.4 |
| Filter G-Scores >= Threshold | True |

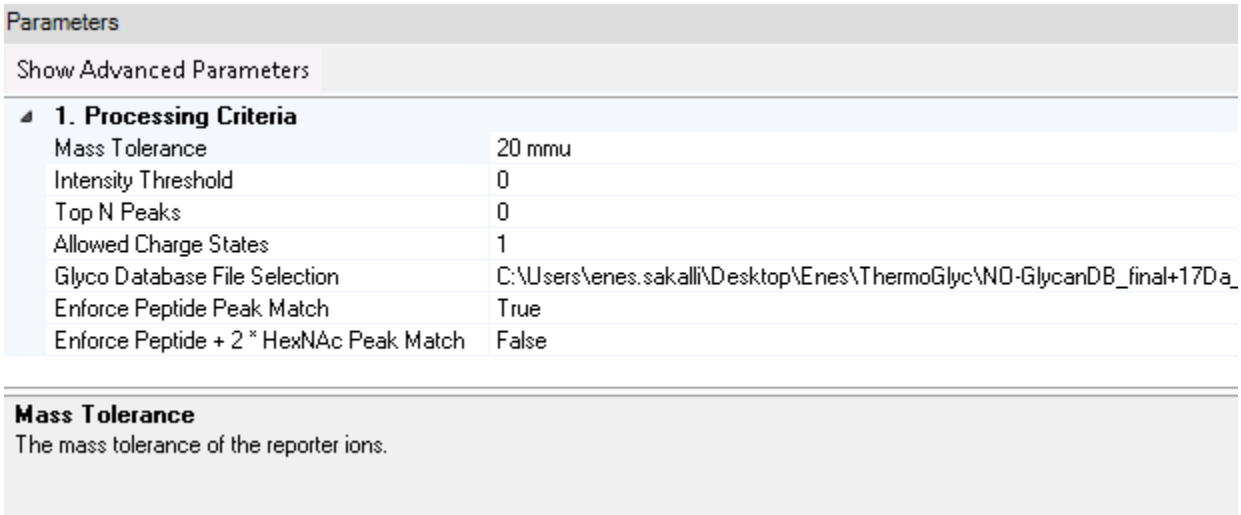
## SugarQb:

The SugarQb Node focuses on the identification of the potential [peptide + HexNAc] + - fragment ions within MS/MS spectra. For this, the precursor-ion masses of a given MS/MS spectrum are iteratively reduced by all masses present in a glycan-composition database, minus the mass of one HexNAc residue (i.e. 203.0794 amu). This approach generates a set of theoretical [peptide + HexNAc] + -fragment ion masses, which are then tried to be matched within the MS/MS spectrum. In cases where an experimental peak matches a theoretical [peptide + HexNAc] + -fragment, the concomitant presence of the corresponding potential [peptide]+ -fragment ion is verified. Only if both peaks are detected, the given spectrum is duplicated with its precursor-ion mass set to the mass of the respective potential [peptide + HexNAc] + fragment-ion (for more details see Stadlmann J., Taubenschmid J., et al. Nature (2017)).

Note, that in this exemplary workflow, charge-deconvoluted MS2 spectra (i.e. all fragment ions are expected to be of charge state 1) are analyzed and thus only charge state 1 is allowed. The .txt Glyco Database File used in this example can be downloaded at:

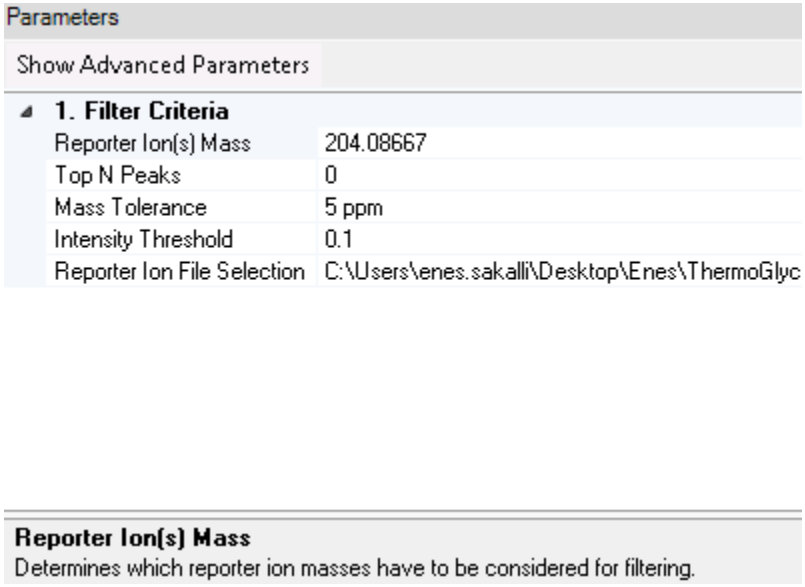
[www.imba.oeaw.ac.at/SugarQb](www.imba.oeaw.ac.at/SugarQb)

The following SugarQb parameter settings are recommended:



## Reporter Ion-Filter(Optional):

This Node enables the filtering/removal of usually highly abundant, glycan-related fragment ions from MS2 spectra. Reporter Ion masses to be completely removed from the MS2 dataset can also be defined in a separate .txt file (i.e. Reporter Ion File Selection). The Reporter Ion File used in this example can be downloaded at: [www.imba.oeaw.ac.at/SugarQb](www.imba.oeaw.ac.at/SugarQb). N.B. the use of this Node is optional.



## MS/MS Search Engine Settings & Parameters:

For the eventual identification of the glycopeptide amino-acid sequences, all MS2 spectra generated by the SugarQb Node (i.e. those with the original and those with the modified precursor-ion masses) are searched against a concatenated forward and decoy database of the Uniprot human reference proteome set, considering HexNAc (and its neutral loss of 203.079373 amu) as a variable modification to any asparagine, serine and threonine residue.

Here, the use of MASCOT and SEQUEST-HT, are exemplified. Of Note, an in-house developed MS/MS search engine, MS Amanda, is freely available at: http://ms.imp.ac.at/?goto=pd-nodes Irrespective of the MS/MS search engine employed, the resulting peptide-spectrum matches (PSMs) are then manually filtered. For this, only the bestscoring PSMs of each spectrum group (i.e. comprising the MS/MS spectrum with the original precursor-ion mass and all its duplicates with the respectively modified precursor ion masses) are kept and filtered to an estimated false discovery rate (FDR) of 1%, employing the standard "target-decoy approach" (Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods 4, 207-214 (2007)).

Importantly, PSM consolidation prior to manual FDR-filtering can be performed using the "Glyco-Filter" Node (see below).

# MASCOT:

Recommended MASCOT search parameter settings are listed below. Of note, MASCOT provides additional options to optimize the search engines performance in the identification of glycopeptide amino-acid sequences (e.g. handling of the dominant neutral loss of the glycan portion upon HCD fragmentation or scoring only singly charged fragment ions). Examples of such adjustments are described in the Annex section.

| | |
|---|---|
| **1. Input Data** | |
| Protein Database | human_uniprot_comb |
| Enzyme Name | Trypsin |
| Maximum Missed Cleavage Sites | 2 |
| Instrument | ESI QUAD 1+ |
| Taxonomy | All entries |
| **2. Tolerances** | |
| Precursor Mass Tolerance | 20 mmu |
| Fragment Mass Tolerance | 20 mmu |
| Use Average Precursor Mass | False |
| **3. Modification Groups** | |
| From Quan Method | |
| **4. Dynamic Modifications** | |
| Show All Modifications | False |
| 1. Dynamic Modification | HexNAc(NL) (NST) |
| 2. Dynamic Modification | Oxidation (C) |
| 3. Dynamic Modification | |
| 4. Dynamic Modification | |
| 5. Dynamic Modification | |
| 6. Dynamic Modification | |
| 7. Dynamic Modification | |
| 8. Dynamic Modification | |
| 9. Dynamic Modification | |
| **5. Static Modifications** | |
| 1. Static Modification | Carbamidomethyl (C) |
| 2. Static Modification | |
| 3. Static Modification | |
| 4. Static Modification | |
| 5. Static Modification | |
| 6. Static Modification | |

## SEQUEST-HT:

Recommended SEQUEST-HT search parameter settings are listed below.

| | |
|---|---|
| **1. Input Data** | |
| Protein Database | |
| Enzyme Name | Trypsin (Full) |
| Max. Missed Cleavage Sites | 2 |
| Min. Peptide Length | 6 |
| Max. Peptide Length | 144 |
| **2. Tolerances** | |
| Precursor Mass Tolerance | 20 ppm |
| Fragment Mass Tolerance | 0.025 Da |
| Use Average Precursor Mass | False |
| Use Average Fragment Mass | False |
| **3. Spectrum Matching** | |
| Use Neutral Loss a Ions | True |
| Use Neutral Loss b Ions | True |
| Use Neutral Loss y Ions | True |
| Use Flanking Ions | True |
| Weight of a Ions | 0 |
| Weight of b Ions | 1 |
| Weight of c Ions | 0 |
| Weight of x Ions | 0 |
| Weight of y Ions | 1 |
| Weight of z Ions | 0 |
| **4. Dynamic Modifications** | |
| Max. Equal Modifications Per Peptide | 3 |
| 1. Dynamic Modification | HexNAc / +203.079 Da (N, S, T) |
| 2. Dynamic Modification | Oxidation / +15.995 Da (M) |
| 3. Dynamic Modification | None |
| 4. Dynamic Modification | None |
| 5. Dynamic Modification | None |
| 6. Dynamic Modification | None |
| **5. Dynamic Modifications (peptide terminus)** | |
| 1. N-Terminal Modification | None |
| 2. N-Terminal Modification | None |
| 3. N-Terminal Modification | None |
| 1. C-Terminal Modification | None |
| 2. C-Terminal Modification | None |
| 3. C-Terminal Modification | None |
| **6. Dynamic Modifications (protein terminus)** | |
| 1. N-Terminal Modification | None |
| 2. N-Terminal Modification | None |
| 3. N-Terminal Modification | None |
| 1. C-Terminal Modification | None |
| 2. C-Terminal Modification | None |
| 3. C-Terminal Modification | None |
| **7. Static Modifications** | |
| Peptide N-Terminus | None |
| Peptide C-Terminus | None |
| 1. Static Modification | Carbamidomethyl / +57.021 Da (C) |
| 2. Static Modification | None |
| 3. Static Modification | None |
| 4. Static Modification | None |
| 5. Static Modification | None |
| 6. Static Modification | None |

## MS-AMANDA:

Recommended SEQUEST-HT search parameter settings are listed below.

Parameters

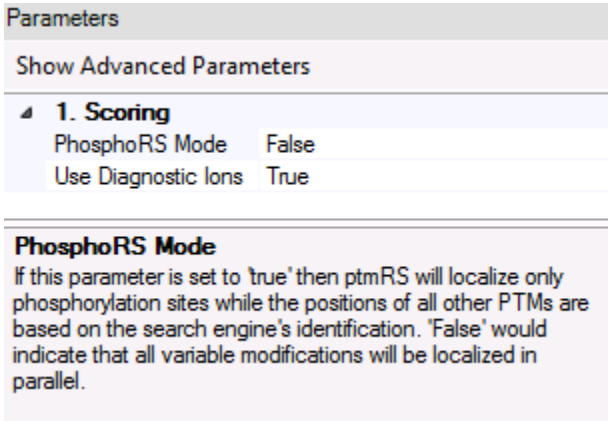
Show Advanced Parameters

| | |
|---|---|
| **1. Input Data** | |
| Protein Database | |
| Enzyme Name | Trypsin (Full) |
| Missed Cleavages | 2 |
| MS1 tolerance | 5 ppm |
| MS2 tolerance | 0.02 Da |
| **2. Static Modifications** | |
| 1. Static Modification | Carbamidomethyl / +57.021 Da (C) |
| 2. Static Modification | None |
| 3. Static Modification | None |
| Static Peptide N-Terminal Modification | None |
| Static Peptide C-Terminal Modification | None |
| Static Protein N-Terminal Modification | None |
| **3. Dynamic Modifications** | |
| 1. Dynamic Modification | Oxidation / +15.995 Da (M) |
| 2. Dynamic Modification | HexNAc / +203.079 Da (N, S, T) |
| 3. Dynamic Modification | None |
| 21. Dynamic Peptide N-Terminal Modification | None |
| 22. Dynamic Peptide C-Terminal Modification | None |
| 23. Dynamic Protein N-Terminal Modification | None |

**Protein Database**
The sequence database to be searched.

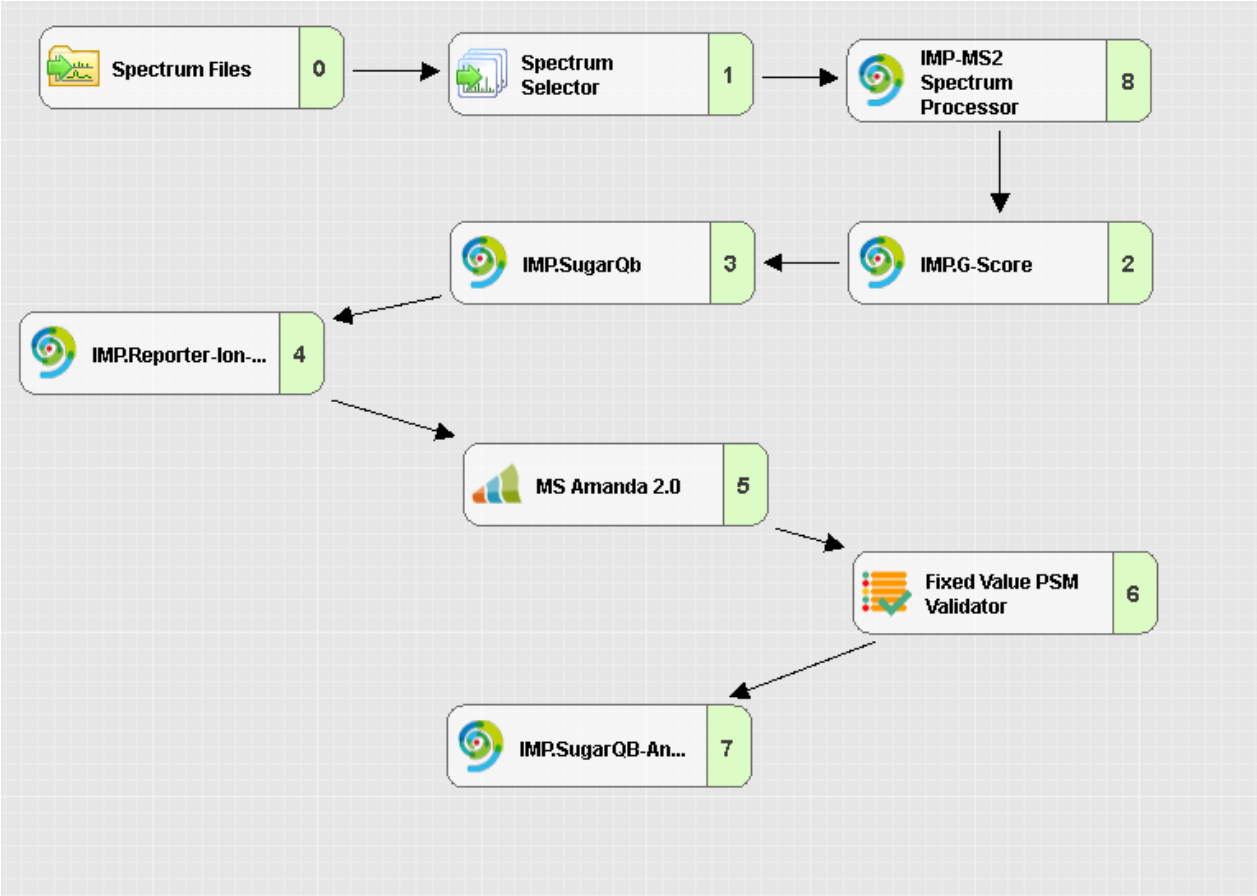
## ptmRS(Optional)

Generally, this tool enables automated and confident localization of modification sites within validated peptide sequences. It calculates individual probability values for each putatively modified site based on the given MS/MS data. ptmRS can also be used to localize N- glycosylation sites. For further information on the algorithm of the software, please refer to Taus T., et al. (2011) *Universal and Confident Phosphorylation Site Localization Using phosphoRS*.



## Workflow with the IMP-Sugar-Annotation-Validation:

The additional Node Glyco-Filter is for annotating the best Psm as Master PSM for Glyco-Data, as well when there are several other Psm which are having the same Ion-Score, it will summarize all the Glycan-IDs of all Psms with the same Ion-Score. Also the node is important for transfering the data from MS/MS layer to the psm layer.




| SugarQB-Is-Master-PSM | Isobaric-SugarQB-IDs | Number-Of-Isobaric-Master-Ps |
|---|---|---|
| Master | id=HexNAc1.Hex1_O-Glycan\|id=HexNAc1.Hex1_O-Glycan\|id=HexNAc1.Hex1_O-Glycan\|id=HexNAc1.Hex1_O-Glycan\|id=HexNAc1.Hex1_O-Glycan\|id=HexNAc1.Hex1_O-Glycan | 6 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
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| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |
| Master | | 1 |

## Anticipated Results using the sample data file provided:

The Thermo Scientific Proteome Discoverer 2.1 result files (.msf), the exported Excel workbook, and a manually filtered result-file (.csv) can be downloaded from:

www.imba.oeaw.ac.at/SugarQb .

Contact: SugarQb@imba.oeaw.ac.at

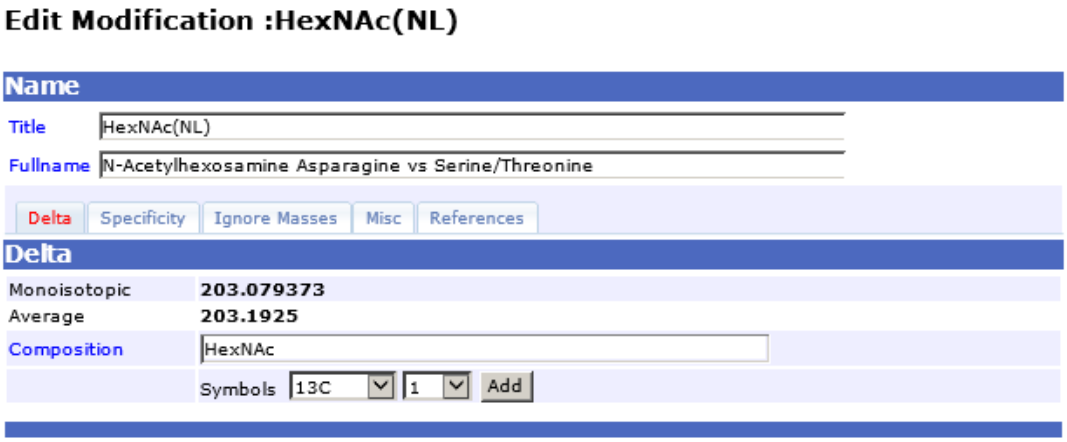
# Annex

## Alternative MASCOT Parameters (optional)

### Instruments Settings:

| Instruments | | |
|---|---|---|
| Ion series | Default | ESI QUAD 1+ |
| 1+ | X | X |
| 2+ | X | |
| 2+ (precursor>3+) | | |
| immonium | | |
| a | X | |
| a* | X | |
| a0 | | |
| b | X | X |
| b* | X | X |
| b0 | | X |
| c | | |
| x | | |
| y | X | X |
| y* | X | X |
| y0 | | X |
| z | | |
| yb | | |
| ya | | |
| y must be significant | | |
| y must be highest score | | |
| z+1 | | |
| d | | |
| v | | |
| w | | |
| z+2 | | |
| Minimum mass | | |
| Max mass | 700 | |
| | Edit | Delete Edit |

## Modification Settings “HexNAc(NL)”:



## Edit Modification :HexNAc(NL)

### Name

Title: HexNAc(NL)

Fullname: N-Acetylhexosamine Asparagine vs Serine/Threonine

Delta | Specificity | Ignore Masses | Misc | References

### Specificity

Specificity — Site: T — Position: Anywhere — Copy | Delete | Hide Details

Classification: Other glycosylation — Hidden ☐ — Group: 1

Notes:

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Monoisotopic: **203.079373** Average: **203.1925**

Composition: HexNAc — Symbols: 13C 1 Add

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Composition: — Symbols: 13C 1 Add

New Neutral Loss

---

Specificity — Site: S — Position: Anywhere — Copy | Delete | Hide Details

Classification: Other glycosylation — Hidden ☐ — Group: 1

Notes:

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Monoisotopic: **203.079373** Average: **203.1925**

Composition: HexNAc — Symbols: 13C 1 Add

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Composition: — Symbols: 13C 1 Add

New Neutral Loss

---

Specificity — Site: N — Position: Anywhere — Copy | Delete | Hide Details

Classification: Other glycosylation — Hidden ☐ — Group: 1

Notes:

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Monoisotopic: **203.079373** Average: **203.1925**

Composition: HexNAc — Symbols: 13C 1 Add

Neutral loss — ◉ Scoring ○ Satellite ○ Peptide ○ Required Peptide — Delete

Composition: — Symbols: 13C 1 Add

New Neutral Loss

Delta | Specificity | Ignore Masses | Misc | References

## Ignore Masses

| | |
|---|---|
| Ignore Mass **1** | Monoisotopic **204.086649** Average **204.1999** Delete |
| Composition | H HexNAc e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **2** | Monoisotopic **186.076084** Average **186.1846** Delete |
| Composition | H HexNAc Water(-1) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **3** | Monoisotopic **168.065519** Average **168.1694** Delete |
| Composition | H HexNAc Water(-2) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **4** | Monoisotopic **144.065519** Average **144.1480** Delete |
| Composition | C(6) H(10) N O(3) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **5** | Monoisotopic **138.054955** Average **138.1434** Delete |
| Composition | C(7) H(8) N O(2) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **6** | Monoisotopic **126.054955** Average **126.1327** Delete |
| Composition | C(6) H(8) N O(2) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **7** | Monoisotopic **163.060100** Average **163.1480** Delete |
| Composition | H Hex e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **8** | Monoisotopic **145.049535** Average **145.1327** Delete |
| Composition | H Hex Water(-1) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **9** | Monoisotopic **127.038970** Average **127.1174** Delete |
| Composition | H Hex Water(-2) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **10** | Monoisotopic **366.139472** Average **366.3405** Delete |
| Composition | H Hex HexNAc e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **11** | Monoisotopic **528.192296** Average **528.4811** Delete |
| Composition | H Hex(2) HexNAc e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **12** | Monoisotopic **292.102693** Average **292.2620** Delete |
| Composition | C(11) H(18) N O(8) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **13** | Monoisotopic **274.092128** Average **274.2467** Delete |
| Composition | C(11) H(18) N O(8) Water(-1) e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **14** | Monoisotopic **657.235438** Average **657.5956** Delete |
| Composition | C(11) H(18) Hex HexNAc N O(8) |
| | Symbols 13C 1 Add |
| Ignore Mass **15** | Monoisotopic **243.026430** Average **243.1279** Delete |
| Composition | H(2) Hex O(3) P e(-1) |
| | Symbols 13C 1 Add |
| Ignore Mass **16** | Monoisotopic **225.015866** Average **225.1126** Delete |
| Composition | H(2) Hex O(3) P Water(-1) e(-1) |
| | Symbols 13C 1 Add |