# Crosslink identification with MS Annika 2.0

MS Annika 2.0 is a cross-linking search engine for use with cleavable crosslinkers and MS2 or MS2-MS3 spectra. It can deal with a wide variety of cleavable crosslinkers and provides robust and transparent FDR control based on a target-decoy approach. MS Annika 2.0 is available as nodes for Proteome Discoverer and can be downloaded and used free of charge.

This tutorial covers the installation of Proteome Discoverer and MS Annika 2.0, how to setup a crosslink search with MS Annika 2.0, which results are provided by MS Annika 2.0, and finally an exemplary down-stream analysis of the MS Annika 2.0 results.

This tutorial will use Proteome Discoverer version 3.0 and MS Annika 2.0 version 1.1.4 but should be applicable to any supported Proteome Discoverer and MS Annika 2.0 version.

A video version of this tutorial can be found at:

https://www.youtube.com/watch?v=L1lVt35PYv4

## References

More information about MS Annika 2.0 is given in the MS Annika and MS Annika 2.0 publications:

- https://doi.org/10.1021/acs.jproteome.0c01000
- https://doi.org/10.1021/acs.jproteome.3c00325

As well as on the MS Annika 2.0 webpage and GitHub repository:

- https://ms.imp.ac.at/?action=ms-annika
- https://github.com/hgb-bin-proteomics/MSAnnika

## Contact


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# Table of Contents

## Data

To run a crosslink search with MS Annika 2.0 you will need at least two files:

- A file containing the recorded mass spectra, either in Thermo RAW format or in Mascot Generic Format (MGF).
- A database containing the sequences of the proteins that you expect in your sample in FASTA format.
- It's also necessary to know which crosslinker was used for the cross-linking experiment and which post-translational modifications you are expecting to see on your proteins.

If you don't have any of those files for yourself but still want to give MS Annika 2.0 a try, you can find example files here:

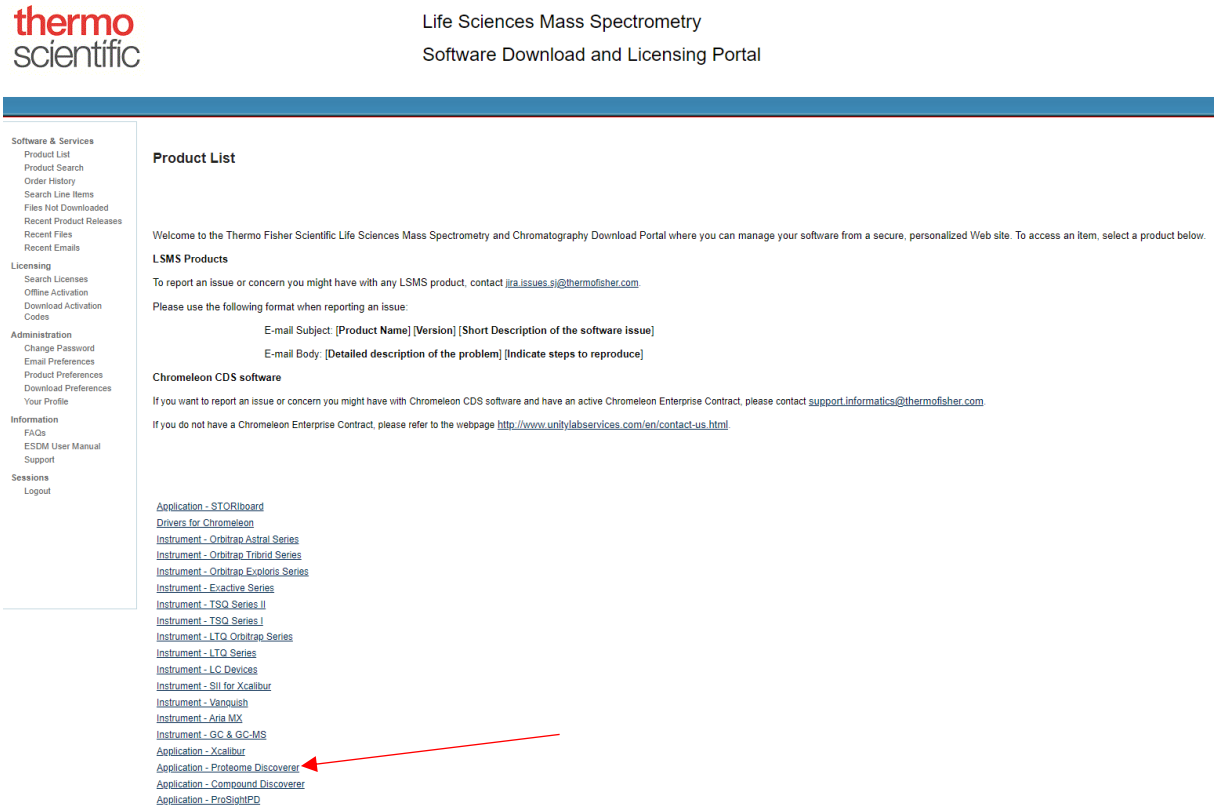
https://github.com/hgb-bin-proteomics/MSAnnika#example-files

# Downloading and installing Proteome Discoverer

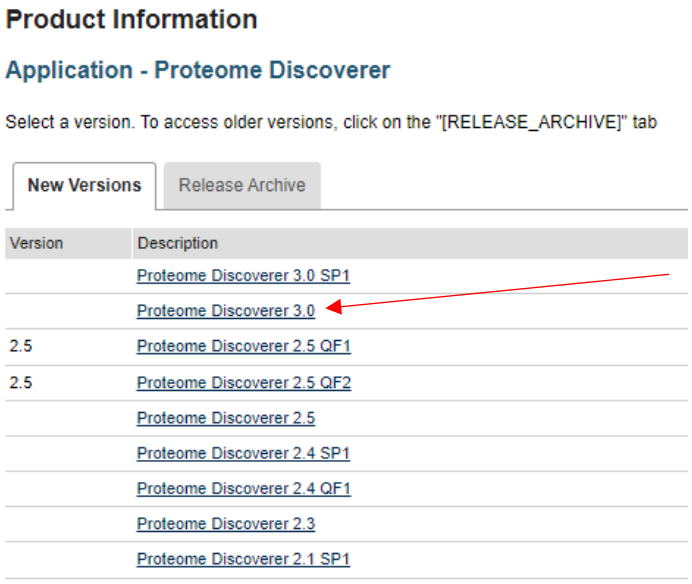
Because MS Annika 2.0 is implemented as nodes within the Proteome Discoverer software suite, the first step is download and install Proteome Discoverer. Proteome Discoverer can be downloaded here:

https://www.thermofisher.com/at/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/proteome-discoverer-software.html

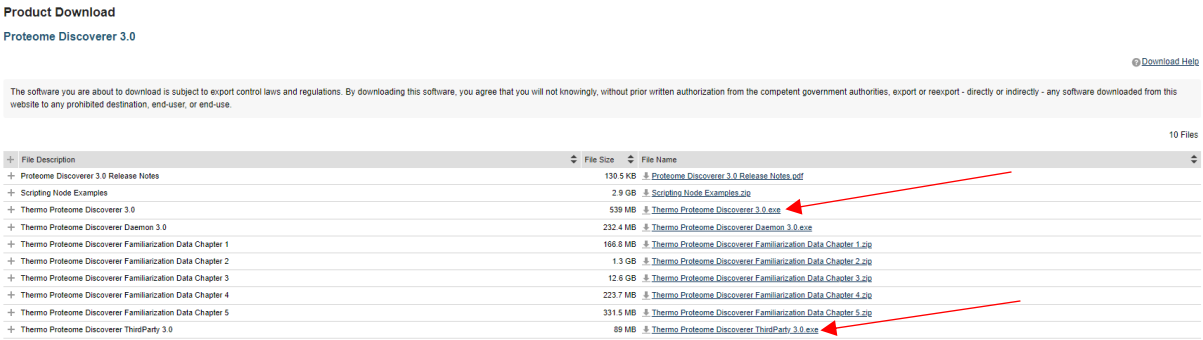

Click on "Free demo download" and you will be forwarded to login with your Thermo Scientific account. In case you don't have an account, you can sign up for free. You will be presented with a list of available software, select "Proteome Discoverer" from that list.



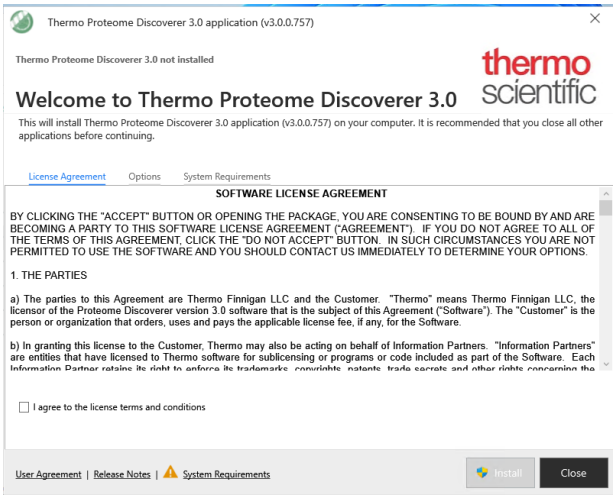
After selecting “Proteome Discoverer” you will be forwarded to the download page where you should select the version that you wish to install. For this tutorial we will go for version 3.0.

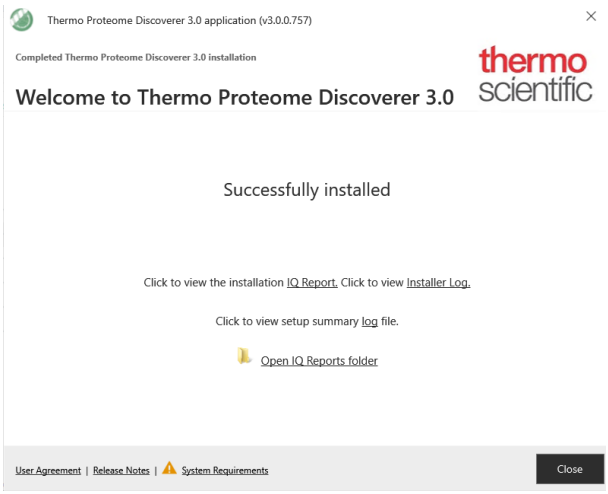



Download “Thermo Proteome Discoverer 3.0” and optionally (but recommended) “Thermo Proteome Discoverer ThirdParty 3.0”.




Install Proteome Discoverer. The installation process requires administrative privileges.

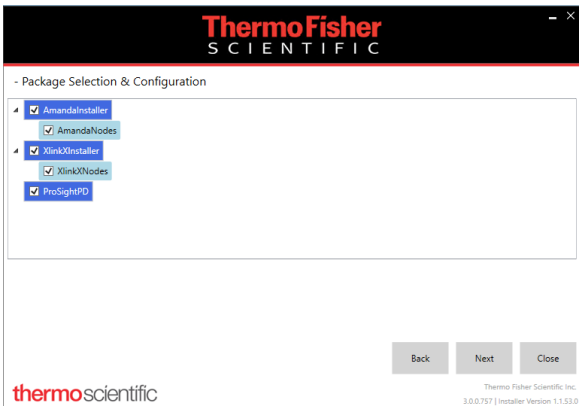


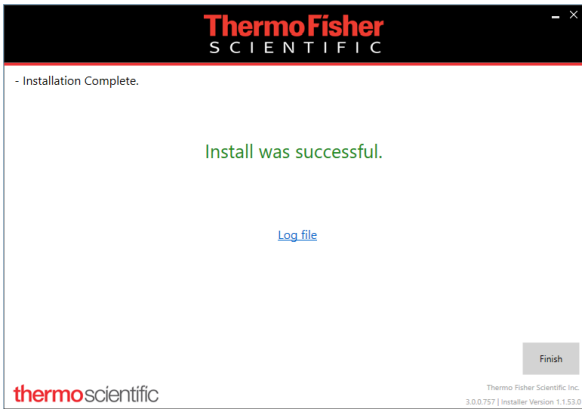



**Optional:** Install the Proteome Discoverer Third Party nodes. In case you are not installing the Proteome Discoverer Third Party nodes it is highly recommended to install MS Amanda. Potentially, there is a newer version of MS Amanda available than provided in the Third Party Nodes, you can download the latest version here:

https://ms.imp.ac.at/?goto=msamanda



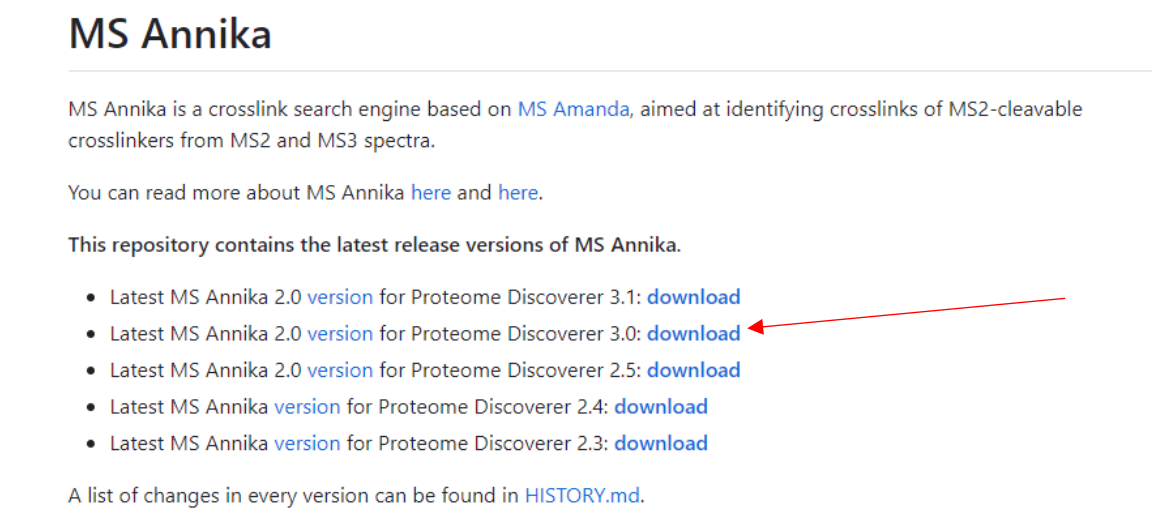



If the installation was successful, you should be able to start Proteome Discoverer now! Make sure to exit Proteome Discoverer before installing MS Annika 2.0!

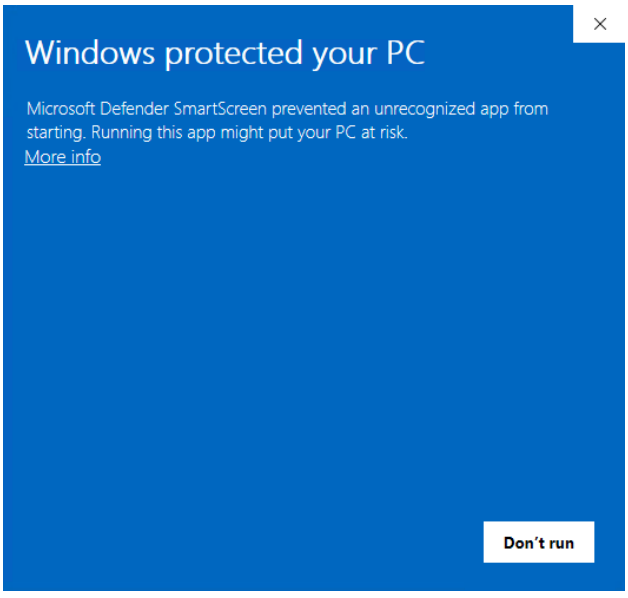
## Downloading and installing MS Annika 2.0

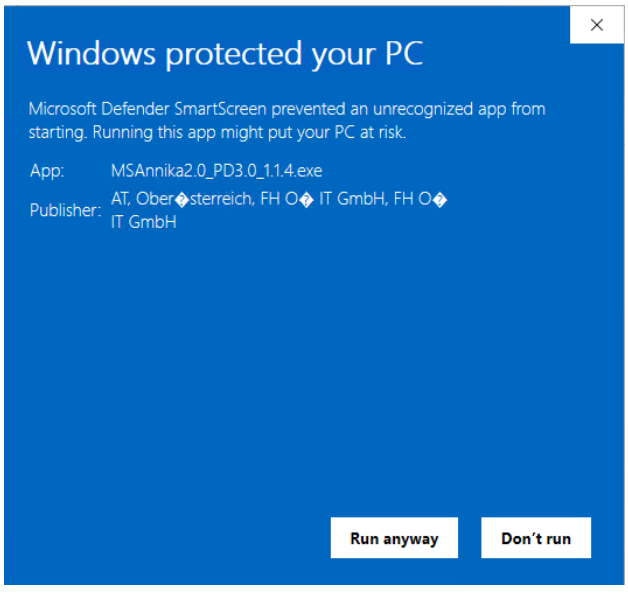
MS Annika 2.0 can be either downloaded from the MS Annika webpage at https://ms.imp.ac.at/?action=ms-annika which also includes some additional information, or alternatively from the MS Annika GitHub repository at https://github.com/hgb-bin-proteomics/MSAnnika which we will use for this tutorial. Clicking the download link for MS Annika 2.0 for Proteome Discoverer 3.0 will download the latest MS Annika 2.0 version and the user manual compressed as a ZIP folder to your computer.




Make sure that Proteome Discoverer is not running during the installation of MS Annika 2.0!

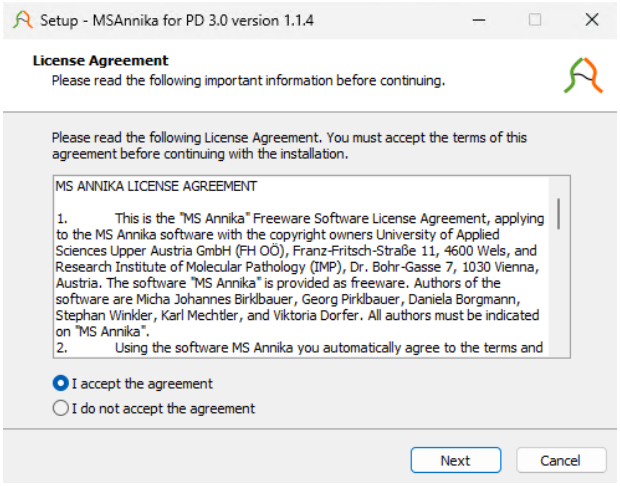
Extract the ZIP file and double click the MS Annika 2.0 installer, in case a Microsoft Defender warning pops up, click on “More info” and “Run anyway”.

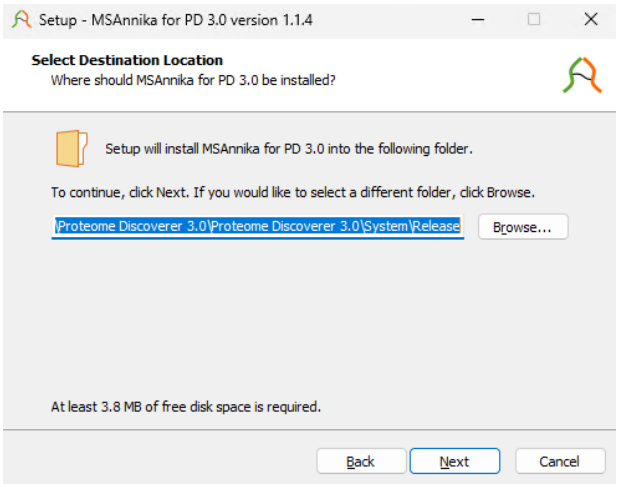


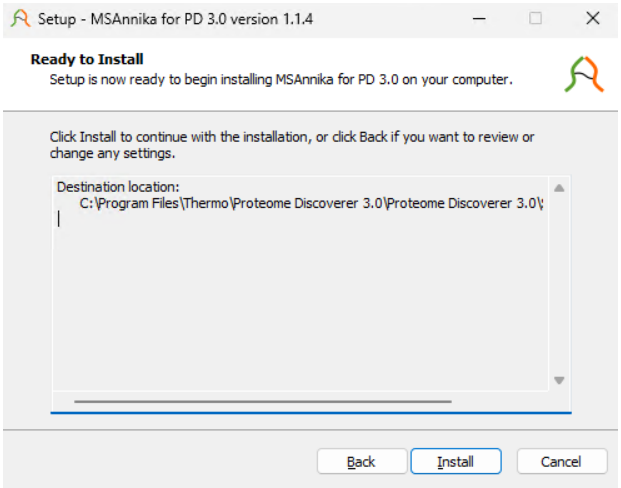



In case any other antivirus software is blocking the application, make sure to whitelist the MS Annika 2.0 installer.

Installing MS Annika 2.0 also requires administrative privileges, therefore a user-account-control prompt should pop up before the MS Annika 2.0 installer launches. After that simply follow the instructions on screen. The installation path should be pre-set and no change should be needed.

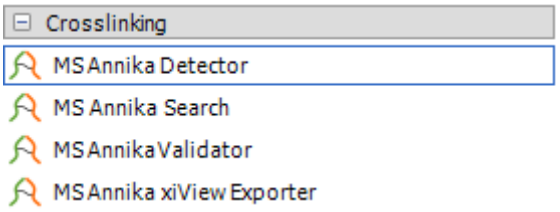


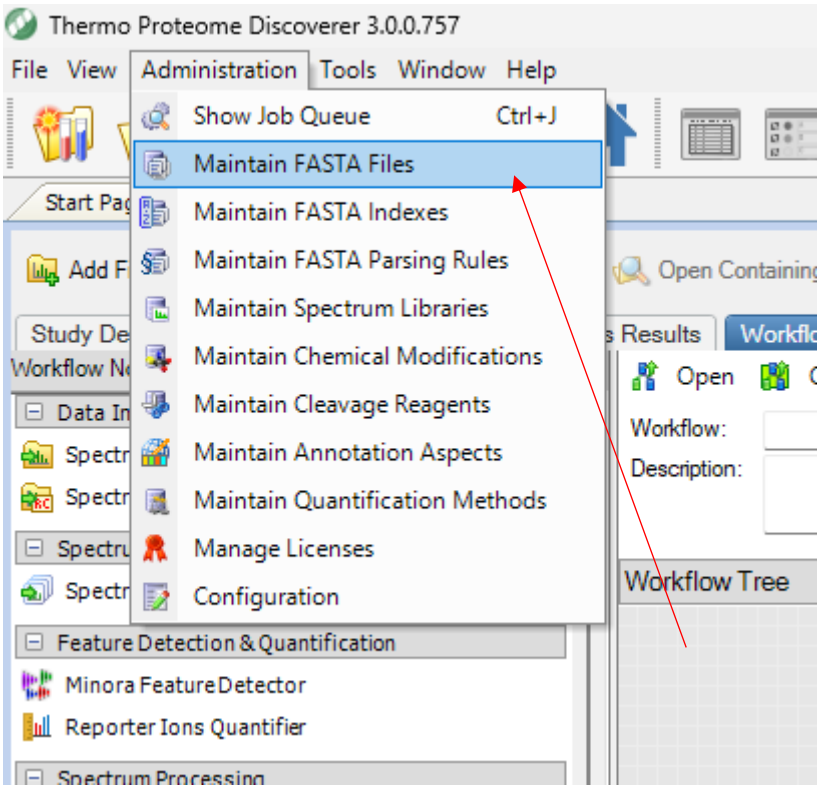


If the installation was successful, the MS Annika 2.0 nodes should now be available in Proteome Discoverer.

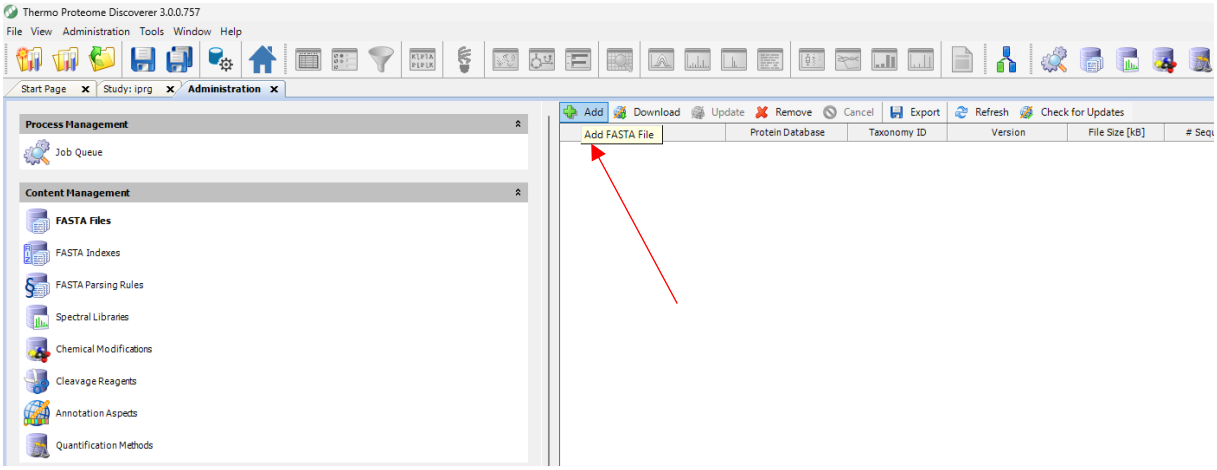


## Importing the FASTA database in Proteome Discoverer

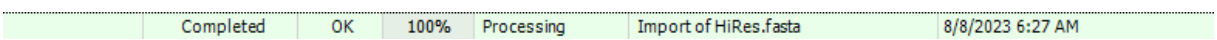
In Proteome Discoverer go to the "Administration" menu in the top left and select "Maintain FASTA Files".




Click the "Add" button to add a FASTA file.

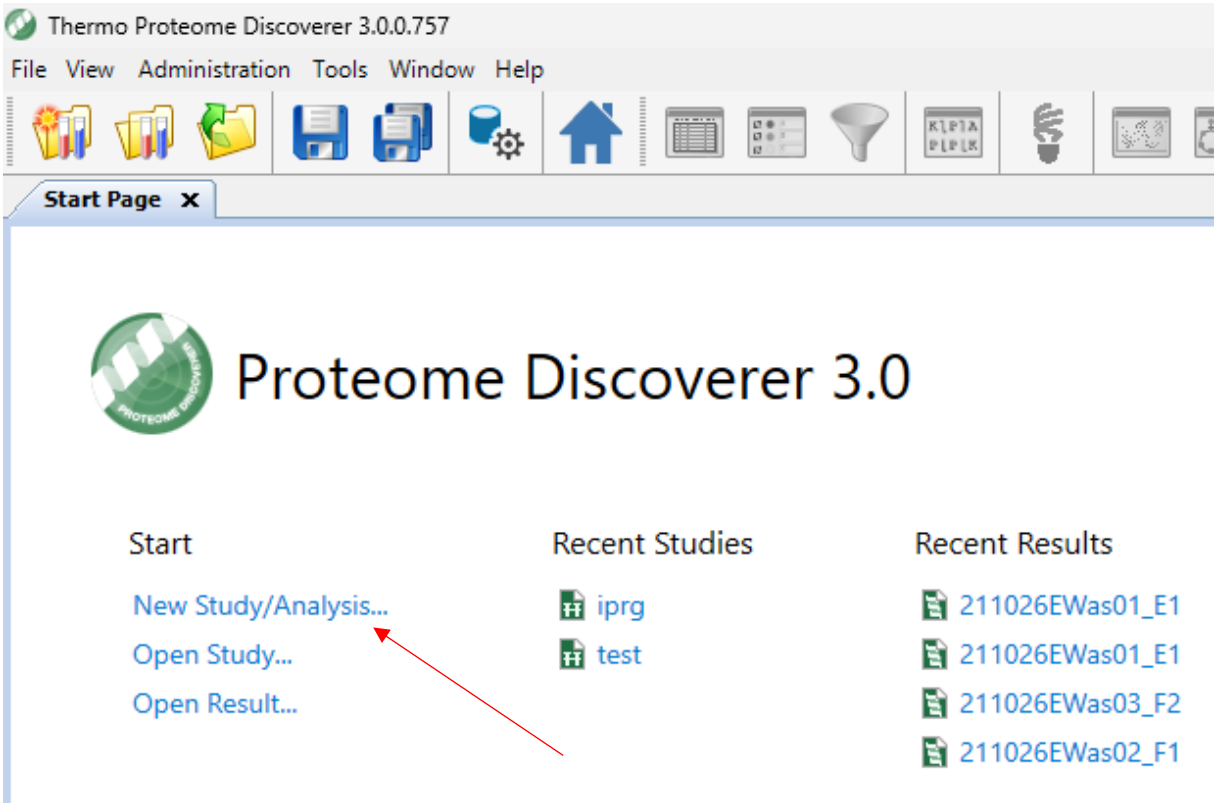



You will need to select the FASTA file in the explorer window that pops up. After clicking "Open", Proteome Discover will switch to the Job Queue were an import job for the FASTA file will be running. If the job successfully completes the FASTA file will be available in Proteome Discoverer.

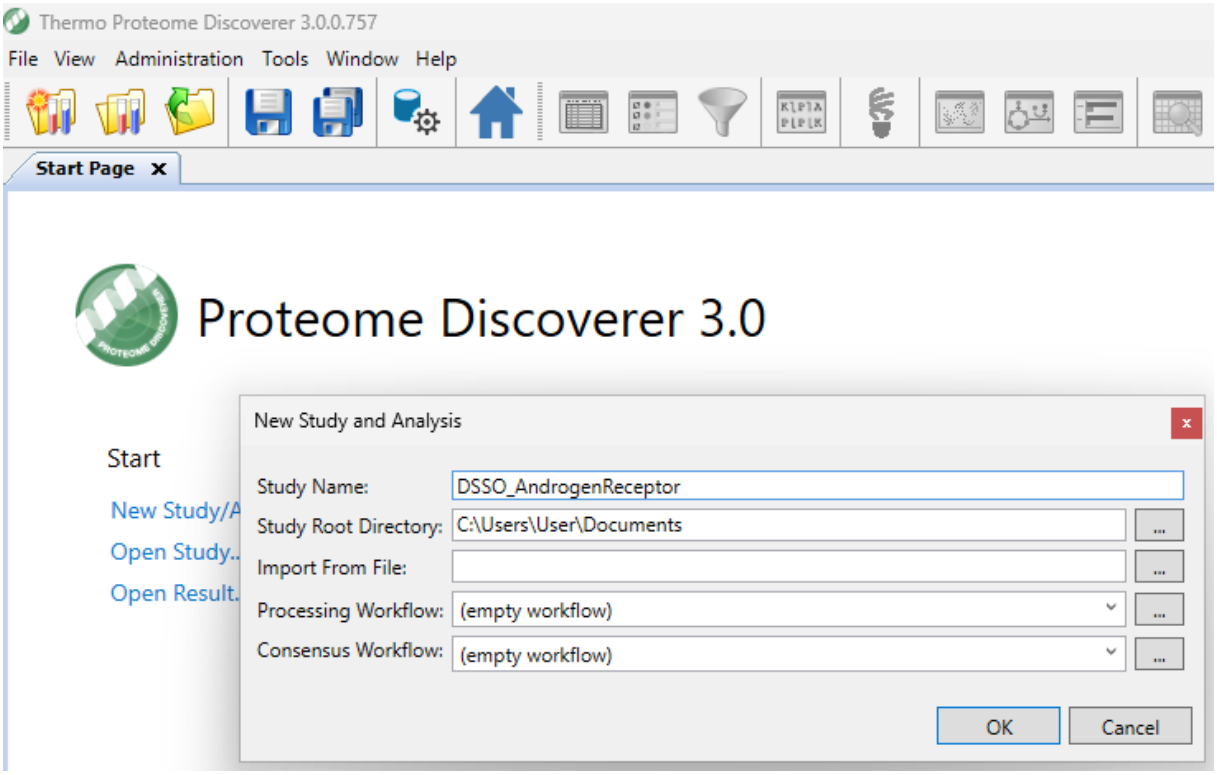


## Creating a new study in Proteome Discoverer

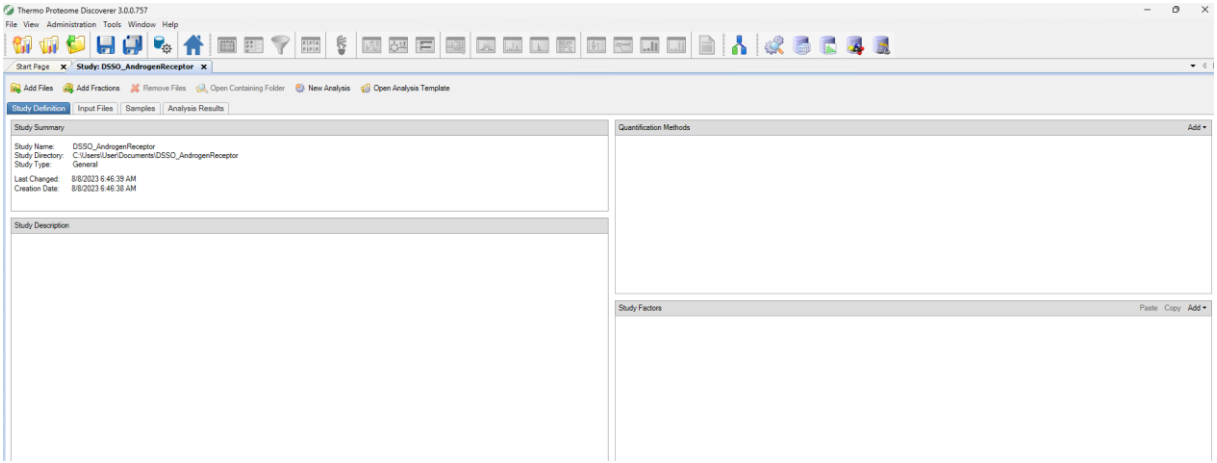
Go to the Start Page of Proteome Discoverer and select “New Study/Analysis…”




Choose a name and the path for your study and hit “OK”.

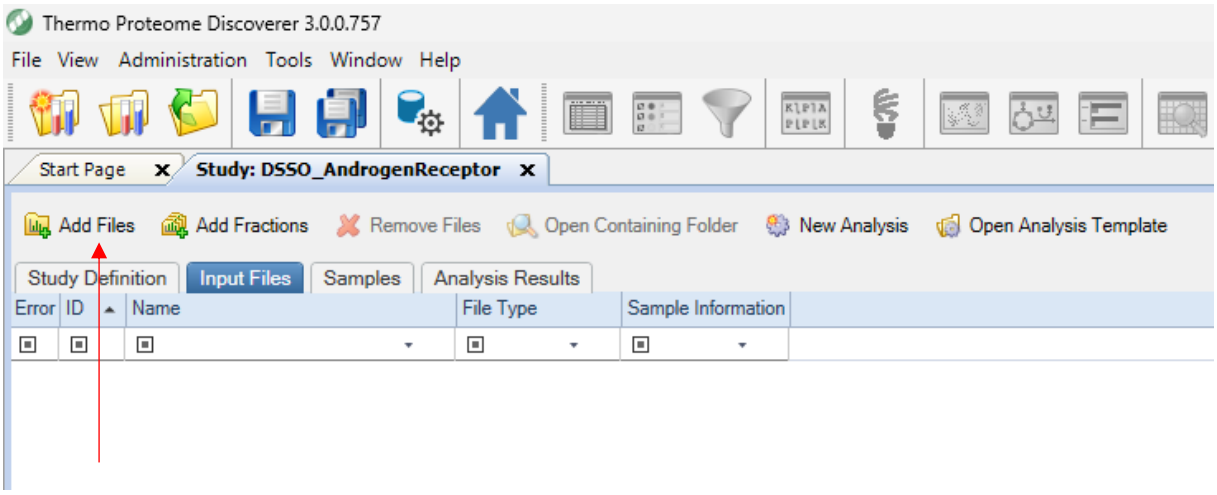


A new tab with your study should open that gives a basic overview of the study.

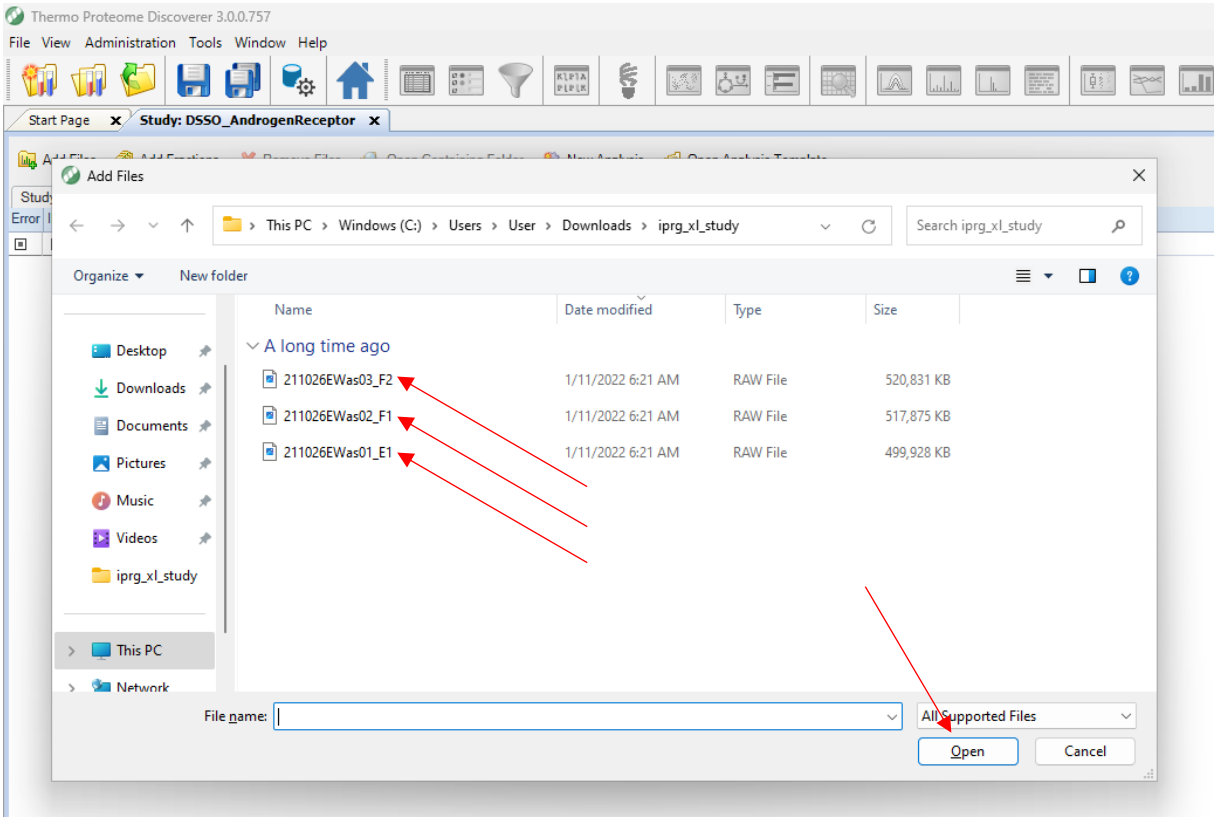


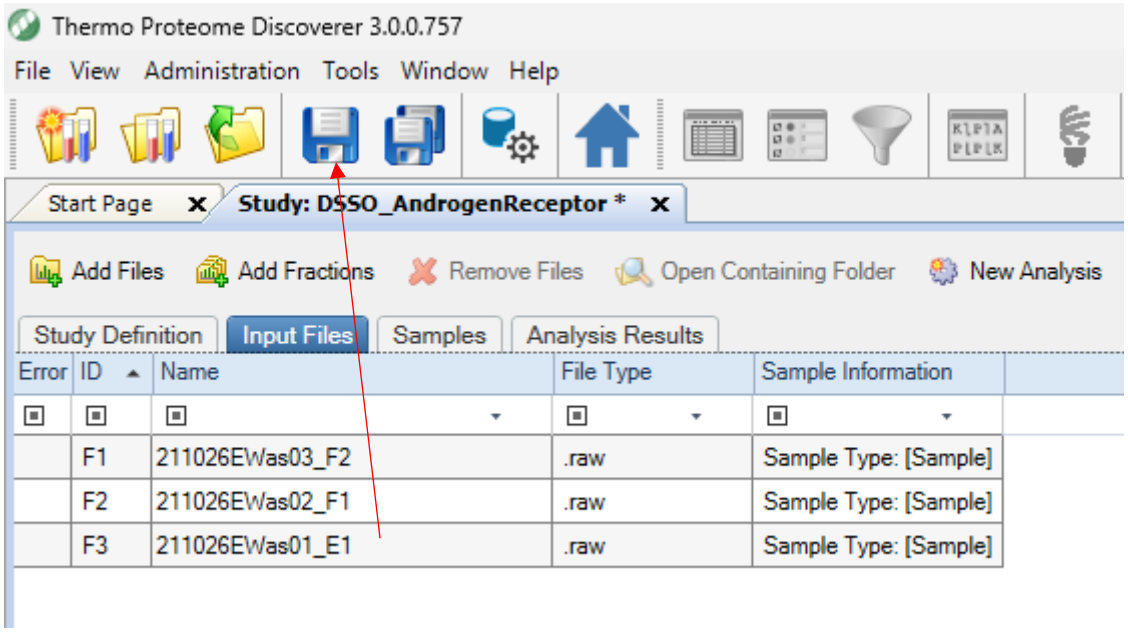
## Importing MS files in Proteome Discoverer

Next you want to import your RAW files in Proteome Discoverer. For that go to the tab “Input Files” in your study and click “Add Files” in the top left corner of your study.




An explorer window will open where you can select all RAW files that you want to import. After confirming with “Open” they will appear in your “Input Files” tab.






Don’t forget to also save your study every other step!

## Working with MGF files

Importing an MGF file works the same way as importing a RAW file. Please do note, that not all MS Annika 2.0 versions support MGF file input, for a full list of MS Annika versions that support processing of MGF files please refer to the according section of the MS Annika 2.0 ReadMe file:

https://github.com/hgb-bin-proteomics/MSAnnika#support-for-mgf-and-timstof-data

Moreover, MGF files are only supported for MS2 searches in MS Annika. This is due to the fact that MGF lacks required information that is necessary to perform an MS3 search.

## Working with timsTOF files

All MS Annika 2.0 versions that support MGF file input also support processing of timsTOF ion mobility data. Again, the full list of MS Annika versions that support timsTOF data input can be found in the MS Annika 2.0 ReadMe file here:

https://github.com/hgb-bin-proteomics/MSAnnika#support-for-mgf-and-timstof-data

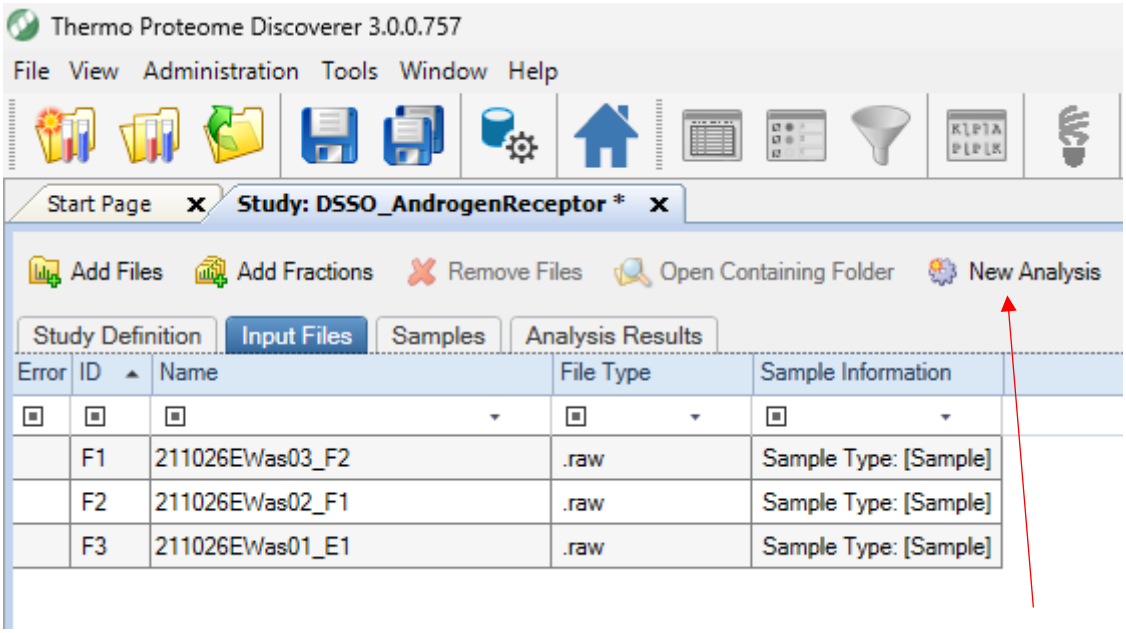
However, it should be noted that reading ion mobility data requires installation of the Bruker Ion Mobility Reader node in Proteome Discoverer which is not publicly available. Please contact us via

- micha.birklbauer@fh-hagenberg.at or
- viktoria.dorfer@fh-hagenberg.at

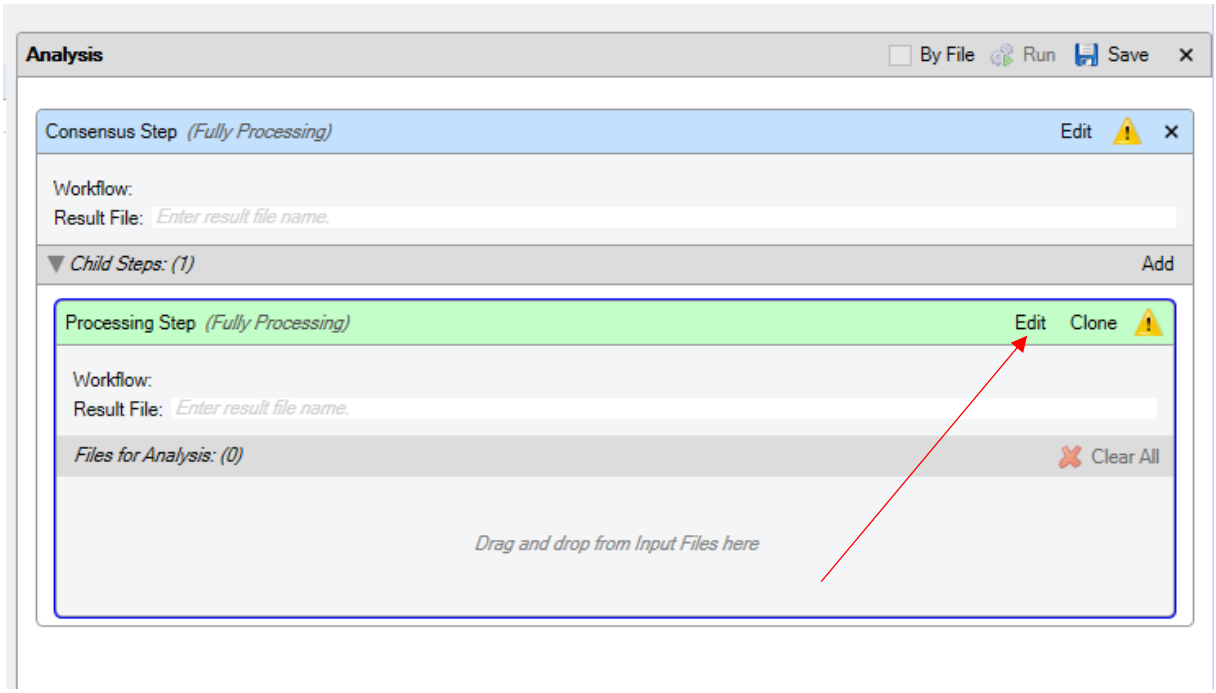
if you want to use MS Annika 2.0 with timsTOF data. Furthermore, only MS2 search is supported for timsTOF data.

## Creating an MS Annika 2.0 workflow in Proteome Discoverer

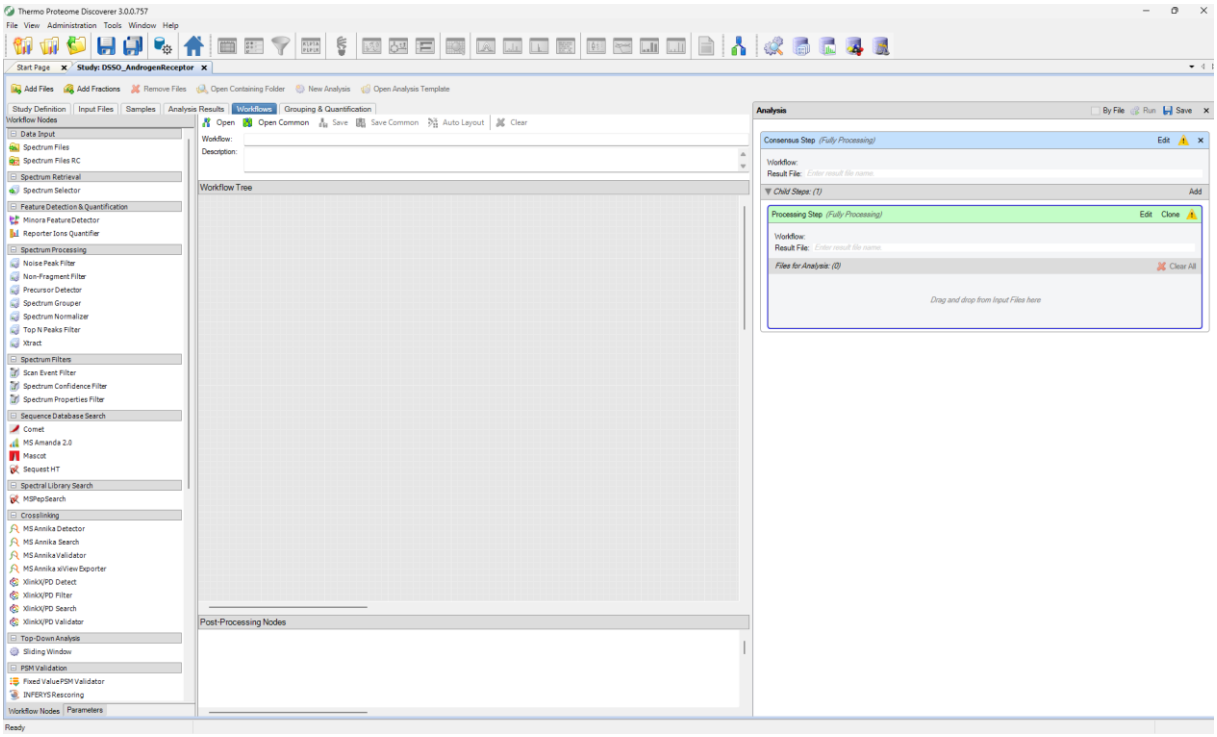
There are two ways to create an MS Annika 2.0 workflow, either creating a new analysis from scratch or opening an analysis template. This section will cover the first approach. In your study click on "New Analysis".



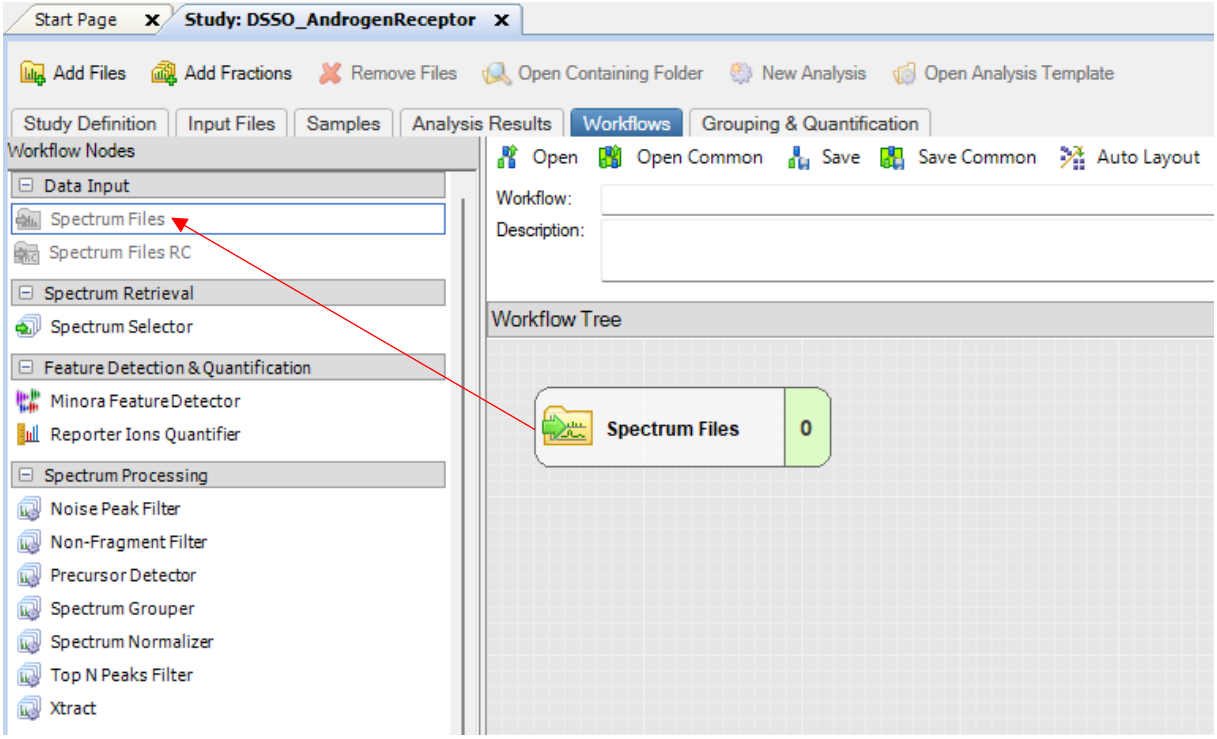

A new window will appear on the right side with two sub-windows called "Consensus Step" and "Processing Step". We will look at the "Processing Step" first, for that click on "Edit" at the top right of the "Processing Step" box.



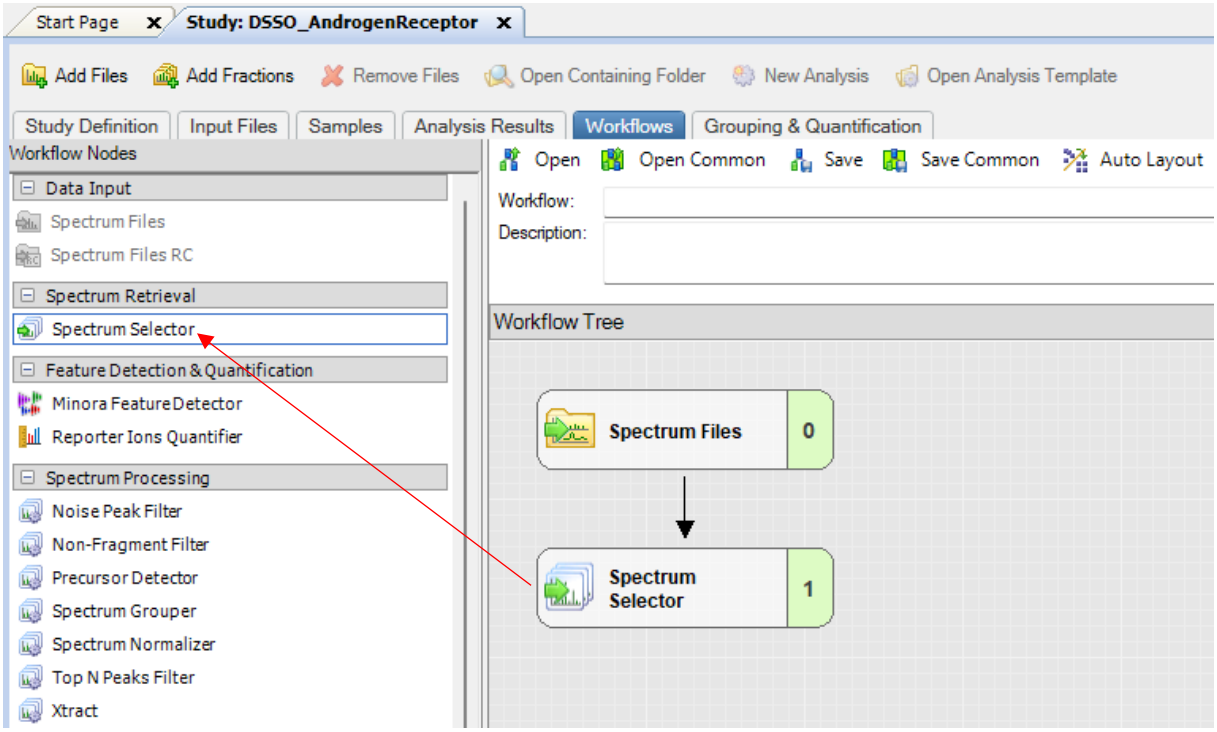
The “Workflow Tree” window should open where we are now able to create a workflow for the identification of crosslinks from mass spectra with MS Annika 2.0.



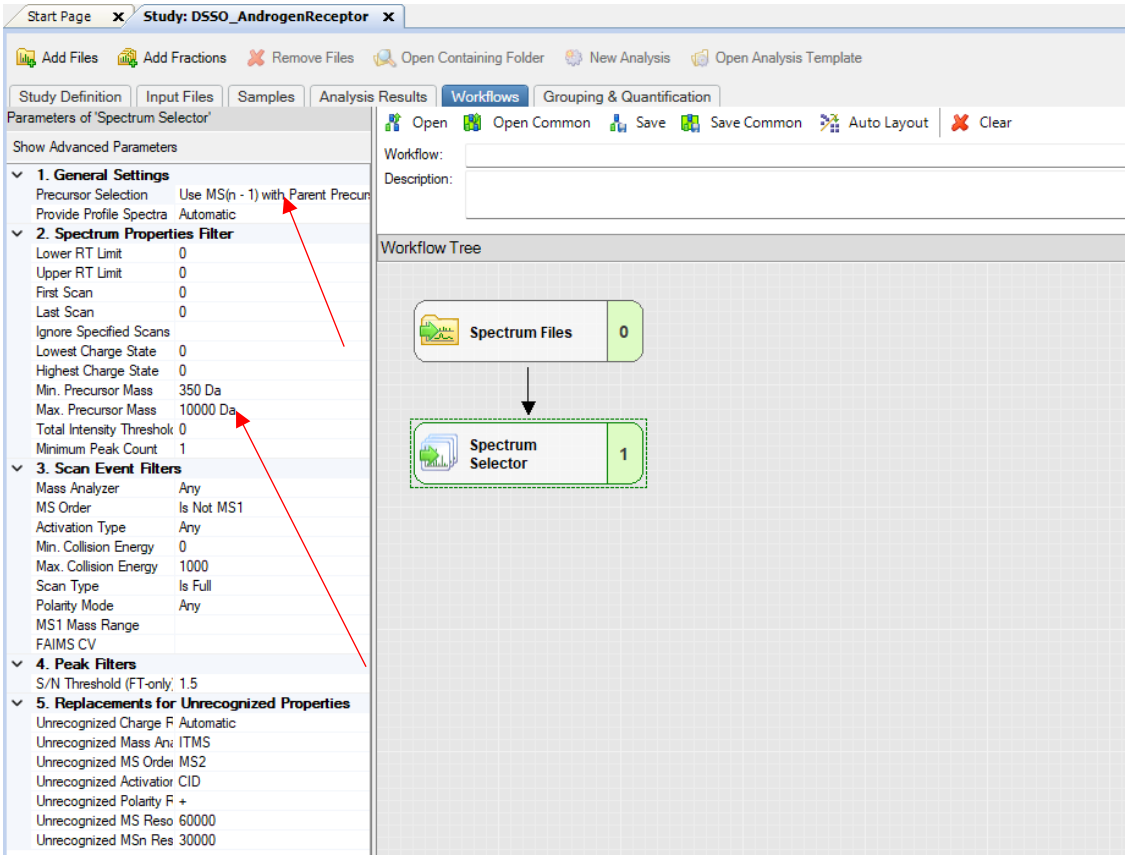
First, we are going to drag-and-drop the “Spectrum Files” node into the “Workflow Tree” window.



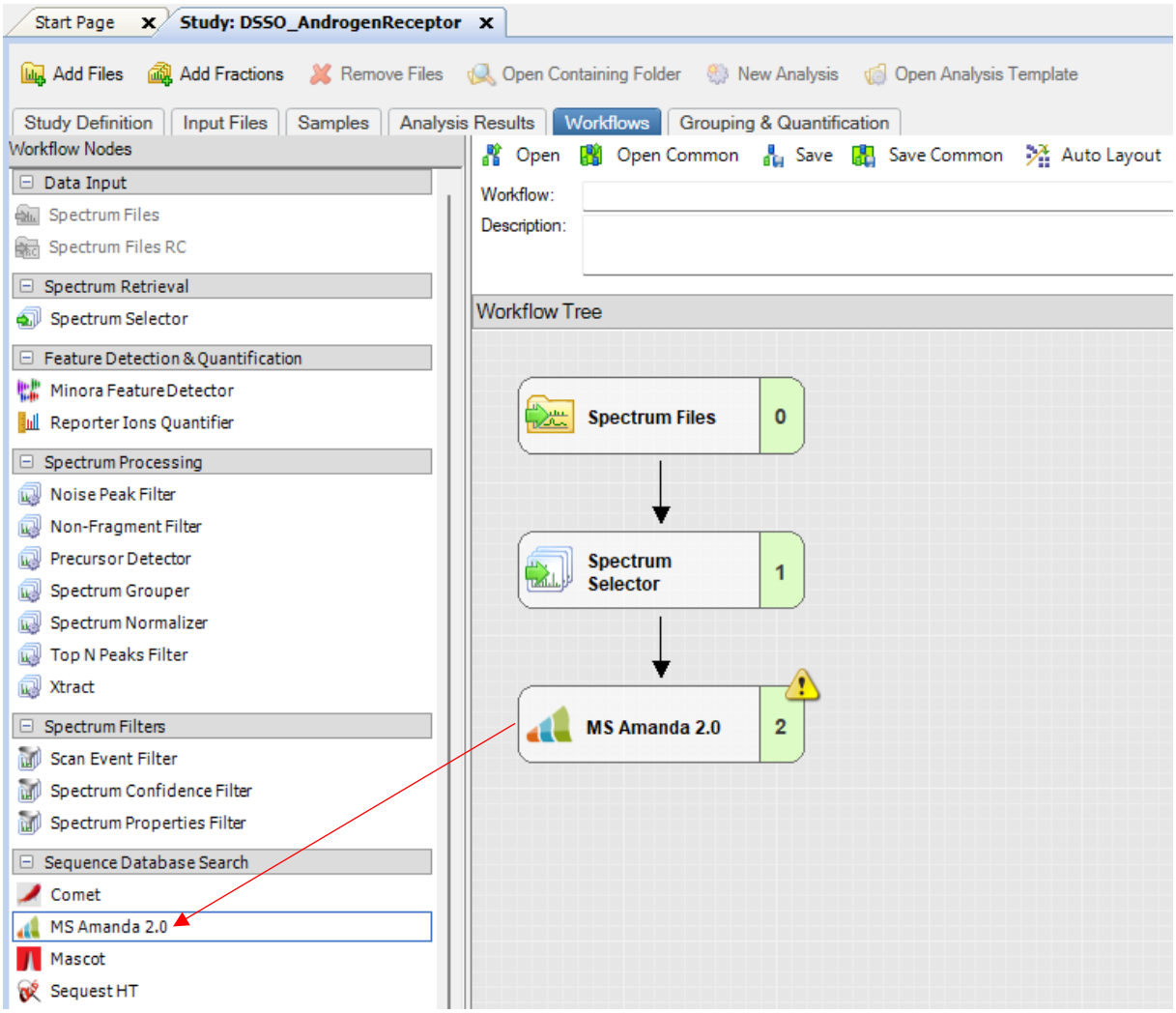
Secondly, drag-and-drop the “Spectrum Selector” node into the workflow window.




Then left click on the “Spectrum Selector” node to bring up the parameters of the node. Change “Precursor Selection” to “Use MS(n - 1) with Parent Precursors” and “Max. Precursor Mass” to “10000 Da”.

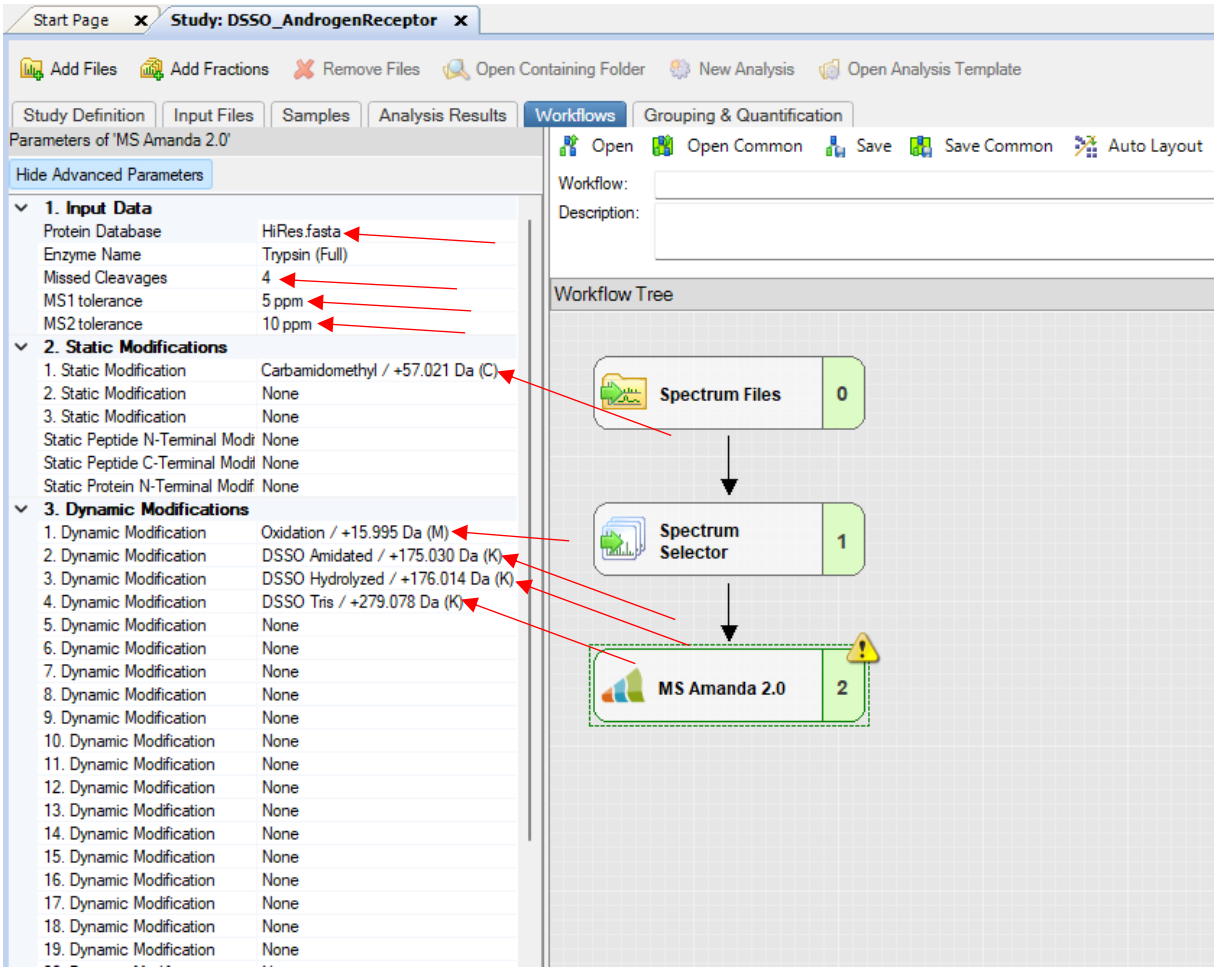


The next node we need is the "MS Amanda 2.0" node: drag-and-drop it from the "Workflow Nodes" window into the workflow pane. Connect the "Spectrum Selector" node to the "MS Amanda 2.0" node by hovering over the "Spectrum Selector" node and dragging one of the appearing white rectangles onto the "MS Amanda 2.0" node.

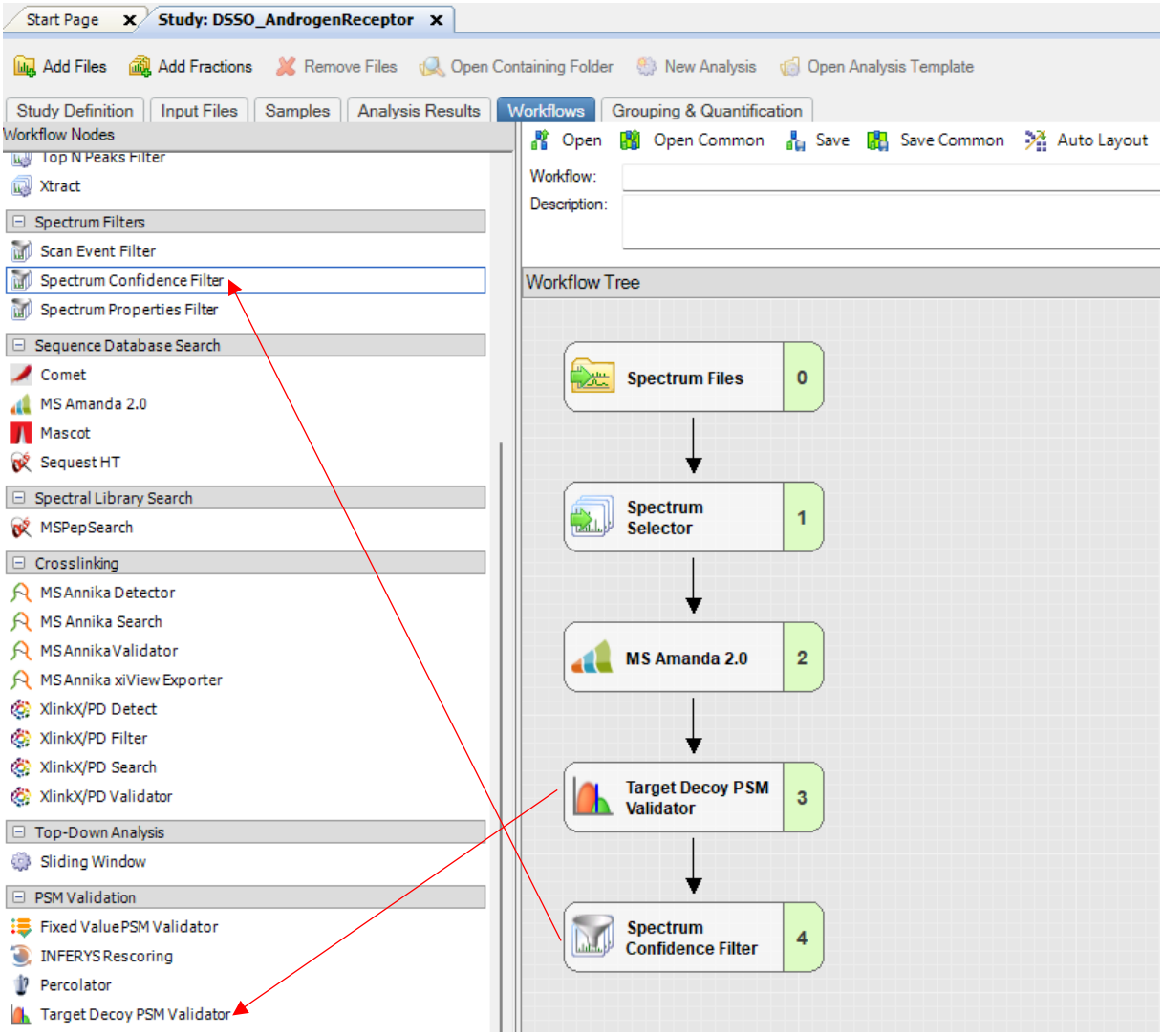



Left click the "MS Amanda 2.0" node to bring up its parameters. Click on "Show Advanced Parameters" in the top left corner of the parameter window to display all available parameters. First you would want to set the "Protein Database" which should let you select your previously imported FASTA file from a drop-down menu. Usually, we also specify a database of common contaminants here, for example as can be found here: https://www.thegpm.org/crap/. For this workflow, the imported FASTA database already contains some contaminants, so we skip this step. Secondly, specify the enzyme used for digestion in the field "Enzyme Name". Most cross-linking workflows use Trypsin, so the default is fine. The next parameter that usually needs changing is "Missed Cleavages": in cross-linking workflows the maximum number of missed cleavages considered is usually higher, in our lab we mostly consider 3 or 4 to be the maximum. The parameters "MS1 tolerance" and "MS2 tolerance" depend on the used MS instrument. With most modern MS instruments values of 5 ppm "MS1 tolerance"

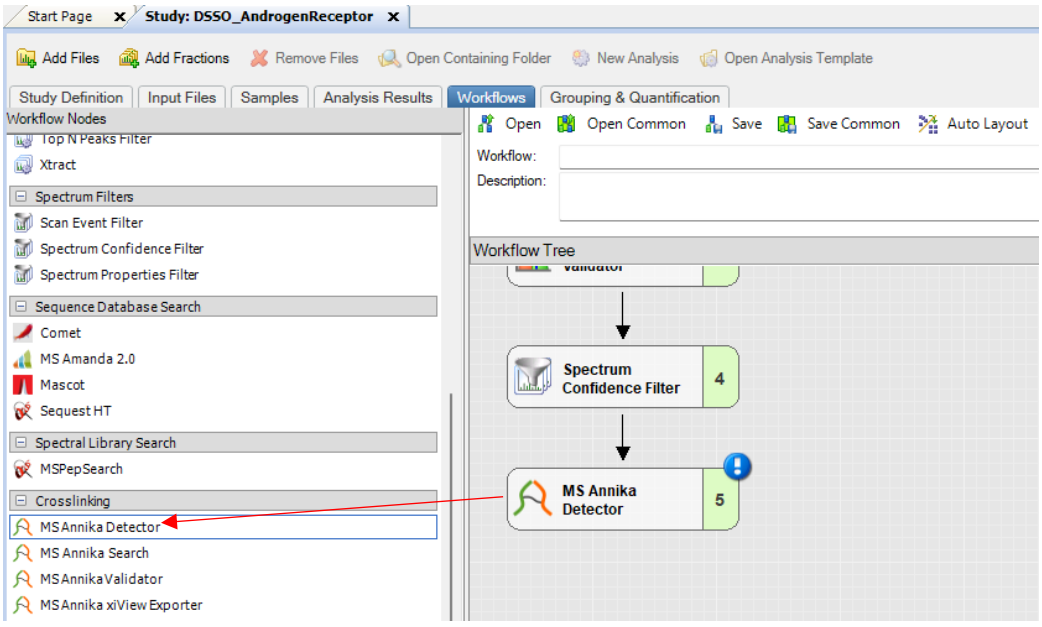
and 10 ppm “MS2 tolerance” are good choices, considering MS1 resolutions greater than/equal to 60K and MS2 resolutions greater than/equal to 30K. For older MS instruments (e.g., Q Exactive or older) or lower resolutions values of 10 ppm “MS1 tolerance” and 20 ppm “MS2 tolerance” are more suitable. Last but not least fixed and variable modifications need to be specified, this highly depends on your crosslinker and sample preparation. Every modification that is expected to be found on linear or monolinked peptides should be specified. For DSSO workflows the considered fixed modification is carbamidomethylation of Cysteine and the considered variable modifications are oxidation of Methionine as well as the monolink forms of DSSO.



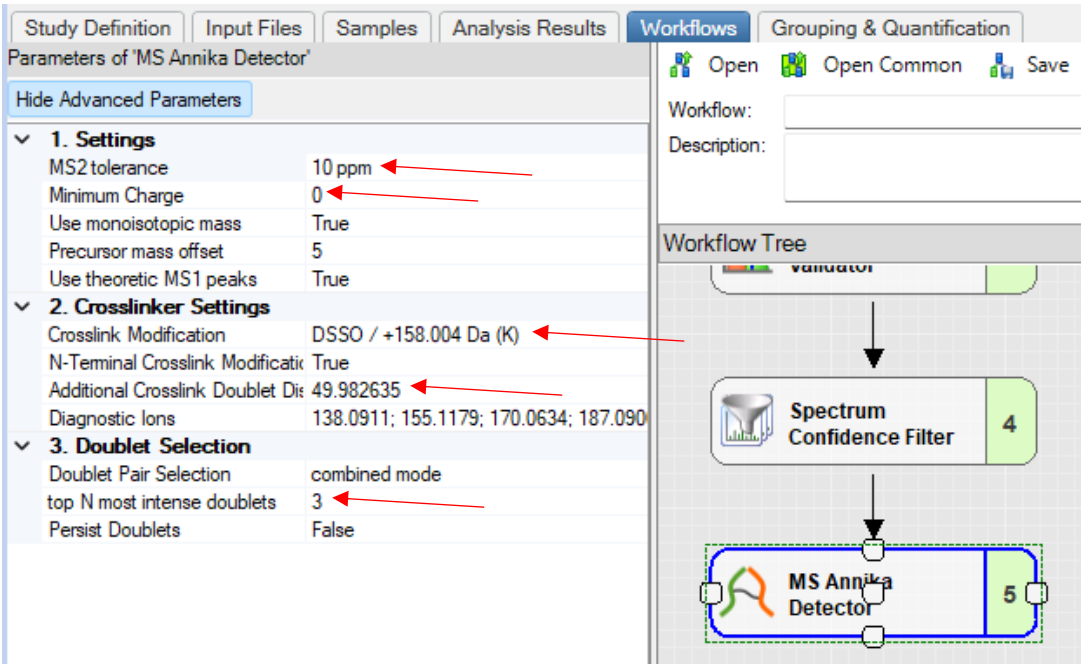

After the identification of linear and monolinked peptides the resulting peptide spectrum matches (PSMs) need to be validated, for that the “Target Decoy PSM Validator” node is added to the workflow and connected to the “MS Amanda 2.0” node. Any spectrum that has a high-confidence PSM should be filtered out and not be considered for crosslink search and we can achieve that by adding the “Spectrum Confidence Filter” node to the workflow and connecting it to the “Target Decoy PSM Validator” node. The default parameters for both nodes are usually fine, there is no need to change them.



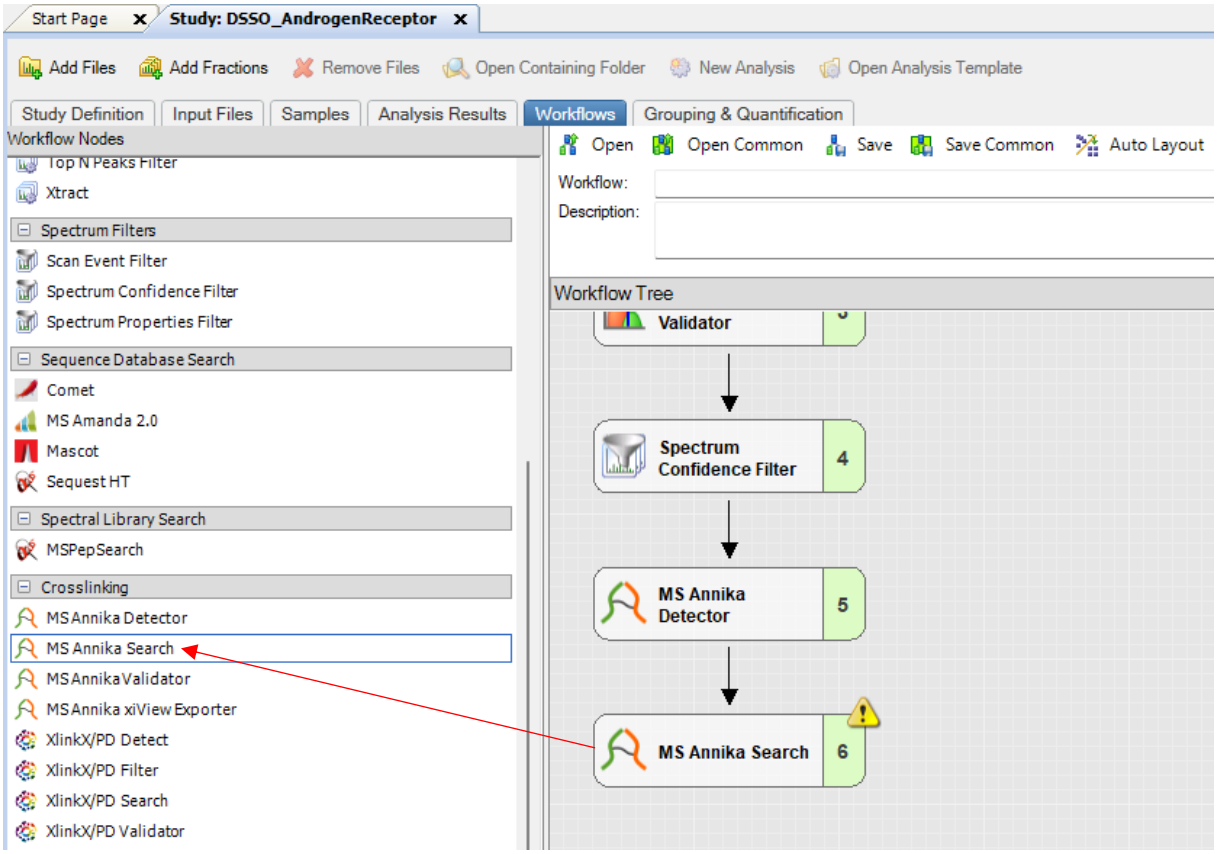

After that the components for the crosslink search are added, first and foremost the "MS Annika Detector" node which you will need to connect to the "Spectrum Confidence Filter" node.



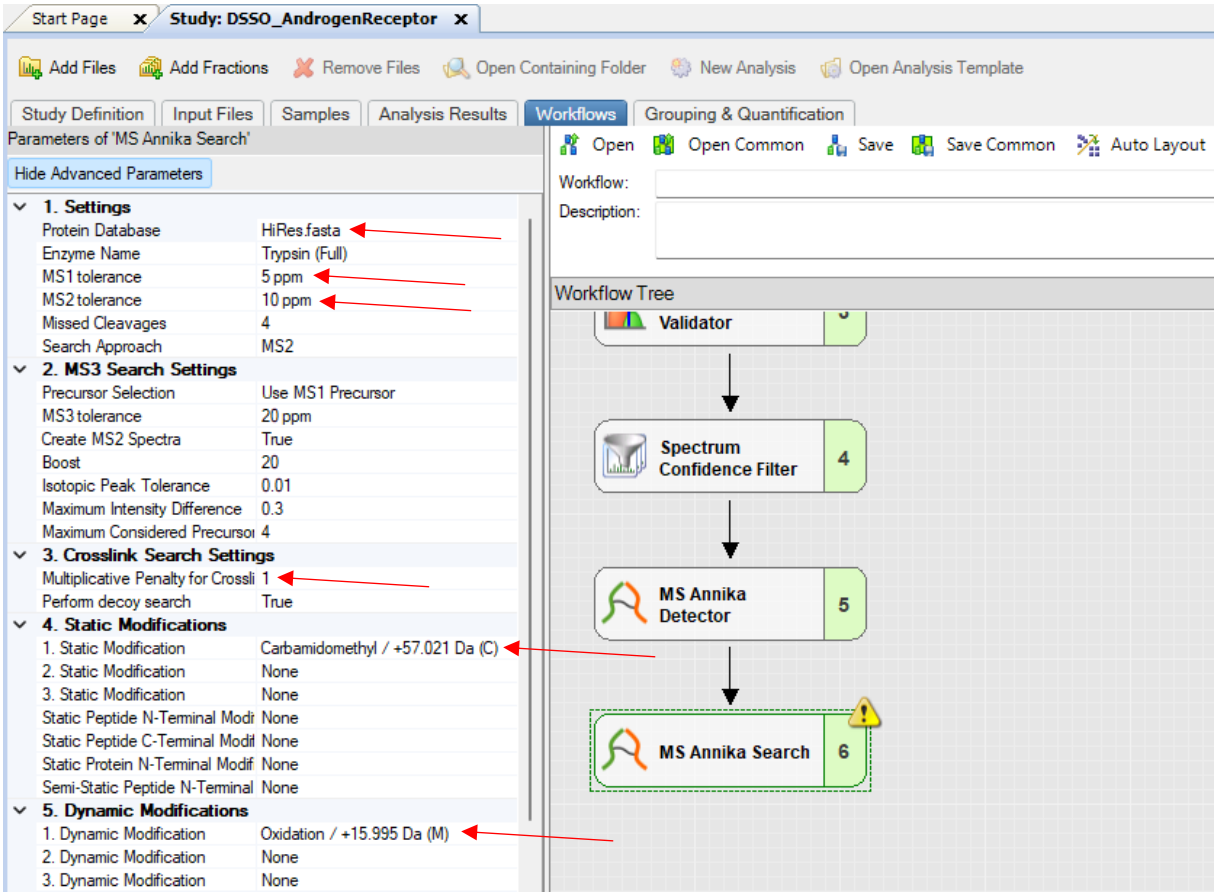
Left click on the "MS Annika Detector" node to access its parameters. The first parameter "MS2 tolerance" should be set to the value also chosen in the "MS Amanda 2.0" node, for this workflow we will use 10 ppm again. Optionally, "Minimum Charge" can be set to 0 which means that any ions with no assigned charge state will be treated as singly charged ions. Next the crosslinker needs to be specified: from the drop-down menu of the parameter "Crosslink Modification" select the crosslinker and target amino acid. If the crosslinker of your sample is not in the list you need to first define it in Proteome Discoverer, this will be covered in a later chapter of the tutorial. If the crosslinker cannot react with the peptide's N-terminus make sure to set "N-Terminal Crosslink Modification" to "False". In case your crosslinker yields more than two possible fragments after cleavage you will also need to set the parameter "Additional Crosslink Doublet Distances". This only applies to a small number of crosslinkers, for example sulfoxy-based linkers like DSSO and DSBSO. DSSO primarily yields an alkene and a thiol fragment upon cleavage that exert a mass difference of ~32 Da, however there is also a chance that the crosslinker forms an alkene and a sulfenic acid fragment upon cleavage which exert a different delta mass of ~50 Da. This second case needs to be specified in the "Additional Crosslink Doublet Distances" parameter. If you are unsure how the crosslinker in your sample fragments, please consult the crosslinker vendor or the people synthesizing the crosslinker. It is also possible to use MS Annika 2.0 for crosslinkers that yield more than three different fragments, please consult the section about defining your own crosslinker in this tutorial. The next important parameter is "Diagnostic Ions" where any masses of diagnostic ions should be set (if any are observed). For DSSO the default selection is fine. Optionally, you can increase the number of "top N most intense doublets" which controls how many of the doublets with highest intensity should be considered for search in cases where only doublets for one of the two peptides are found. In our lab we usually set this value to 3.



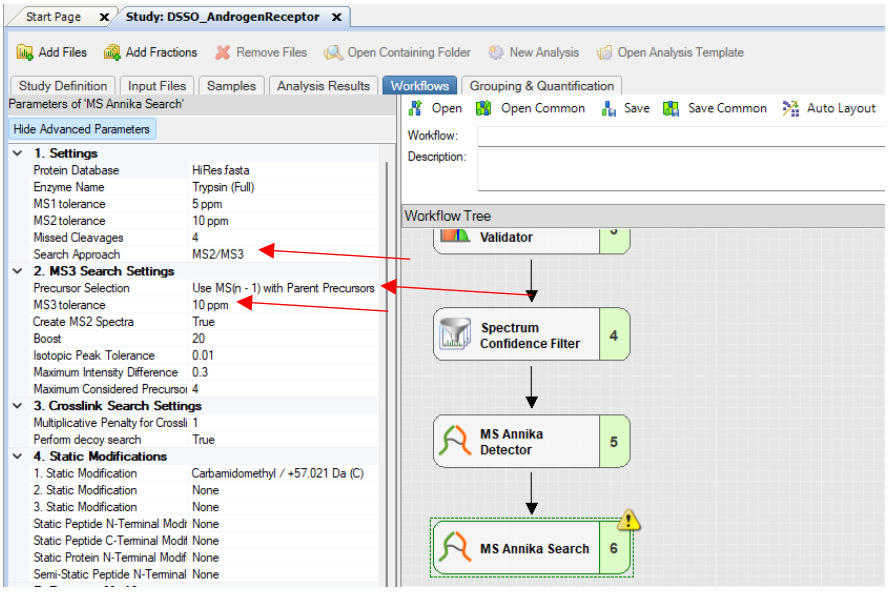
Next you need to add the "MS Annika Search" node to the workflow. It should automatically connect to the "MS Annika Detector" node.



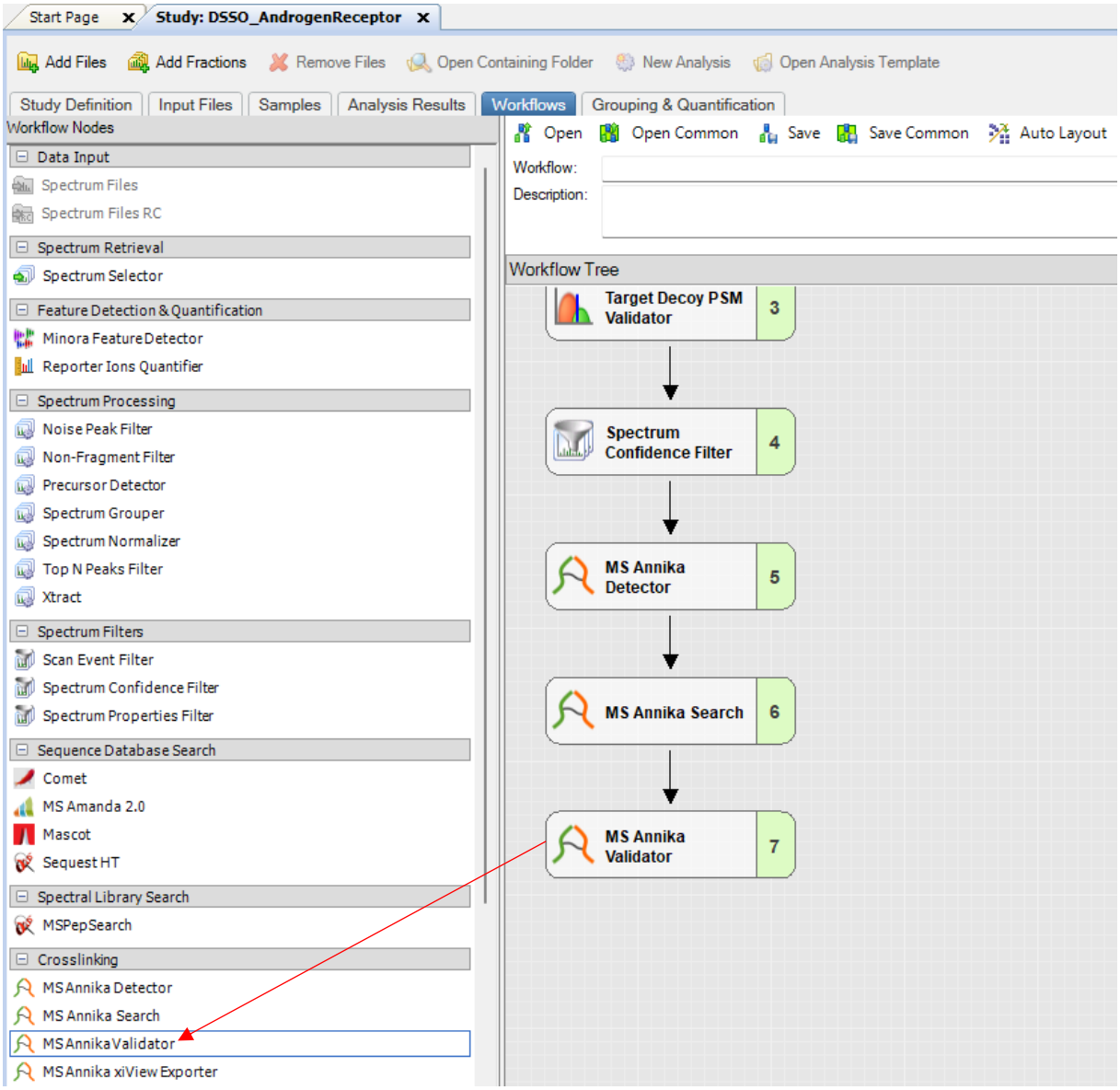

Left click on the "MS Annika Search" node to set its parameters. The "MS Annika Search" node is very similarly structured to the "MS Amanda 2.0" node. The first parameter to set is the "Protein Database". This should be the imported FASTA file that contains the sequences of all cross-linked proteins in your sample. Additionally, one should also consider adding a database of common contaminants again. The digestion enzyme needs to be set under "Enzyme Name" which for most cross-linking workflows can be left at the default of Trypsin. "MS1 tolerance" and "MS2 tolerance" are again the same as in the "MS Amanda 2.0" and the "MS Annika Detector" node. The same goes for the maximum number of "Missed Cleavages". The parameter "Search Approach" can be left at the default of "MS2" for any kind of MS2 workflows (MS2/MS2-MS2/stepped HCD MS2, CID/ETD/HCD). If MS2-MS3 acquisition was used the "Search Approach" should be set to "MS2/MS3". The next parameter that usually needs to be adjusted is "Multiplicative Penalty for Crosslinker with Equal Sequences" which usually should be set to 1 which means that crosslinks where the two peptides have the same sequence are not penalized. Finally set the fixed and variable modifications again, for standard cross-linking workflows we consider carbamidomethylation of Cysteine as a fixed modification and oxidation of Methionine as a variable modification.



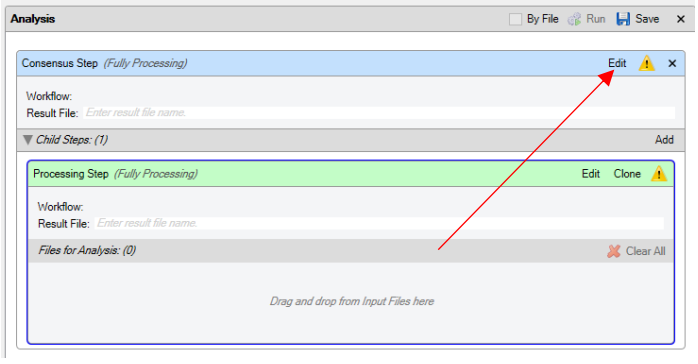

**[Settings for MS2-MS3 workflows]** If your cross-linking workflow used MS2-MS3 acquisition, some additional settings need to be considered: the parameter "Precursor Selection" should be set to the same value that was used in the "Spectrum Selector" node. It is recommended to use "Use MS(n - 1) with Parent Precursors" in both nodes. Secondly, the MS3 tolerance (fragment ion tolerance for MS3 spectra) should be adjusted to an appropriate value. For MS3 spectra recorded in the orbitrap this should be set to 10-20 ppm, for MS3 spectra recorded in the ion trap a higher tolerance like 0.5 Da is recommended.



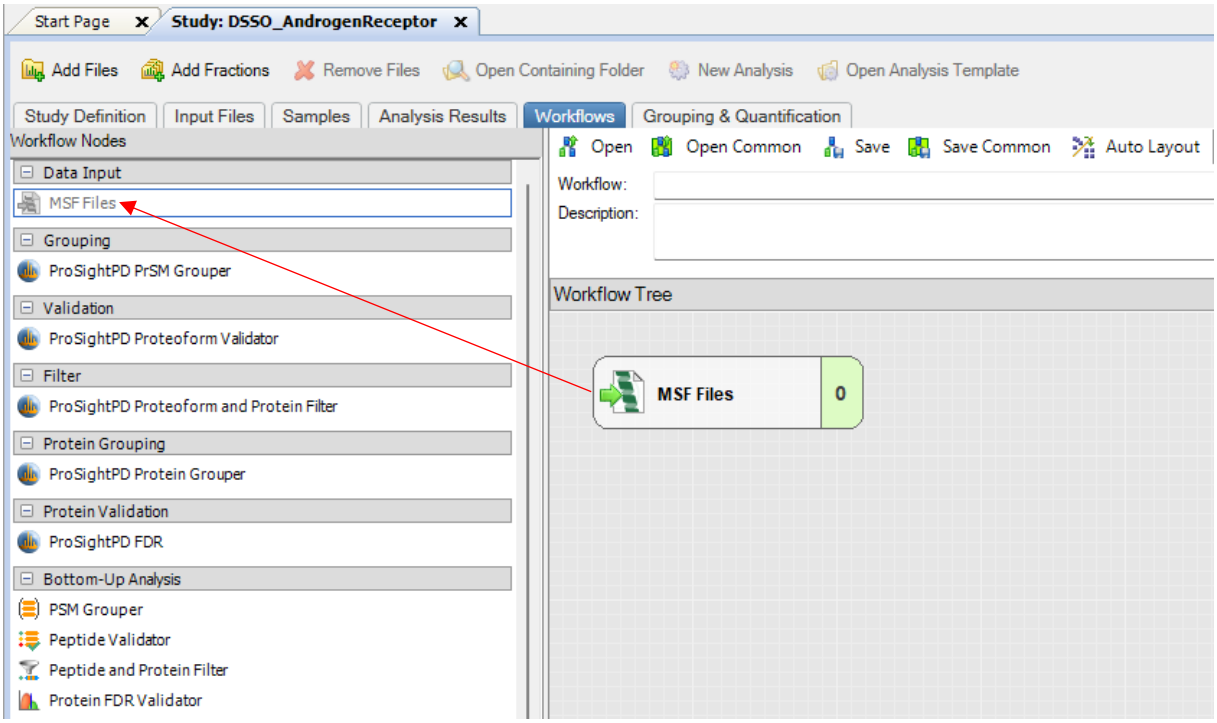
The last step in the processing workflow is to add a validator node for crosslinks and crosslink-spectrum-matches (CSMs). This is the task of the "MS Annika Validator" node. Add it to your workflow pane and it should automatically connect to the "MS Annika Search" node. No parameters need to be changed there.



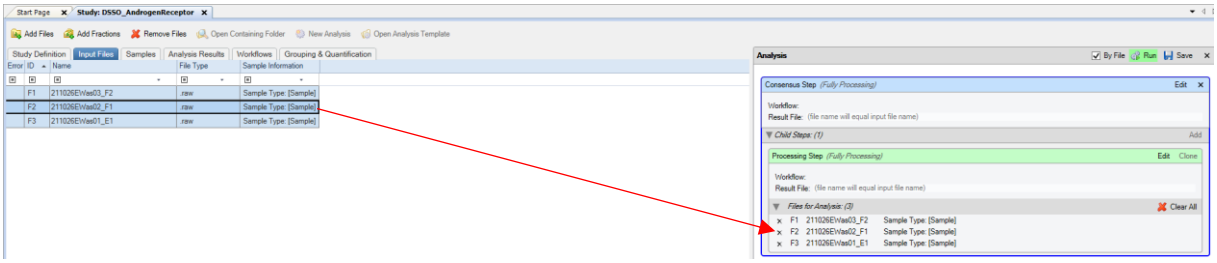

Now open the "Consensus Step" by clicking "Edit" in the top right corner of the blue box.



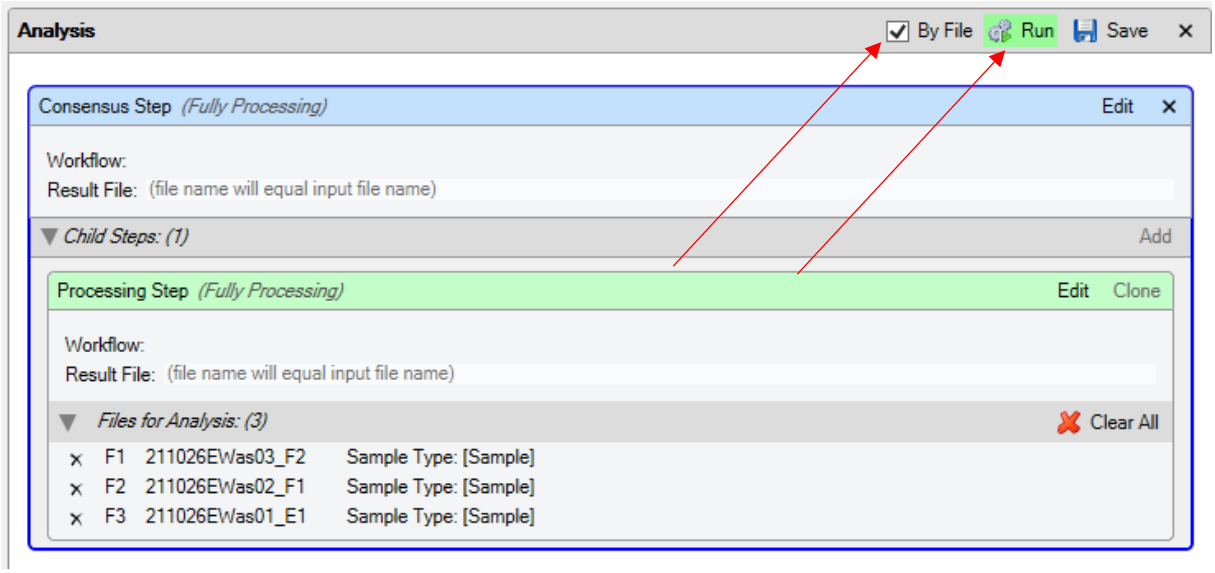
Drag-and-drop the “MSF Files” node into the “Workflow Tree” of the “Consensus Step”. That’s the minimal setup to get MS Annika 2.0 result files. Adjust the consensus workflow to your need, e.g., by opening a common consensus workflow.



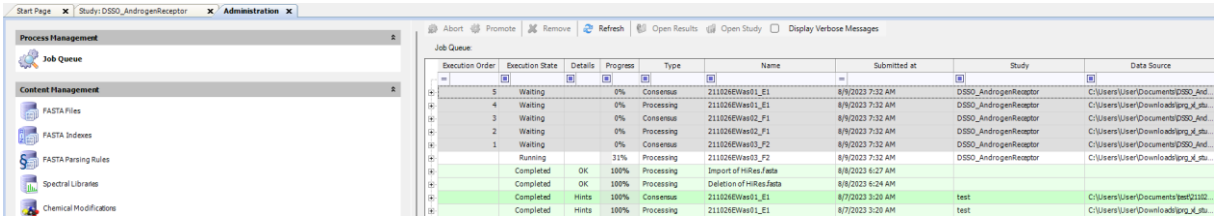

Now all that’s left to do is to add the imported MS files to the workflow. Go to the “Input Files” tab and select all files you want to analyze, then drag-and-drop them into the “Files for Analysis” area of the “Processing Step” (green box).



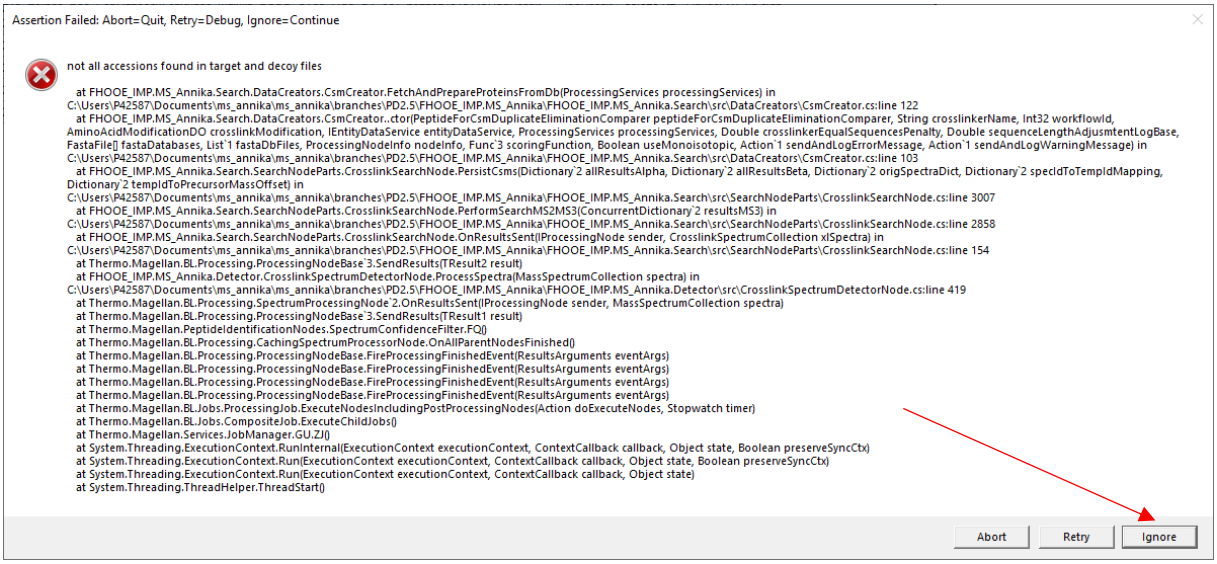
If you are analyzing technical replicates, make sure to tick the “By File” checkbox in the top right corner of the “Analysis” window. If your files are not replicates but e.g., different fractions of your chromatography you should not check this box! For this tutorial our files are technical triplicates, so we tick the “By File” checkbox. After that press “Run” in the top right corner of the “Analysis” window.




The analysis should be started and you should be redirected to the "Job Queue" view of the "Administration" window. The job queue should display the current process of the analysis. If everything works correctly the job will be highlighted in green and get the status "Completed" after it is finished.



In some rare cases (usually when the FASTA headers are not in a standard format) the job might be interrupted by the following assertion warning. If it pops up just press "Ignore" and the job will complete successfully. This issue only happens in Proteome Discoverer version <= 3.0.



## Loading a template MS Annika 2.0 analysis workflow

Analysis templates for MS Annika 2.0 can be found in the MS Annika 2.0 GitHub repository at https://github.com/hgb-bin-proteomics/MSAnnika.

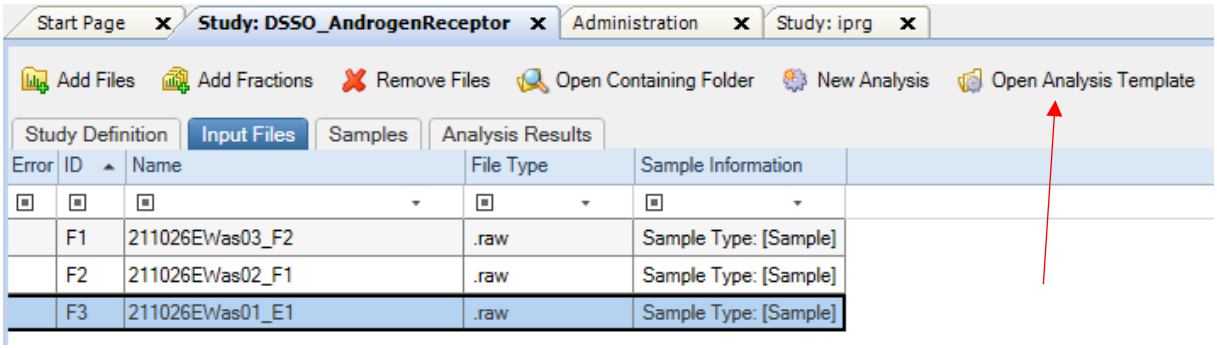
### Example Workflows

Example workflows that can be used in Proteome Discoverer*:

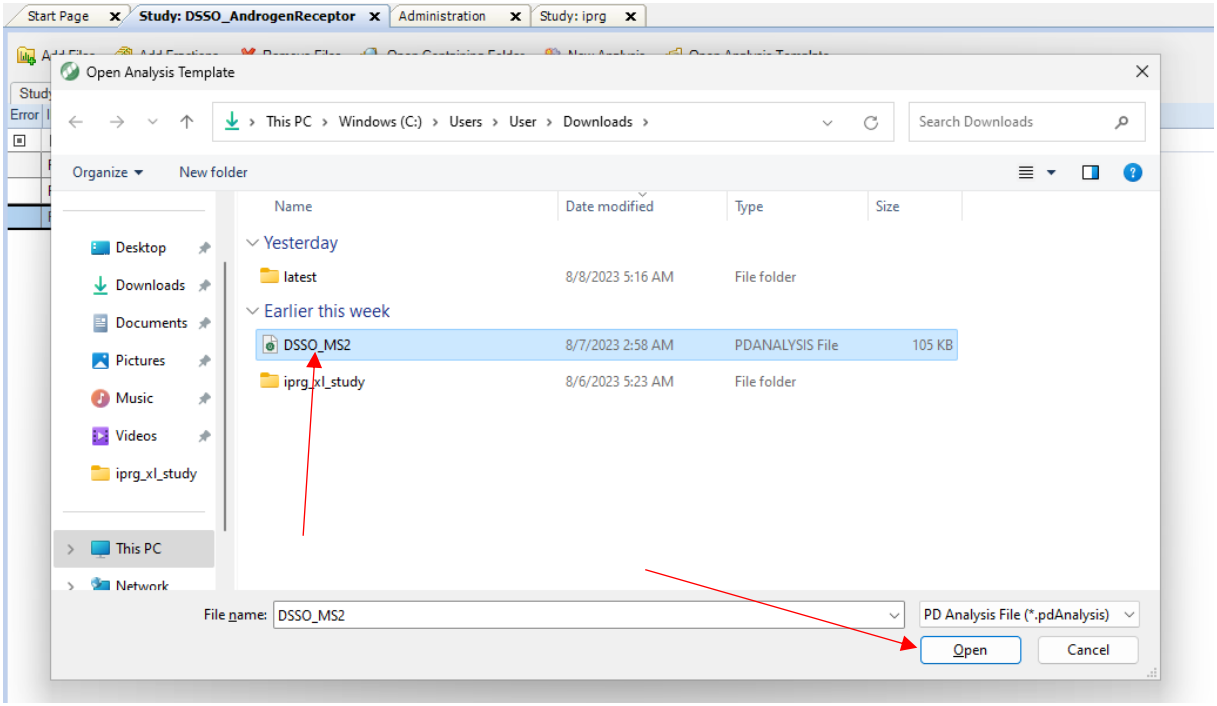
- DSSO MS2 search (CID, ETD, HCD, stepped HCD)
- DSSO MS2-MS3 search (MS3 recorded in the orbitrap)
- DSSO MS2-MS3 search (MS3 recorded in the ion trap)

*The provided workflows also require the installation of MS Amanda which can be downloaded here.

They can be optionally used ***instead*** of creating your own workflow. Download the workflow of your choice to your computer, then select "Open Analysis Template" in Proteome Discoverer. **This will close any currently open analysis!**




Selected the analysis template that you want to load and then click "Open".

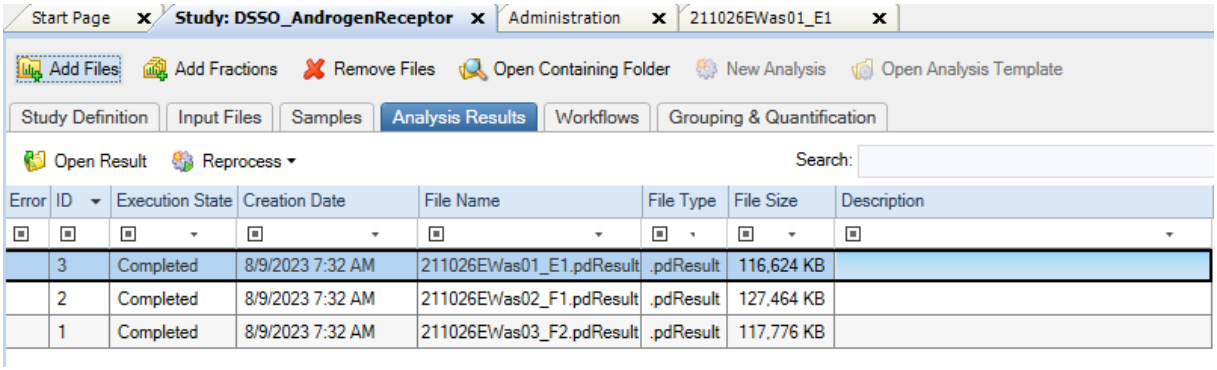


The “Analysis” window should open. You will still need to set the parameter “Protein Database” in both the “MS Amanda 2.0” and the “MS Annika Search” node. You should also check if all other parameters are suitable for you!

Lastly you need to add your MS files and start the analysis, exactly as described in the previous section.

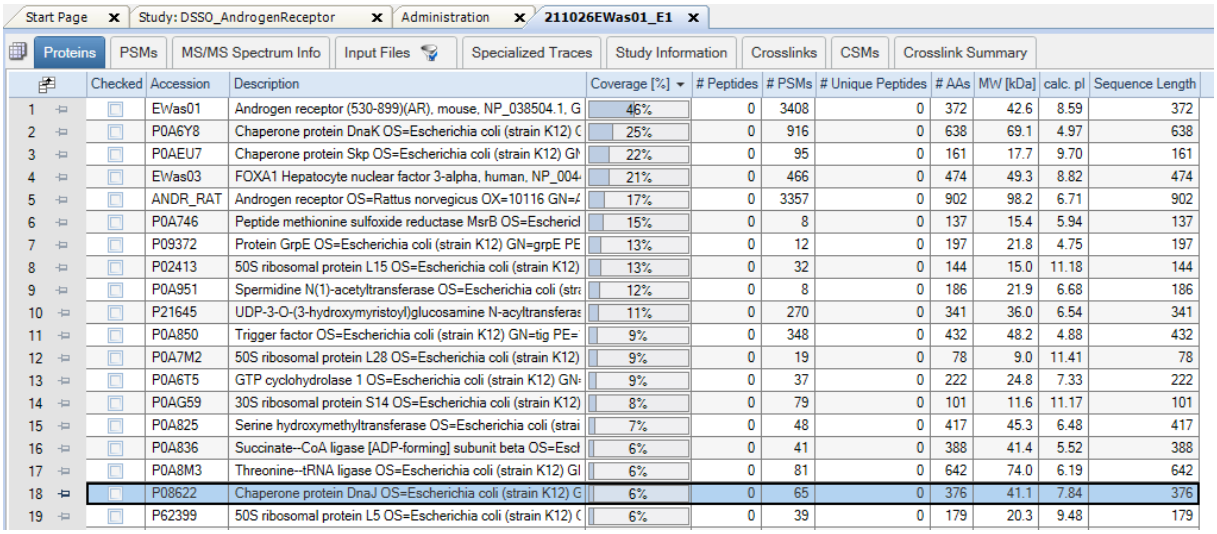
# Inspecting MS Annika 2.0 results

In the “Analysis Results” tab double click on the result that you want to open.



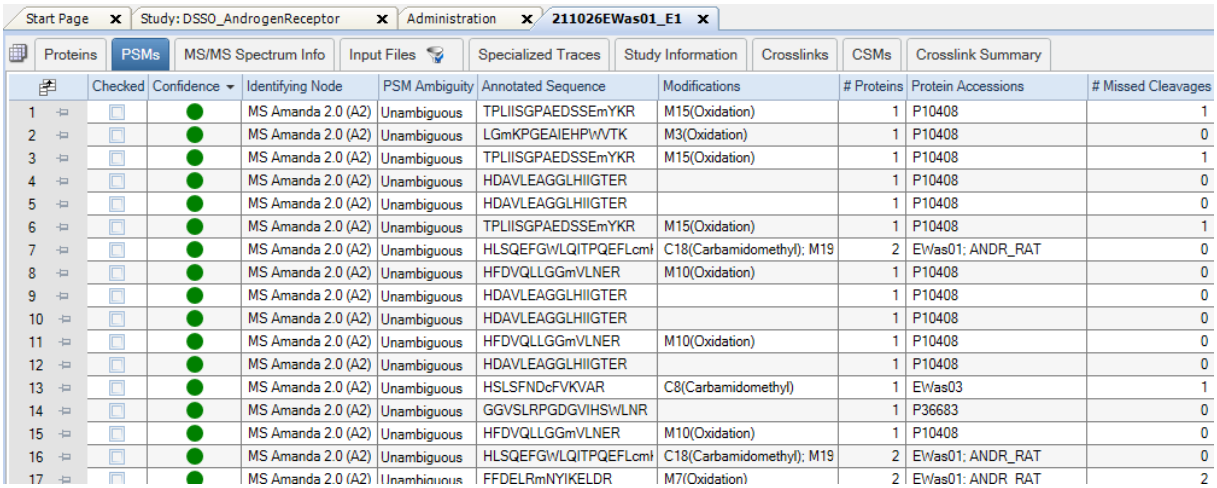

| Error | ID | Execution State | Creation Date | File Name | File Type | File Size | Description |
|---|---|---|---|---|---|---|---|
| | 3 | Completed | 8/9/2023 7:32 AM | 211026EWas01_E1.pdResult | .pdResult | 116,624 KB | |
| | 2 | Completed | 8/9/2023 7:32 AM | 211026EWas02_F1.pdResult | .pdResult | 127,464 KB | |
| | 1 | Completed | 8/9/2023 7:32 AM | 211026EWas03_F2.pdResult | .pdResult | 117,776 KB | |

A new tab with the file name should open. By default, it will display the “Proteins” tab which shows protein coverage.



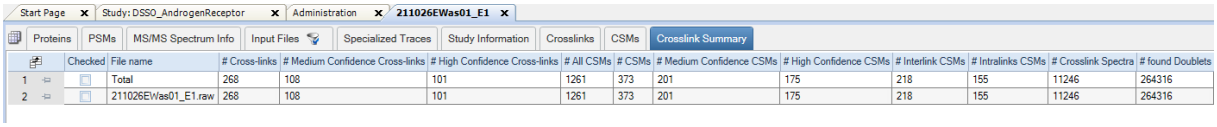

| | Checked | Accession | Description | Coverage [%] | # Peptides | # PSMs | # Unique Peptides | # AAs | MW [kDa] | calc. pI | Sequence Length |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | EWas01 | Androgen receptor (530-899)(AR), mouse, NP_038504.1, G | 46% | 0 | 3408 | 0 | 372 | 42.6 | 8.59 | 372 |
| 2 | | P0A6Y8 | Chaperone protein DnaK OS=Escherichia coli (strain K12) ( | 25% | 0 | 916 | 0 | 638 | 69.1 | 4.97 | 638 |
| 3 | | P0AEU7 | Chaperone protein Skp OS=Escherichia coli (strain K12) GI | 22% | 0 | 95 | 0 | 161 | 17.7 | 9.70 | 161 |
| 4 | | EWas03 | FOXA1 Hepatocyte nuclear factor 3-alpha, human, NP_004- | 21% | 0 | 466 | 0 | 474 | 49.3 | 8.82 | 474 |
| 5 | | ANDR_RAT | Androgen receptor OS=Rattus norvegicus OX=10116 GN=A | 17% | 0 | 3357 | 0 | 902 | 98.2 | 6.71 | 902 |
| 6 | | P0A746 | Peptide methionine sulfoxide reductase MsrB OS=Escherici | 15% | 0 | 8 | 0 | 137 | 15.4 | 5.94 | 137 |
| 7 | | P09372 | Protein GrpE OS=Escherichia coli (strain K12) GN=grpE PE | 13% | 0 | 12 | 0 | 197 | 21.8 | 4.75 | 197 |
| 8 | | P02413 | 50S ribosomal protein L15 OS=Escherichia coli (strain K12) | 13% | 0 | 32 | 0 | 144 | 15.0 | 11.18 | 144 |
| 9 | | P0A951 | Spermidine N(1)-acetyltransferase OS=Escherichia coli (str | 12% | 0 | 8 | 0 | 186 | 21.9 | 6.68 | 186 |
| 10 | | P21645 | UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferas | 11% | 0 | 270 | 0 | 341 | 36.0 | 6.54 | 341 |
| 11 | | P0A850 | Trigger factor OS=Escherichia coli (strain K12) GN=tig PE= | 9% | 0 | 348 | 0 | 432 | 48.2 | 4.88 | 432 |
| 12 | | P0A7M2 | 50S ribosomal protein L28 OS=Escherichia coli (strain K12) | 9% | 0 | 19 | 0 | 78 | 9.0 | 11.41 | 78 |
| 13 | | P0A6T5 | GTP cyclohydrolase 1 OS=Escherichia coli (strain K12) GN | 9% | 0 | 37 | 0 | 222 | 24.8 | 7.33 | 222 |
| 14 | | P0AG59 | 30S ribosomal protein S14 OS=Escherichia coli (strain K12) | 8% | 0 | 79 | 0 | 101 | 11.6 | 11.17 | 101 |
| 15 | | P0A825 | Serine hydroxymethyltransferase OS=Escherichia coli (strai | 7% | 0 | 48 | 0 | 417 | 45.3 | 6.48 | 417 |
| 16 | | P0A836 | Succinate--CoA ligase [ADP-forming] subunit beta OS=Escl | 6% | 0 | 41 | 0 | 388 | 41.4 | 5.52 | 388 |
| 17 | | P0A8M3 | Threonine--tRNA ligase OS=Escherichia coli (strain K12) GI | 6% | 0 | 81 | 0 | 642 | 74.0 | 6.19 | 642 |
| 18 | | P08622 | Chaperone protein DnaJ OS=Escherichia coli (strain K12) G | 6% | 0 | 65 | 0 | 376 | 41.1 | 7.84 | 376 |
| 19 | | P62399 | 50S ribosomal protein L5 OS=Escherichia coli (strain K12) ( | 6% | 0 | 39 | 0 | 179 | 20.3 | 9.48 | 179 |

The PSMs tab gives an overview of PSMs of both the linear, monolink and crosslink search.



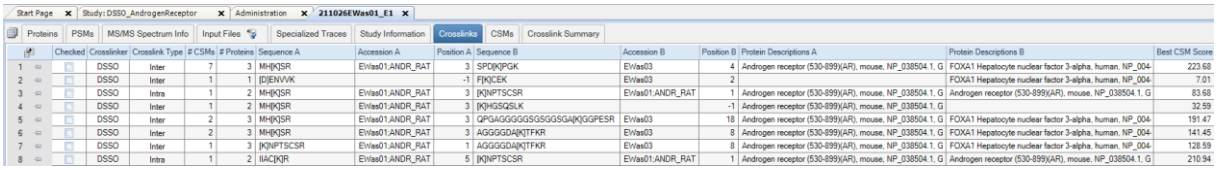

| | Checked | Confidence | Identifying Node | PSM Ambiguity | Annotated Sequence | Modifications | # Proteins | Protein Accessions | # Missed Cleavages |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | | MS Amanda 2.0 (A2) | Unambiguous | TPLIISGPAEDSSEmYKR | M15(Oxidation) | 1 | P10408 | 1 |
| 2 | | | MS Amanda 2.0 (A2) | Unambiguous | LGmKPGEAIEHPWVTK | M3(Oxidation) | 1 | P10408 | 0 |
| 3 | | | MS Amanda 2.0 (A2) | Unambiguous | TPLIISGPAEDSSEmYKR | M15(Oxidation) | 1 | P10408 | 1 |
| 4 | | | MS Amanda 2.0 (A2) | Unambiguous | HDAVLEAGGLHIIGTER | | 1 | P10408 | 0 |
| 5 | | | MS Amanda 2.0 (A2) | Unambiguous | HDAVLEAGGLHIIGTER | | 1 | P10408 | 0 |
| 6 | | | MS Amanda 2.0 (A2) | Unambiguous | TPLIISGPAEDSSEmYKR | M15(Oxidation) | 1 | P10408 | 1 |
| 7 | | | MS Amanda 2.0 (A2) | Unambiguous | HLSQEFGWLQITPQEFLcmK | C18(Carbamidomethyl); M19 | 2 | EWas01; ANDR_RAT | 0 |
| 8 | | | MS Amanda 2.0 (A2) | Unambiguous | HFDVQLLGGmVLNER | M10(Oxidation) | 1 | P10408 | 0 |
| 9 | | | MS Amanda 2.0 (A2) | Unambiguous | HDAVLEAGGLHIIGTER | | 1 | P10408 | 0 |
| 10 | | | MS Amanda 2.0 (A2) | Unambiguous | HDAVLEAGGLHIIGTER | | 1 | P10408 | 0 |
| 11 | | | MS Amanda 2.0 (A2) | Unambiguous | HFDVQLLGGmVLNER | M10(Oxidation) | 1 | P10408 | 0 |
| 12 | | | MS Amanda 2.0 (A2) | Unambiguous | HDAVLEAGGLHIIGTER | | 1 | P10408 | 0 |
| 13 | | | MS Amanda 2.0 (A2) | Unambiguous | HSLSFNDcFVKVAR | C8(Carbamidomethyl) | 1 | EWas03 | 1 |
| 14 | | | MS Amanda 2.0 (A2) | Unambiguous | GGVSLRPGDGVIHSWLNR | | 1 | P36683 | 0 |
| 15 | | | MS Amanda 2.0 (A2) | Unambiguous | HFDVQLLGGmVLNER | M10(Oxidation) | 1 | P10408 | 0 |
| 16 | | | MS Amanda 2.0 (A2) | Unambiguous | HLSQEFGWLQITPQEFLcmK | C18(Carbamidomethyl); M19 | 2 | EWas01; ANDR_RAT | 0 |
| 17 | | | MS Amanda 2.0 (A2) | Unambiguous | FFDELRmNYIKELDR | M7(Oxidation) | 2 | EWas01; ANDR_RAT | 2 |

The “Crosslink Summary” gives an overview of how many crosslinks were identified, how many of them fall within the 1% estimated FDR threshold (High Confidence) and how many of them fall within the 5% estimated FDR threshold (Medium Confidence). It also displays these numbers for CSMs. Additionally, the number of crosslink spectra and the number of identified doublets is shown.

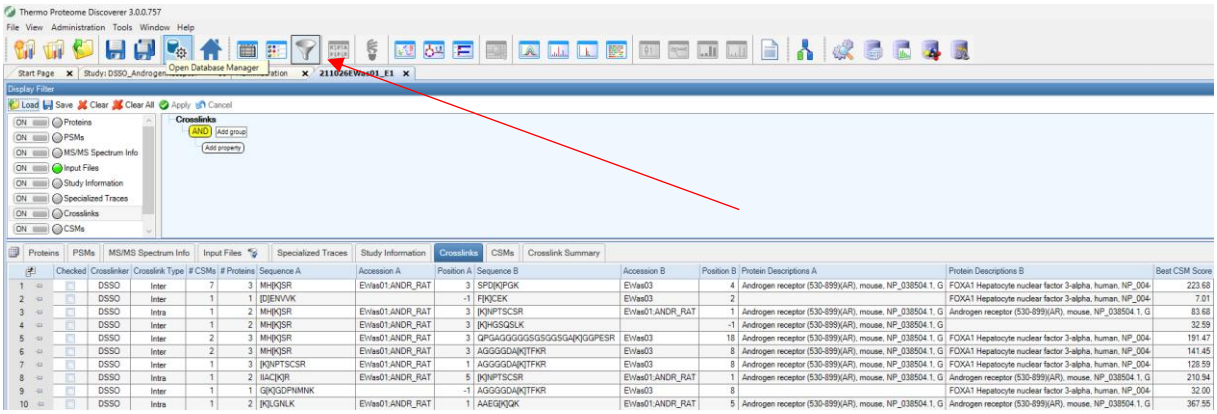


| | Checked | File name | # Cross-links | # Medium Confidence Cross-links | # High Confidence Cross-links | # All CSMs | # CSMs | # Medium Confidence CSMs | # High Confidence CSMs | # Interlink CSMs | # Intralinks CSMs | # Crosslink Spectra | # found Doublets |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | Total | 268 | 108 | 101 | 1261 | 373 | 201 | 175 | 218 | 155 | 11246 | 264316 |
| 2 | | 211026EWas01_E1.raw | 268 | 108 | 101 | 1261 | 373 | 201 | 175 | 218 | 155 | 11246 | 264316 |

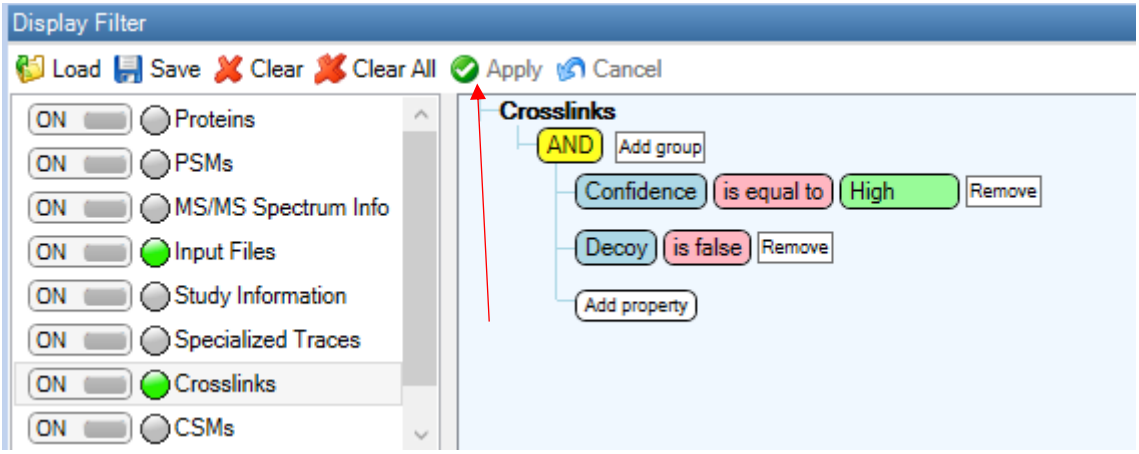
The most important crosslink results are in the “Crosslinks” and “CSMs” tab. The crosslinks tab features a list of all identified crosslinks that can be filtered for high and medium confidence crosslinks.



A filter can be applied by selecting the cone item at the top of Proteome Discoverer which should open the “Display Filter” window.

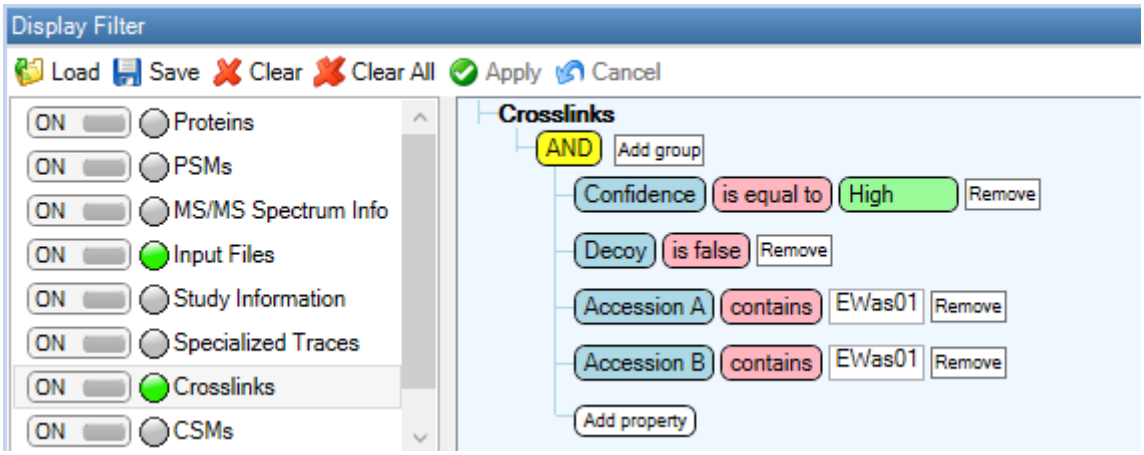


We are going to filter for high confidence crosslinks without decoys by applying “Confidence is equal to High” and “Decoy is false”.

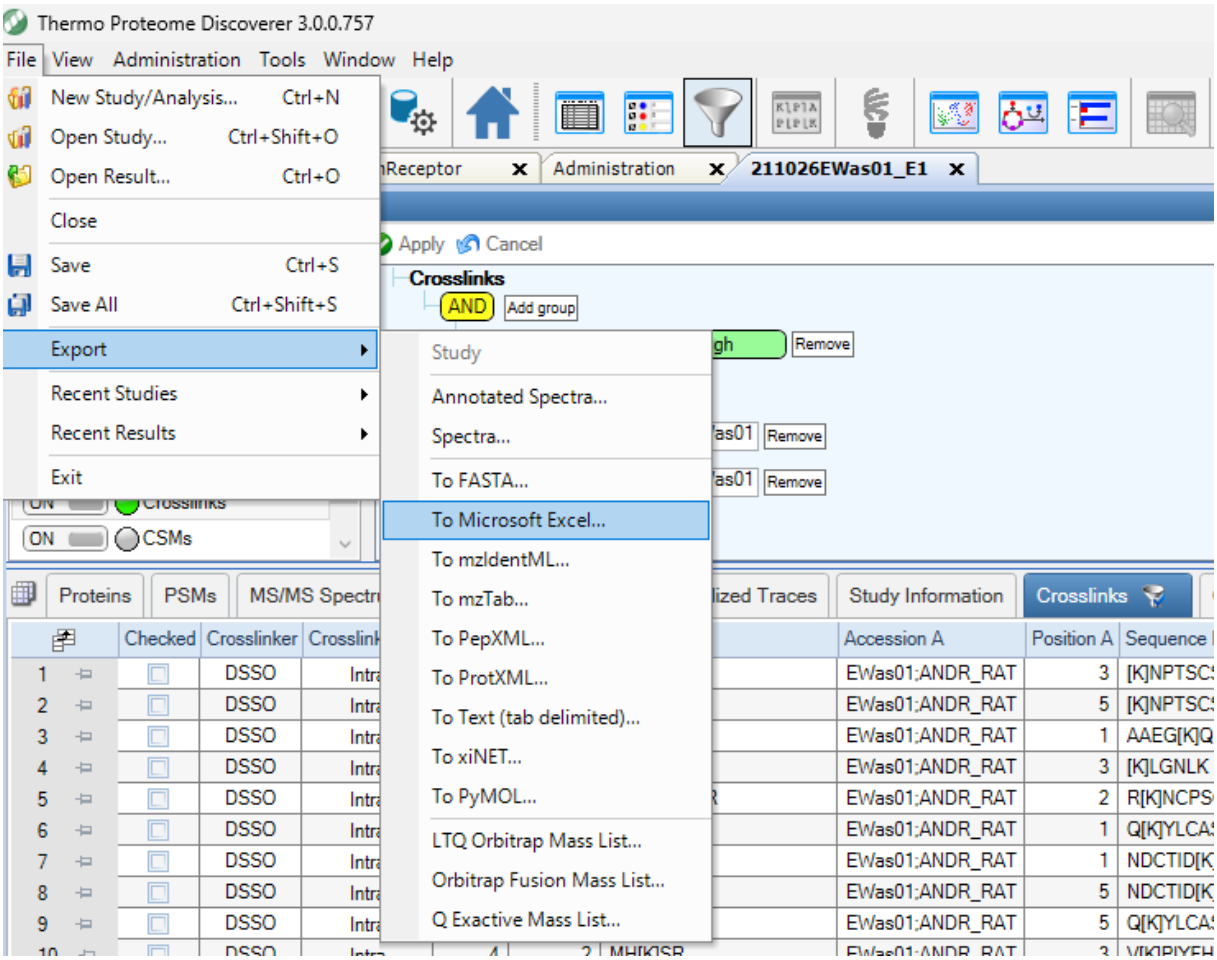


The resulting list of crosslinks contains only high confidence (1% estimated FDR) target-target hits. These can be exported for further analysis.

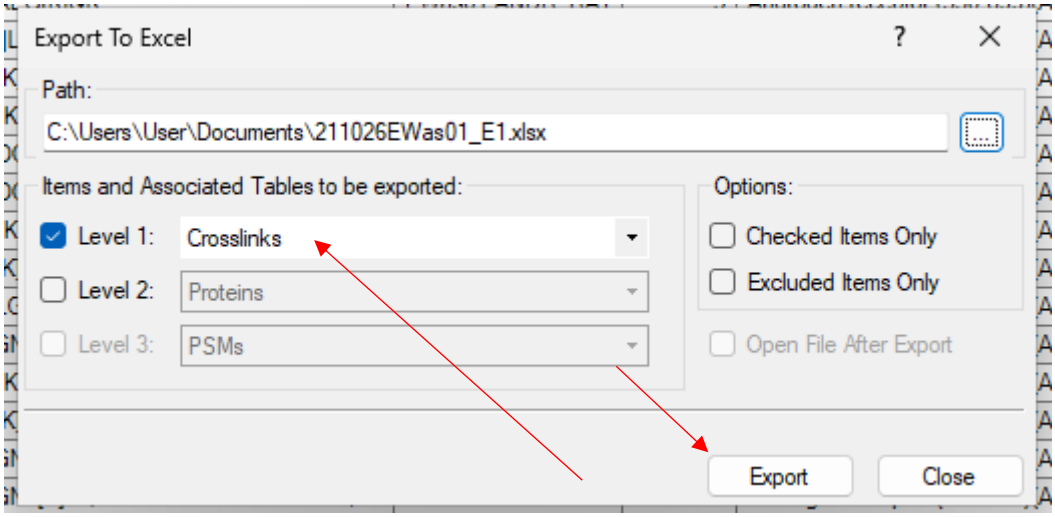
For this tutorial we will also filter out any crosslinks that are not within the mouse androgen receptor because we want to analyze those further later. Applying the filter option "Accession A contains EWas01" and "Accession B contains EWas01" will achieve this.



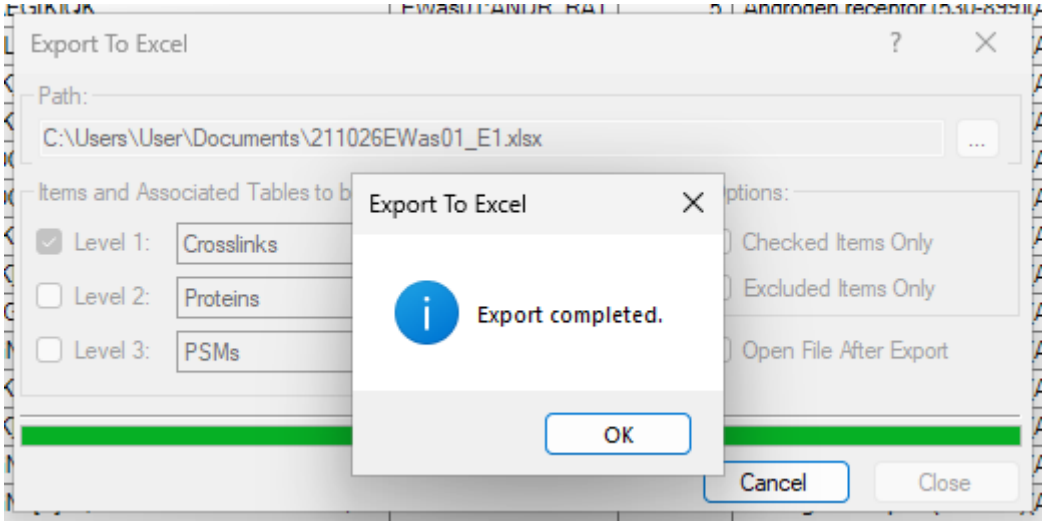

We can export the remaining crosslinks by clicking "File" > "Export" in the top left corner of Proteome Discoverer. We will export to Microsoft Excel format.



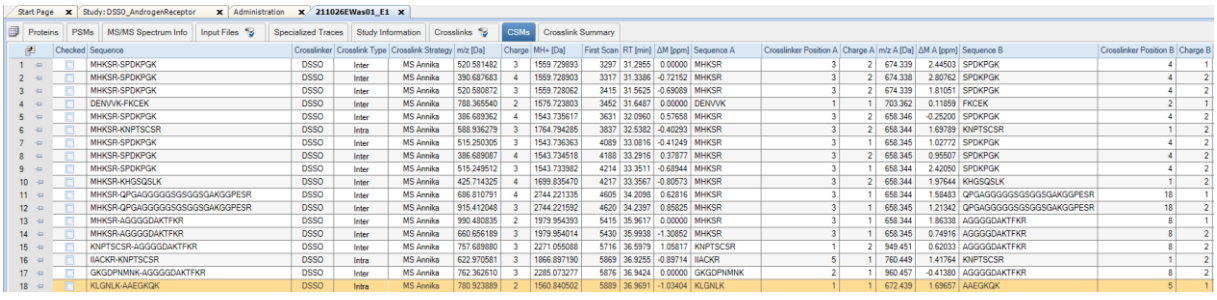
Select the path and which table to export. By default the "Proteins" table is pre-selected, make sure to export the "Crosslinks" table and click "Export".




If everything works correctly an "Export completed." should pop up.




Another important table is the "CSMs" table that shows which crosslink is associated to which spectrum.



## Down-stream analysis of MS Annika 2.0 results

We provide a variety of scripts that aid in the down-stream analysis of MS Annika 2.0 results. Most of them are based on the exported Microsoft Excel file from the previous section. An overview and download options for all scripts is given in the GitHub repository https://github.com/hgb-bin-proteomics/MSAnnika_exporters.

Currently scripts for the export to the following tools are provided:

- xiNET: https://crosslinkviewer.org/
- xiVIEW: https://xiview.org/xiNET_website/index.php
- PyXlinkViewer (pyMOL plugin): https://github.com/BobSchiffrin/PyXlinkViewer
- XMAS (ChimeraX plugin): https://github.com/ScheltemaLab/ChimeraX_bundle
- PAE Viewer: http://www.subtiwiki.uni-goettingen.de/v4/paeViewerDemo

### Quick start

- **Exporting to xiNET**
  Files needed:
  - result.xlsx - MS Annika result file(s) exported to .xlsx
  - seq.fasta - FASTA file containing sequences of the crosslinked proteins

```
python xiNetExporter_msannika.py result.xlsx -fasta seq.fasta
```

- **Exporting to xiVIEW**
  Files needed:
  - result.xlsx - MS Annika result file(s) exported to .xlsx
  - seq.fasta - FASTA file containing sequences of the crosslinked proteins

```
python xiViewExporter_msannika.py result.xlsx -fasta seq.fasta
```

- **Exporting to pyXlinkViewer (pyMOL)**
  Files needed:
  - result.xlsx - MS Annika result file(s) exported to .xlsx
  - structure.pdb - 3D structure of the protein (complex) that crosslinks should be mapped to, alternatively you can also just provide the 4-letter code from the PDB and the script will fetch the structure from internet

```
python pyXlinkViewerExporter_msannika.py result.xlsx -pdb structure.pdb
```

- **Exporting to XMAS (ChimeraX)**
  Visualization of MS Annika results works out of the box with .xlsx files exported from Proteome Discoverer.
- **Exporting to PAE Viewer**
  Files needed:
  - pyXlinkViewer_export.csv - Crosslinks exported from pyXlinkViewer as .csv

```
python PAEViewerExporter_msannika.py pyXlinkViewer_export.csv
```

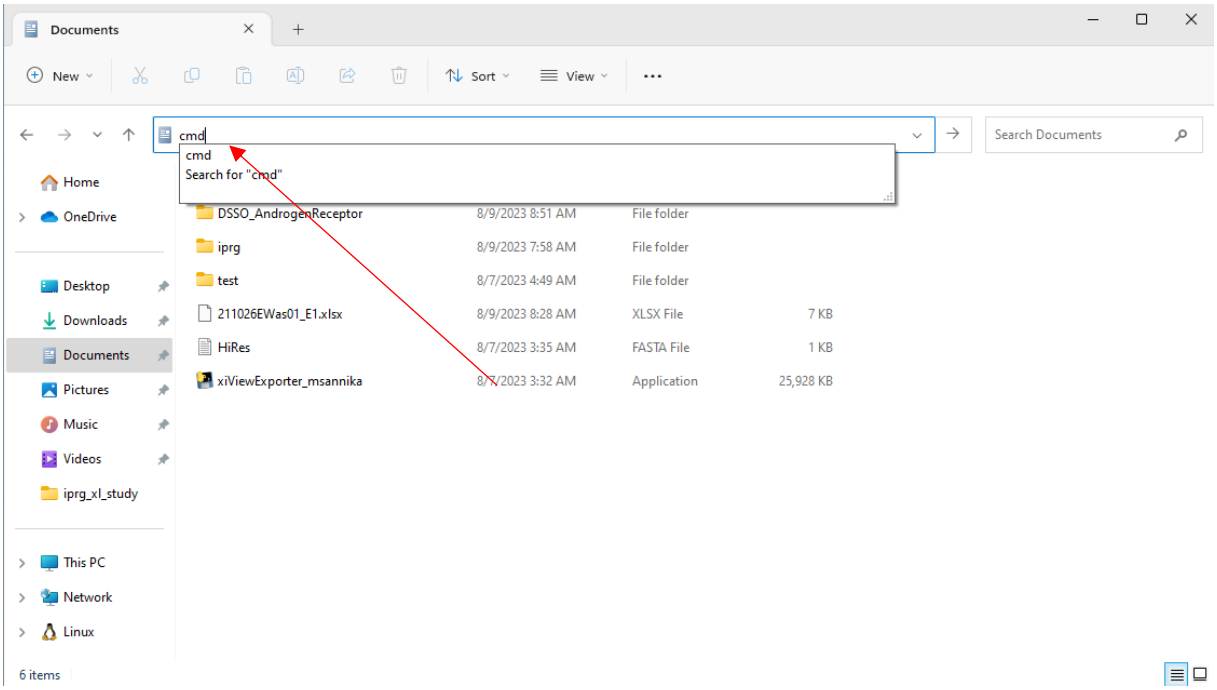
## Example: Export to xiVIEW

This section describes how the previously created Microsoft Excel file of crosslinks identified by MS Annika 2.0 can be used to visualize crosslinks in xiVIEW. The export process is similar for all exporter scripts and should be easily transferable.

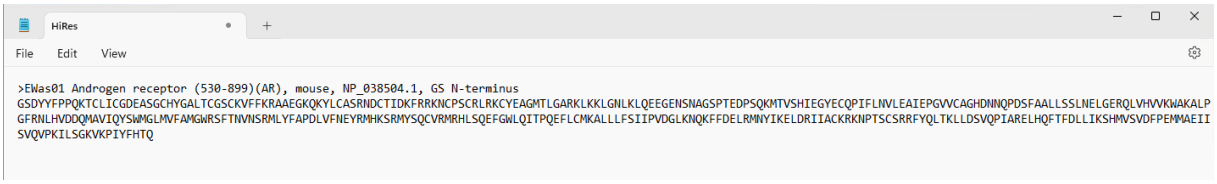
First download the xiVIEW exporter (*xiViewExporter_msannika.exe*) from the GitHub repository https://github.com/hgb-bin-proteomics/MSAnnika_exporters/releases.

Alternatively, you can also download the exporter as python script, the process is the same but obviously knowledge about running python scripts is required. The tutorial will only cover running the executable!

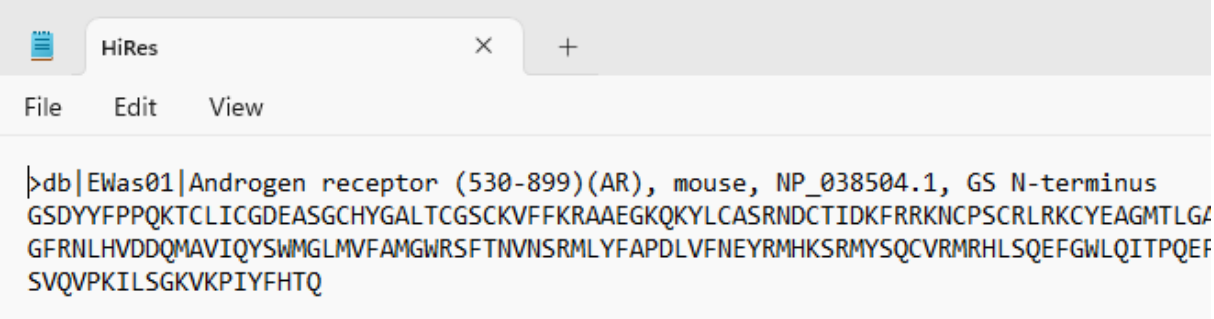
After downloading the exporter, make sure to put the exporter, the used FASTA file and the exported Microsoft Excel file into the same folder. Subsequently open a command line window in that folder. In Windows 10/11 that is for example possible by typing "cmd" into the path.



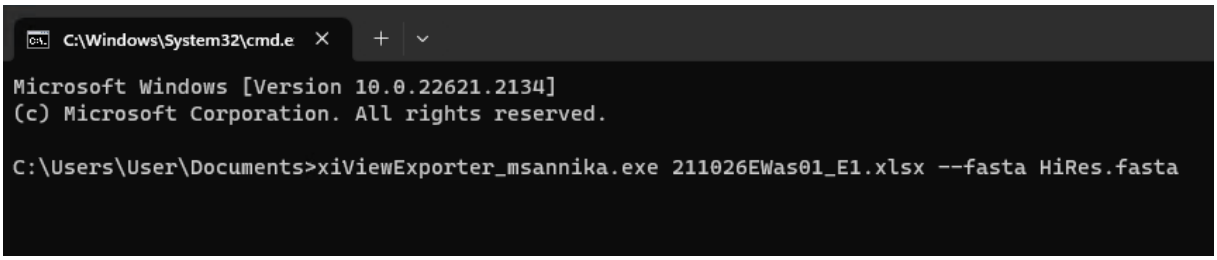

Now before we run the exporter from the command line window, we need to modify the FASTA file. We only want to analyze crosslinks of the mouse androgen receptor, so we are going to delete all other sequences from the FASTA file. After the other sequences are deleted, the FASTA file should look like this:



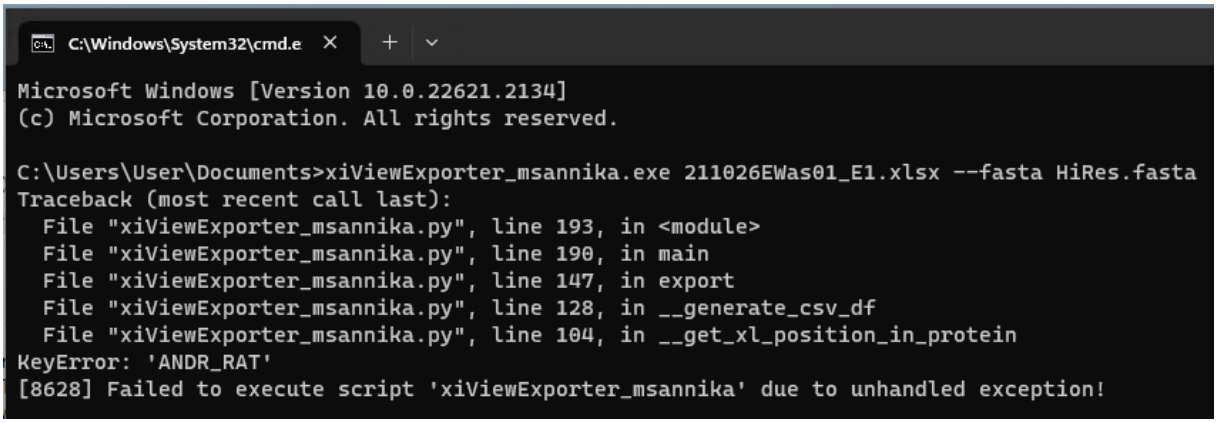
As mentioned in the documentation of the xiVIEW exporter, the FASTA needs to conform to UniProtKB standard formatting, so we still need to change the FASTA header to the following:



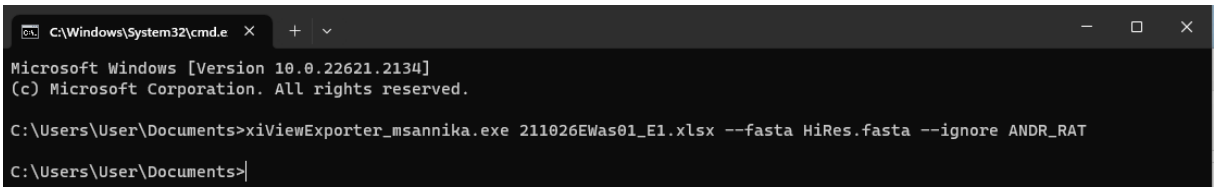

Save the FASTA file and close it. Next you want to open the terminal and type "xiViewExporter_msannika.exe CROSSLINKS_FILE --fasta FASTA_FILE". In our case the crosslinks file is named "211026EWas01_E1.xlsx" and the FASTA file is called "HiRes.fasta" so we will enter the following command:



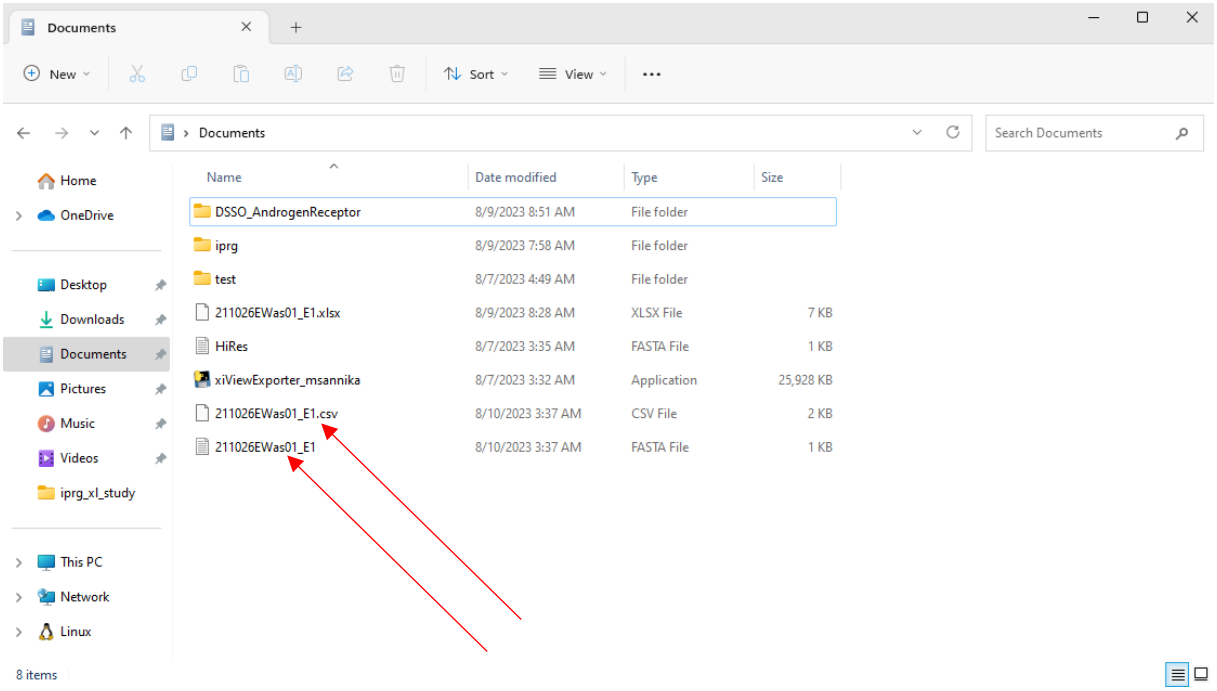

After hitting enter you will get the following error:




This error appears because some of the cross-linked peptides are both part of the mouse androgen receptor and the rat androgen receptor, however we only supplied a FASTA with the mouse androgen receptor. We need to add the flag "--ignore ANDR_RAT" to the command so the exporter knows it should not look for that sequence. With the following command the exporter now runs through successfully.



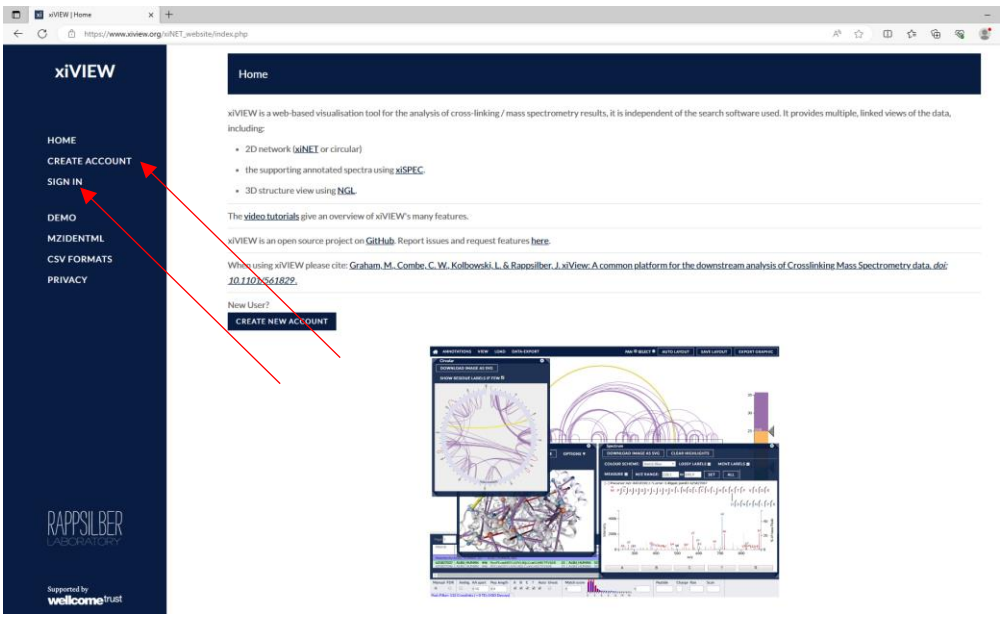

You now should have two files for upload to xiVIEW: “211026EWas01_E1.csv” and “211026EWas01_E1.fasta”.



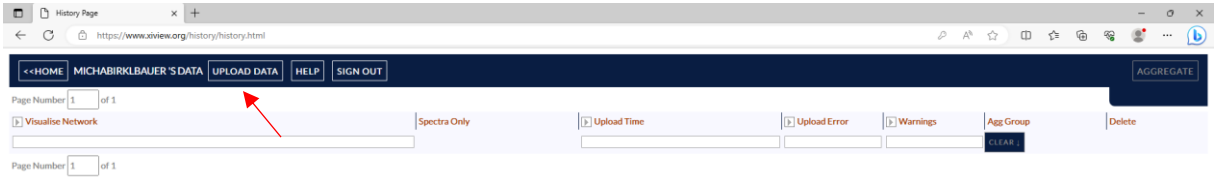

Next open a browser and head over to xiVIEW:

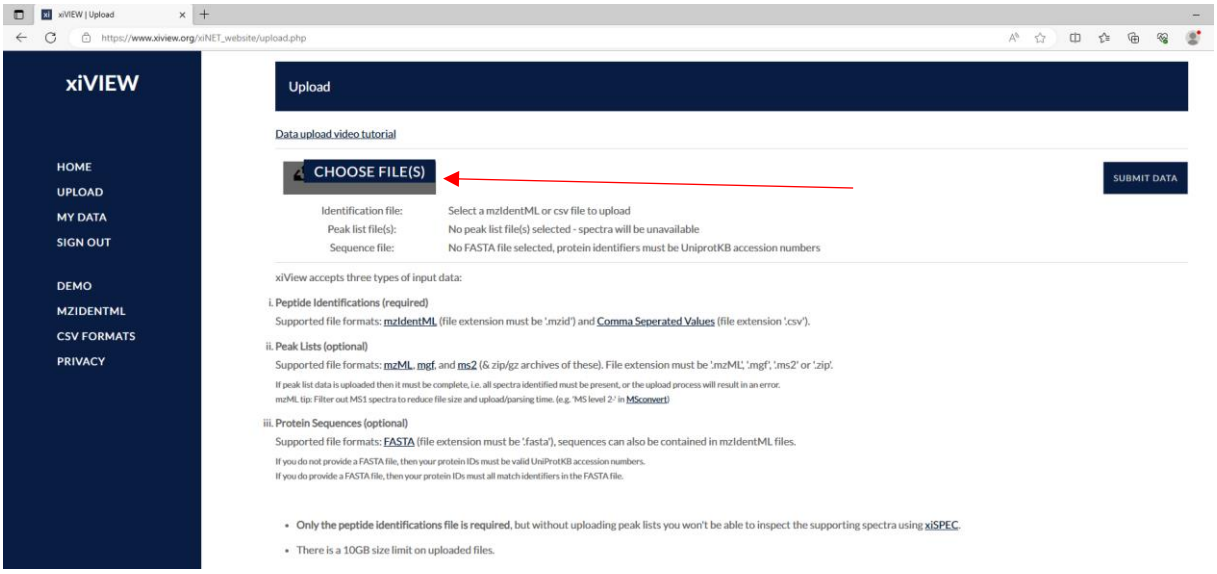
https://www.xiview.org/xiNET_website/index.php

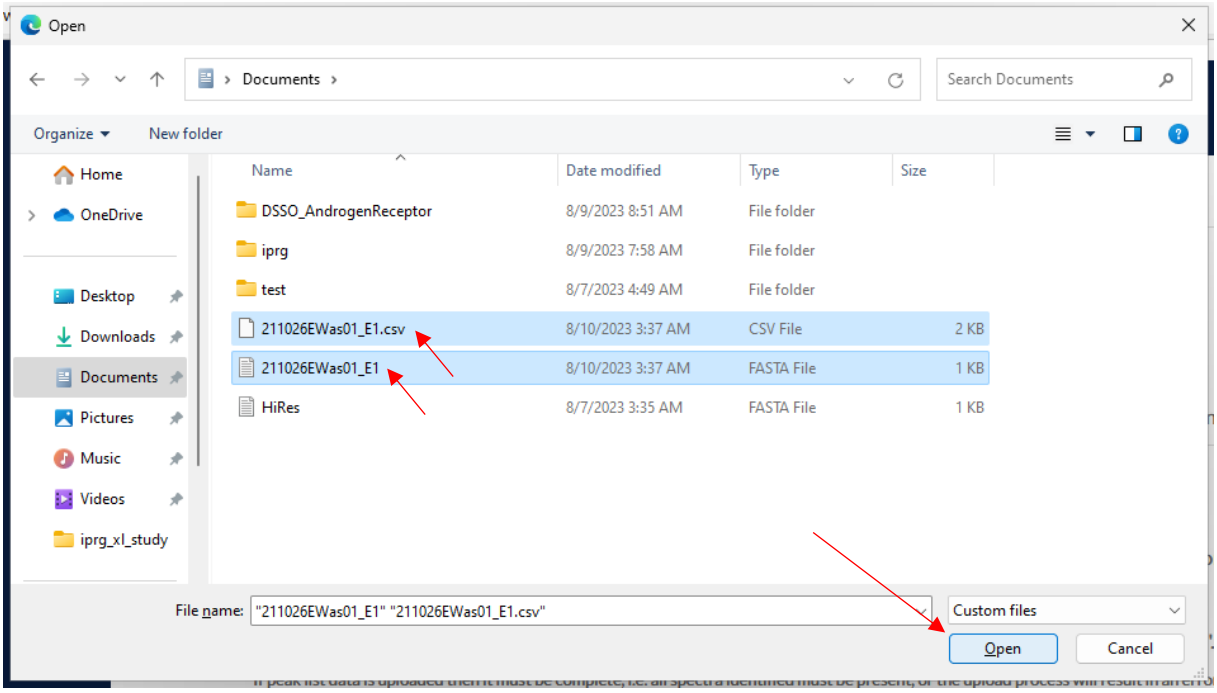
If you don’t have an account yet, create an account and login (it’s free), if you already have an account -> sign in.

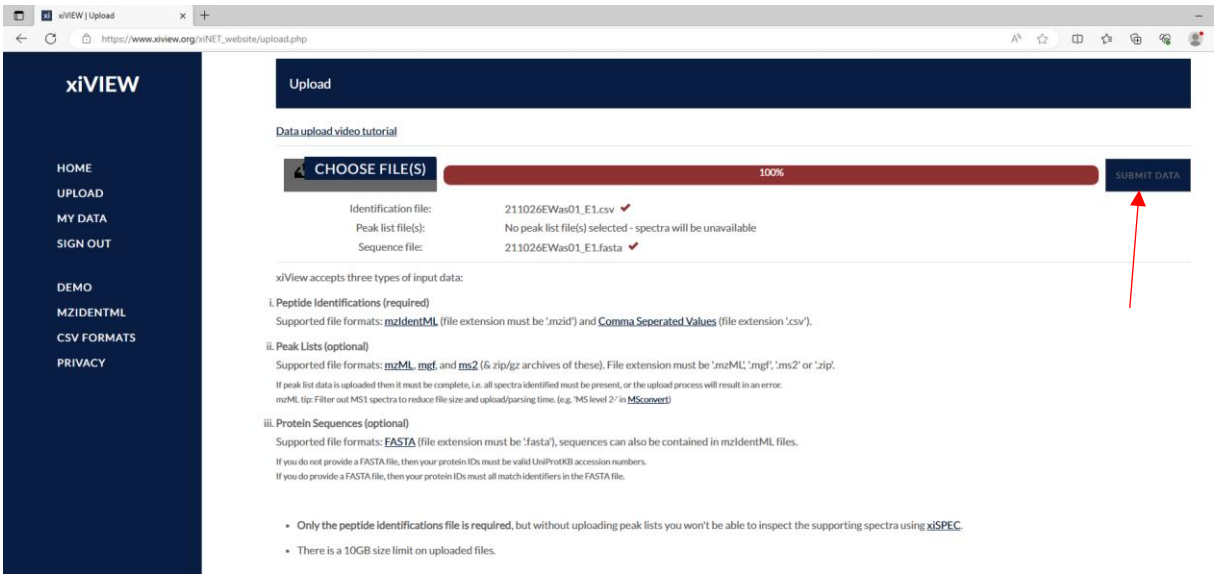


Select “UPLOAD DATA” and upload the two files generated by the exporter.

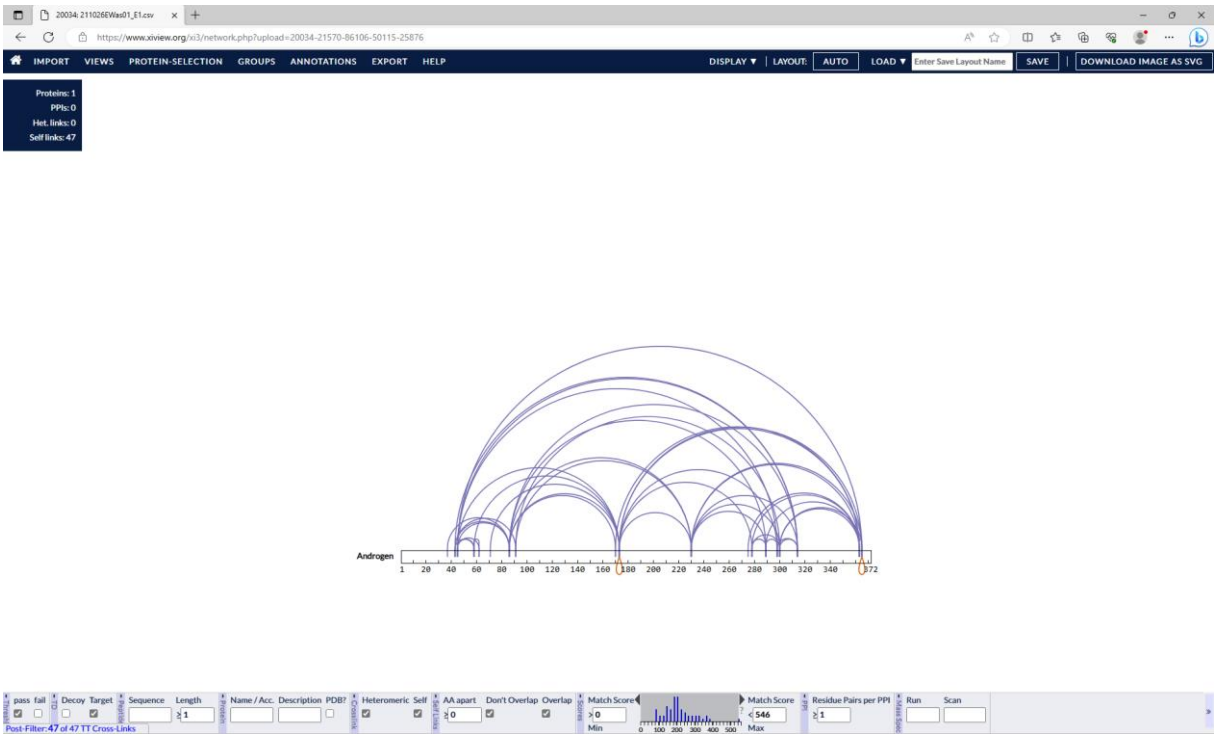


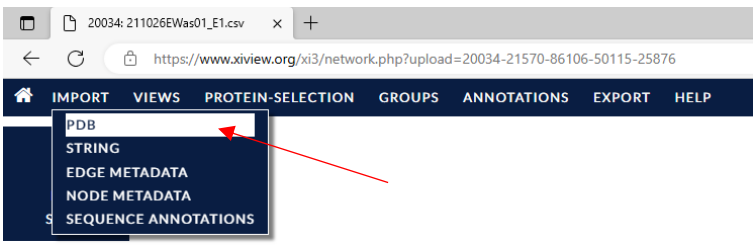





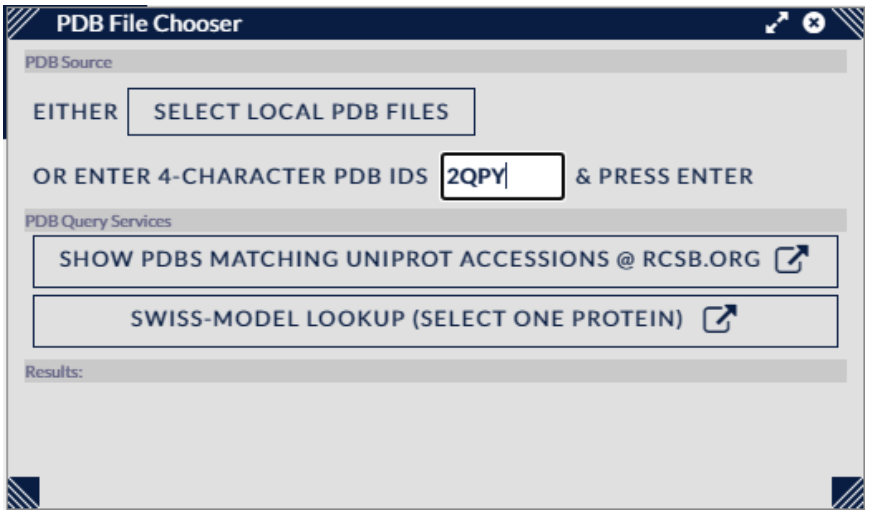

You should be forwarded to the result window that should look like this:



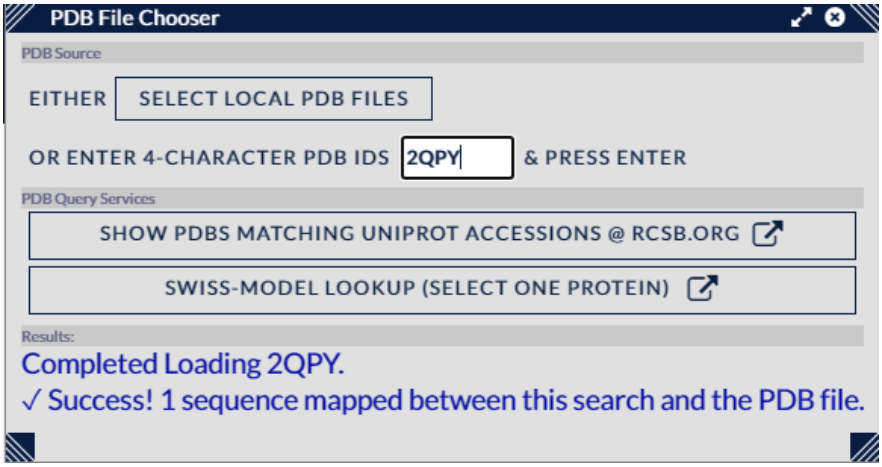

Depicted is the sequence of the mouse androgen receptor and which residues are cross-linked with each other. We can also upload a 3D protein structure to display the crosslinks. For that click on “IMPORT” and “PDB” in the top left corner.



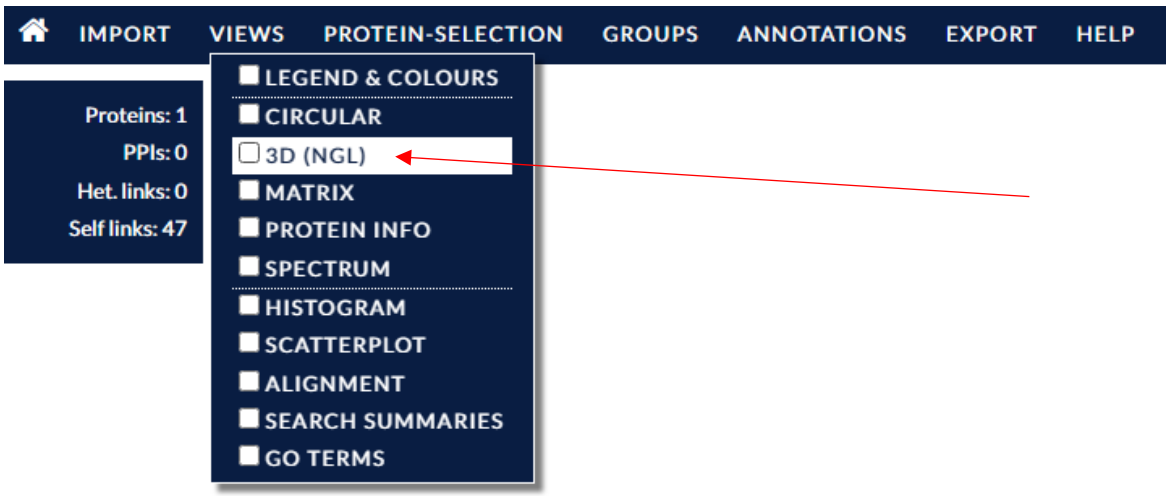
You can either select a local PDB file or enter a 4-character PDB code. We are going to do the later. The PDB short code of the androgen receptor of the mouse is “2QPY”, we enter that into the field and press the enter key.



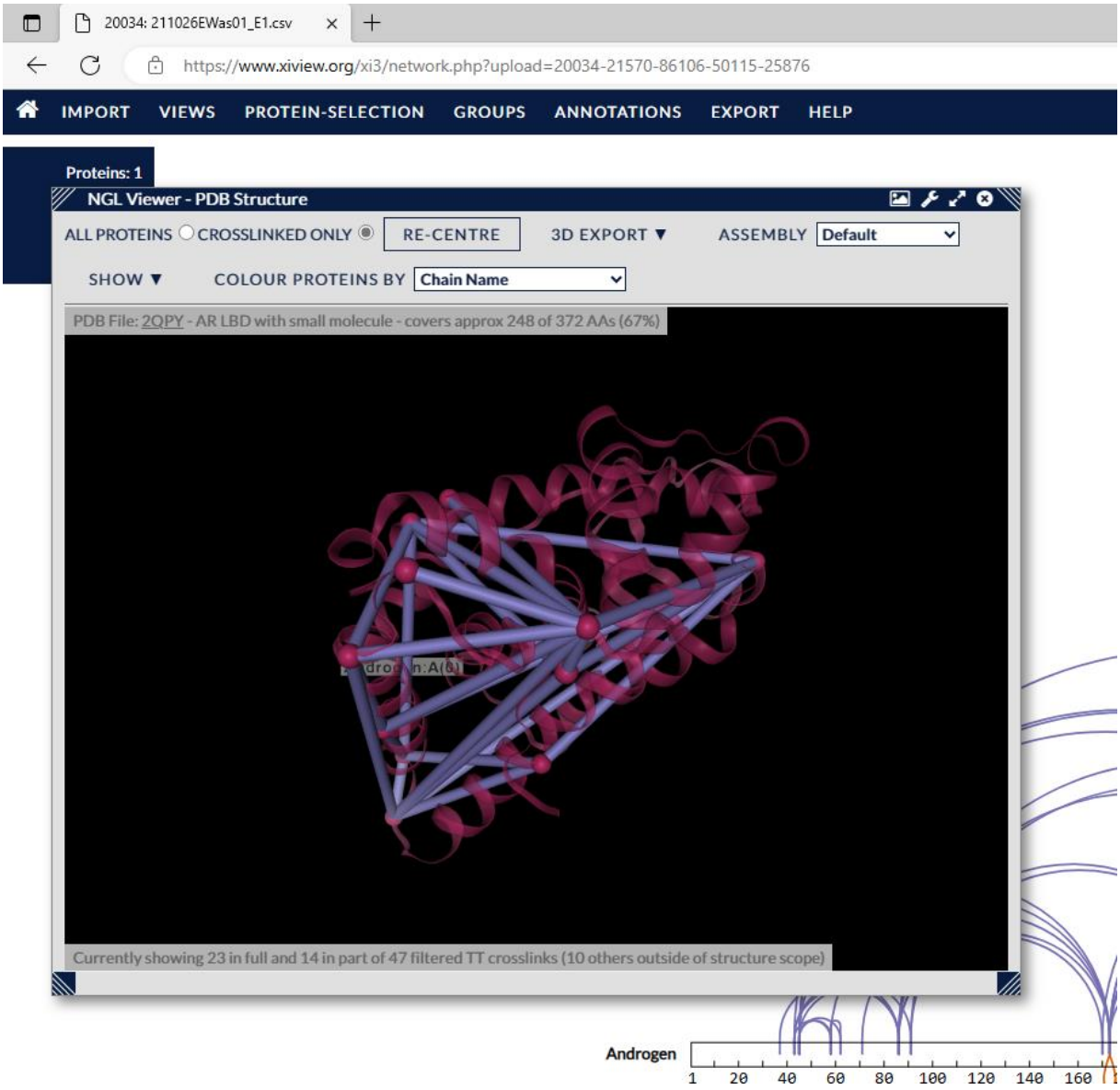

You should get a success message.




Now close the “PDB File Chooser” and select “VIEWS” and “3D (NGL)” in the top left corner.

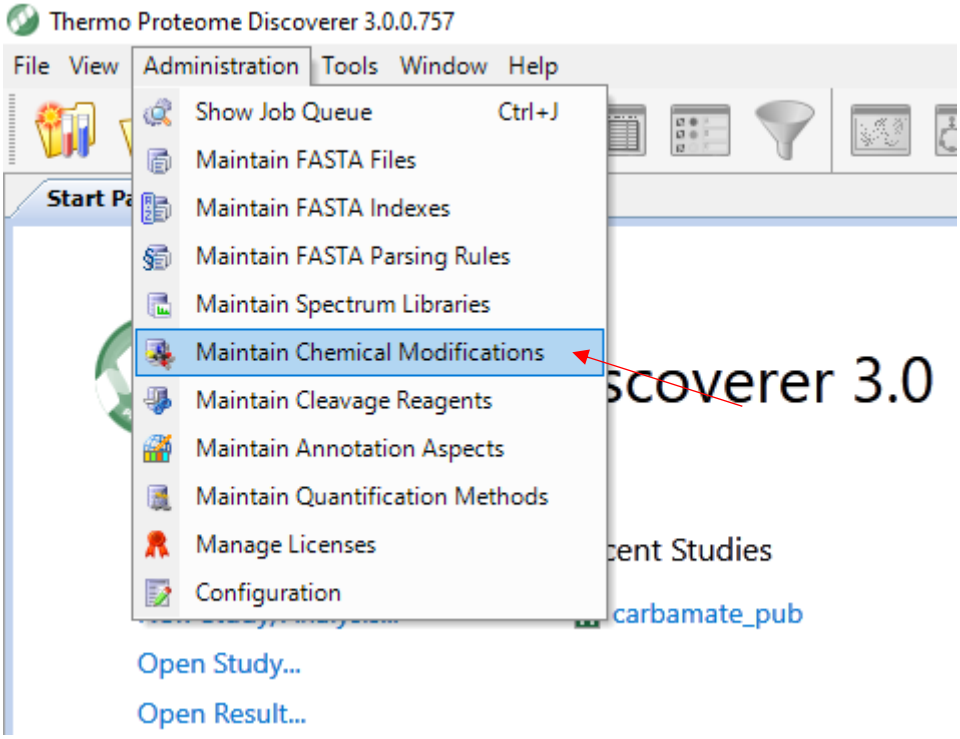


You should be presented with the 3D structure of the protein and the mapped crosslinks.

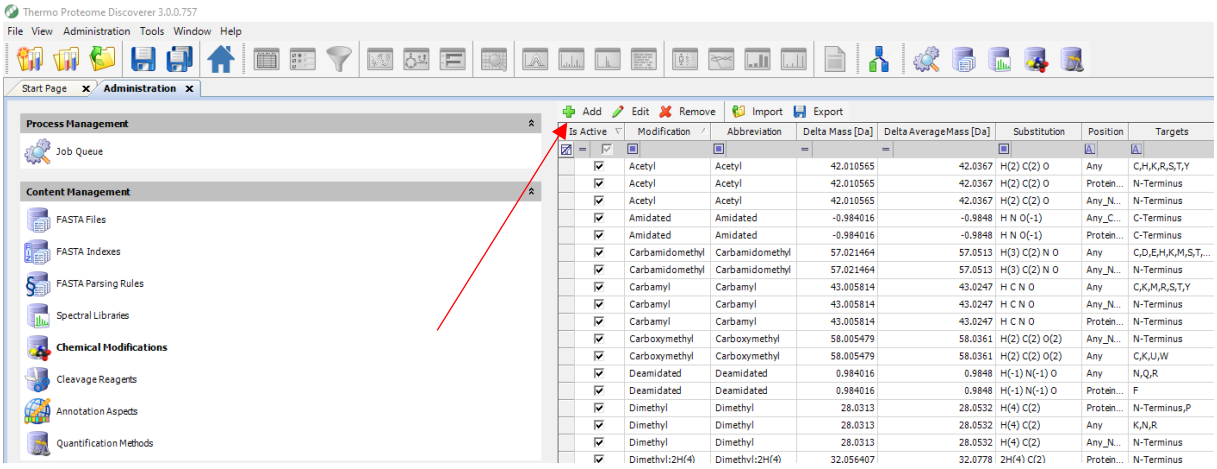


## Defining a crosslinker in Proteome Discoverer

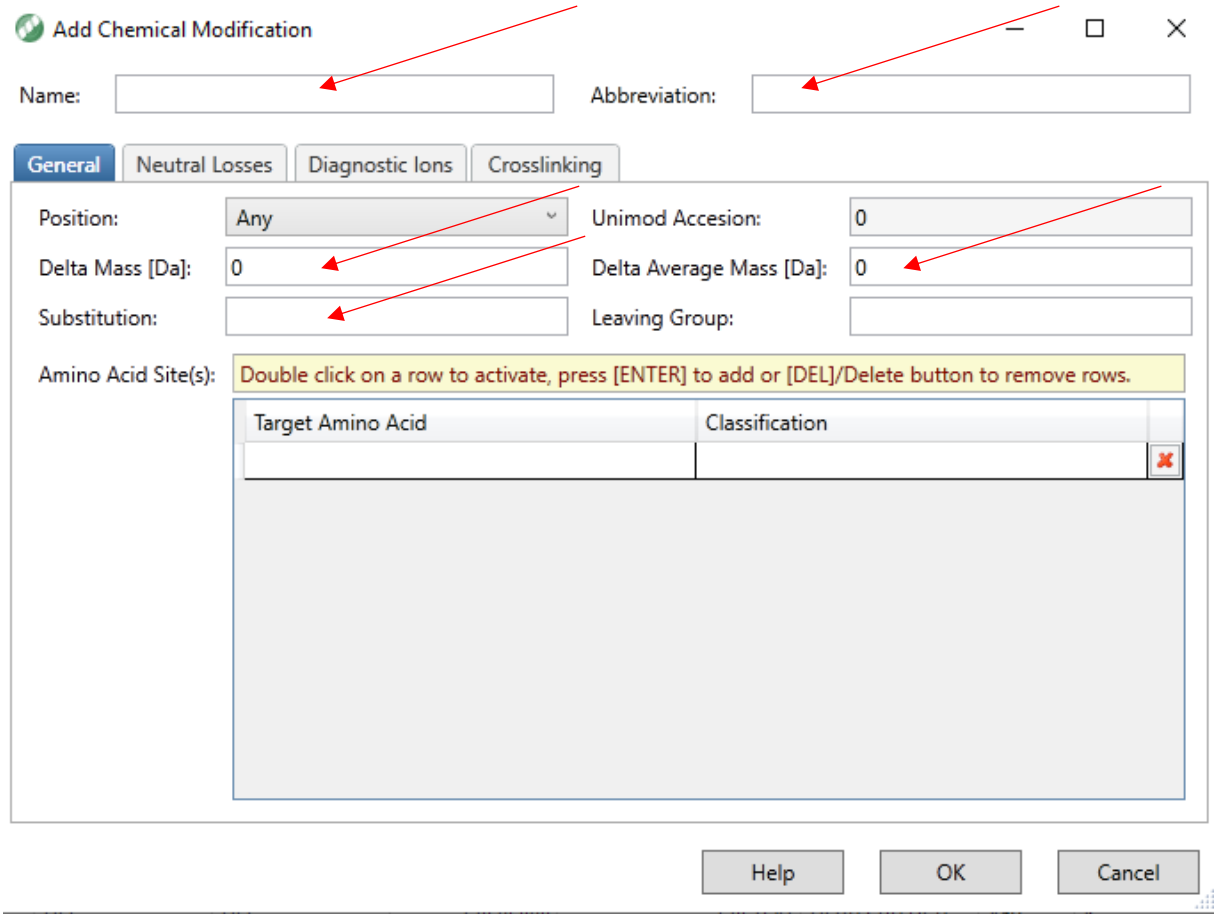
If you need to define your own crosslinker in Proteome Discoverer, open the “Maintain Chemical Modifications” window from the “Administration” menu.



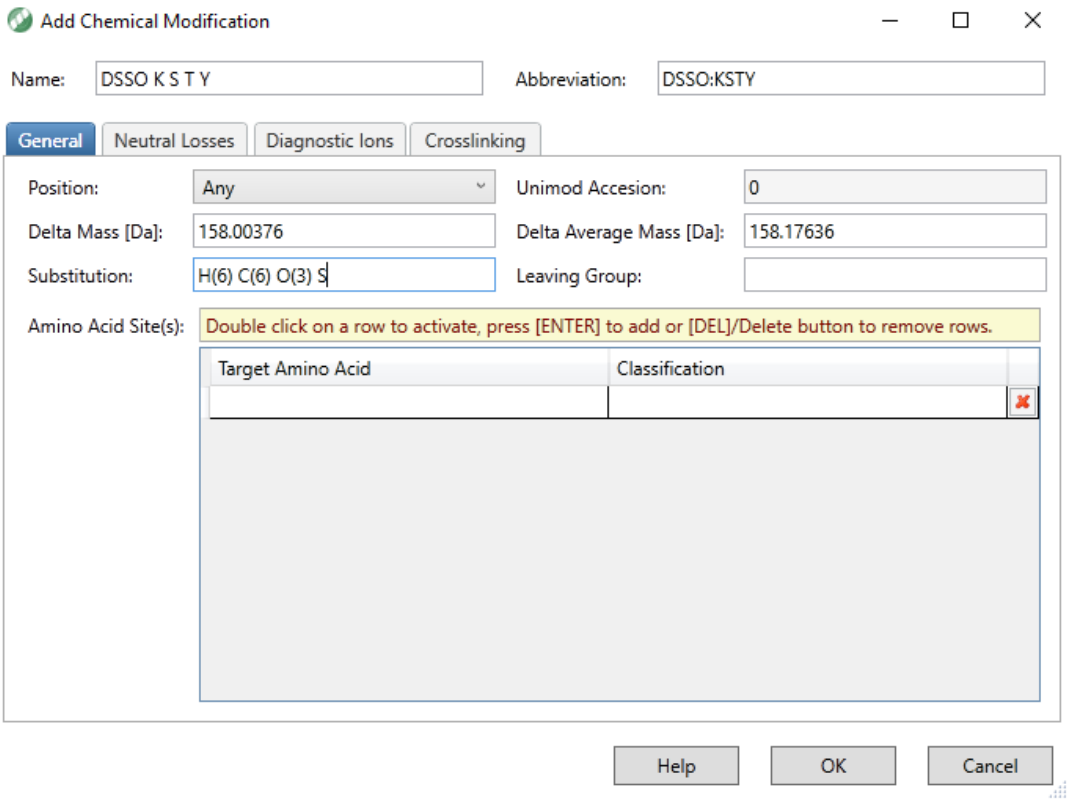

Click on “Add” in the top middle of the screen to add a new modification.



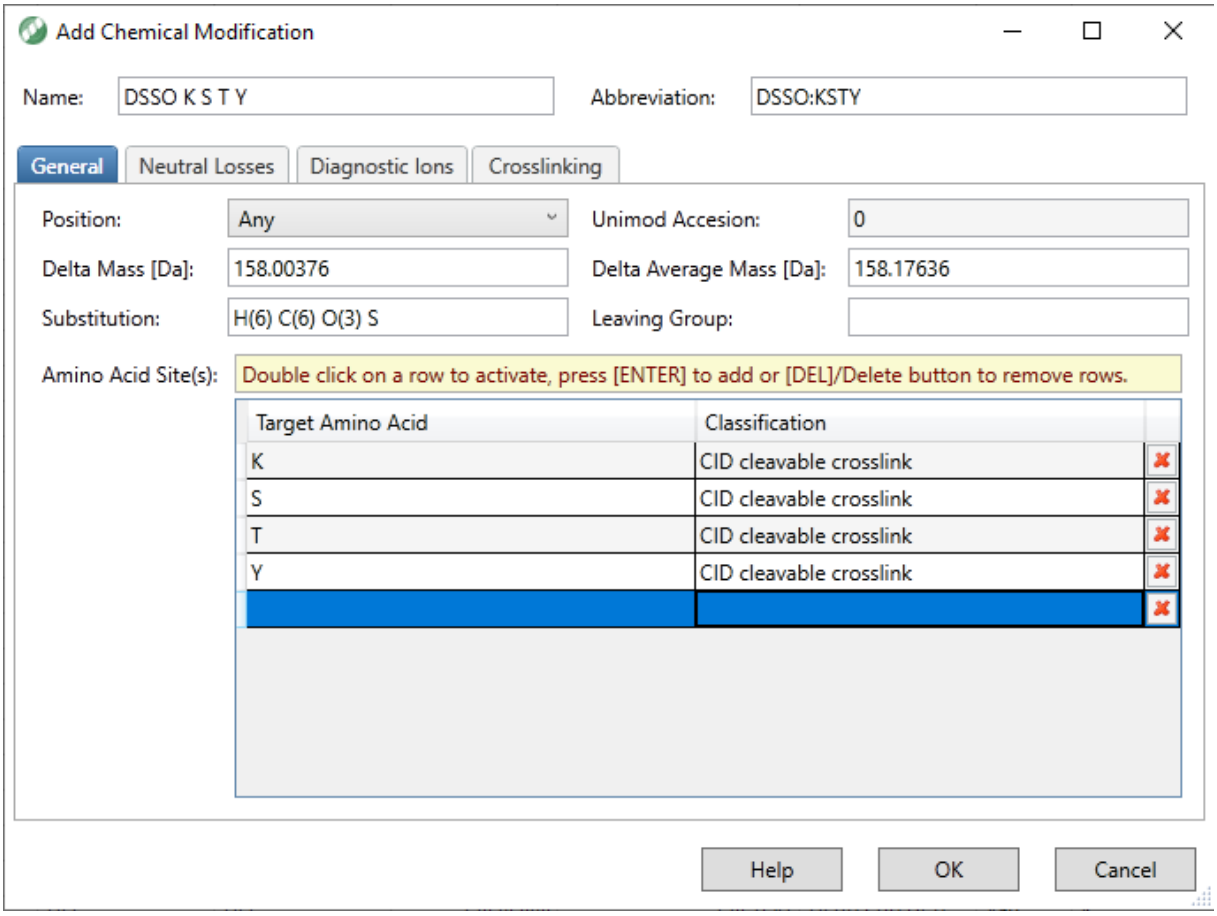

First you have to enter a name, abbreviation, delta mass, delta average mass and the atomic composition in the “General” tab of the “Add Chemical Modification” window that pops up.



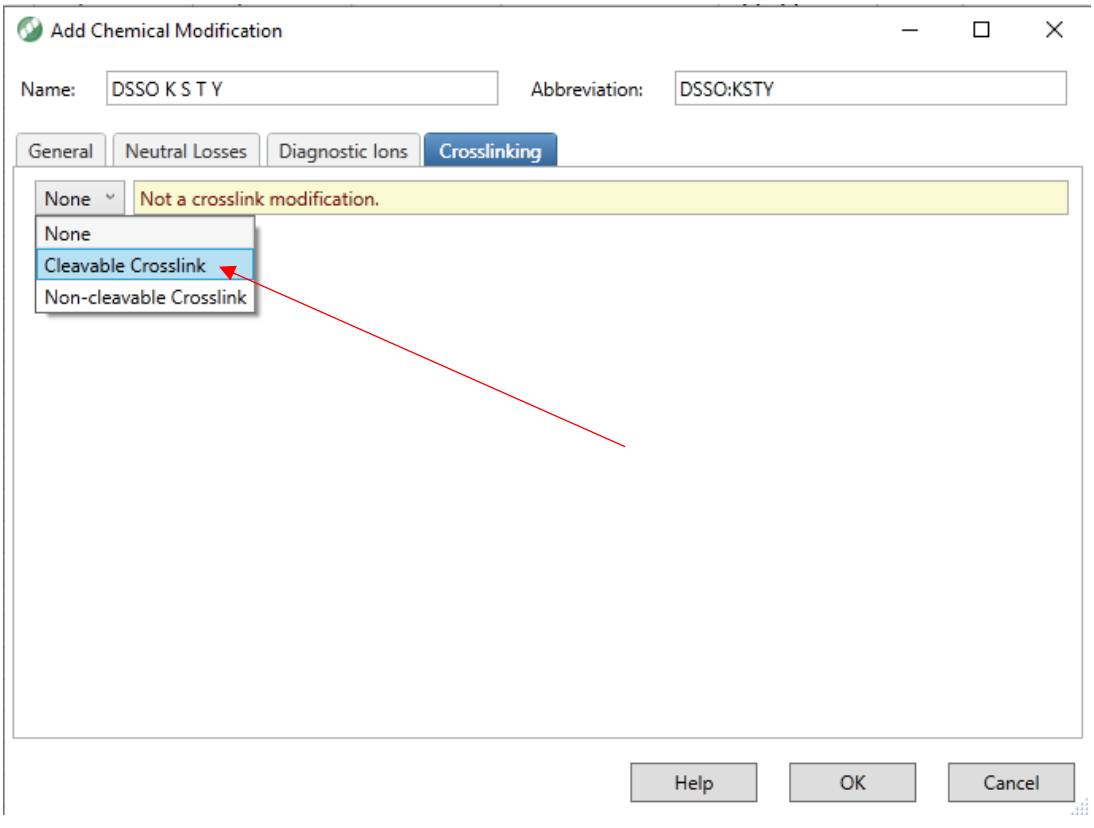

For this example, we are going to define a new DSSO variant that reacts with K, S, T, and Y.



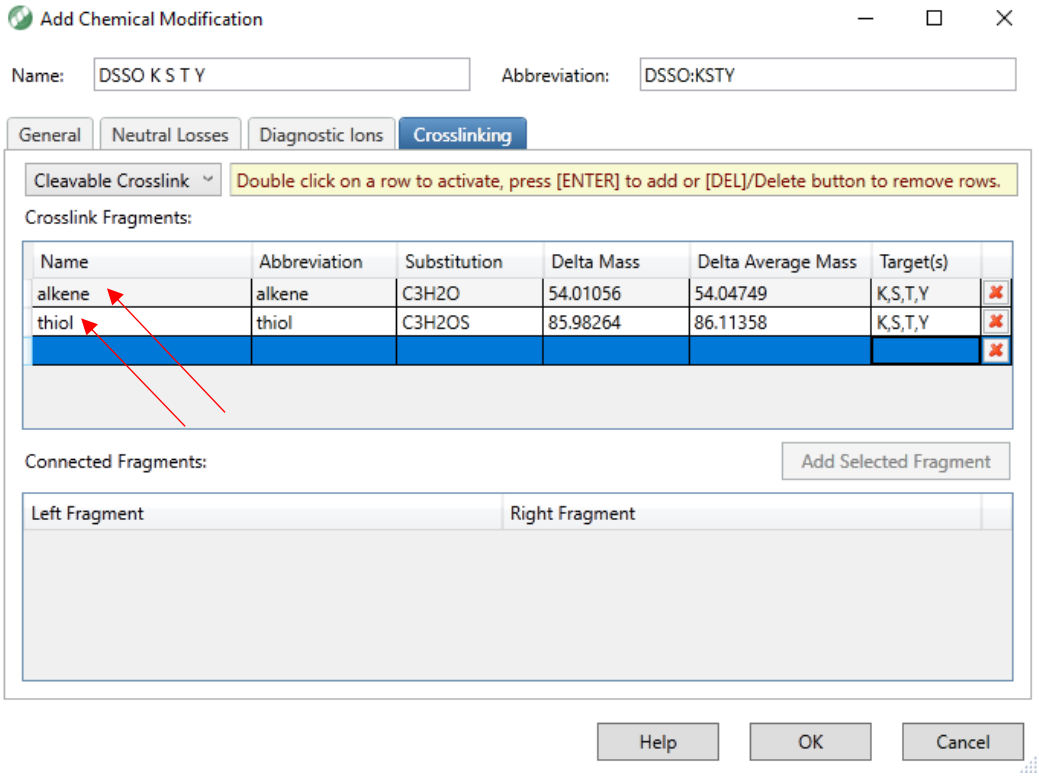
Next, we are going to define the target amino acids in the “Amino Acid Site(s)” box.



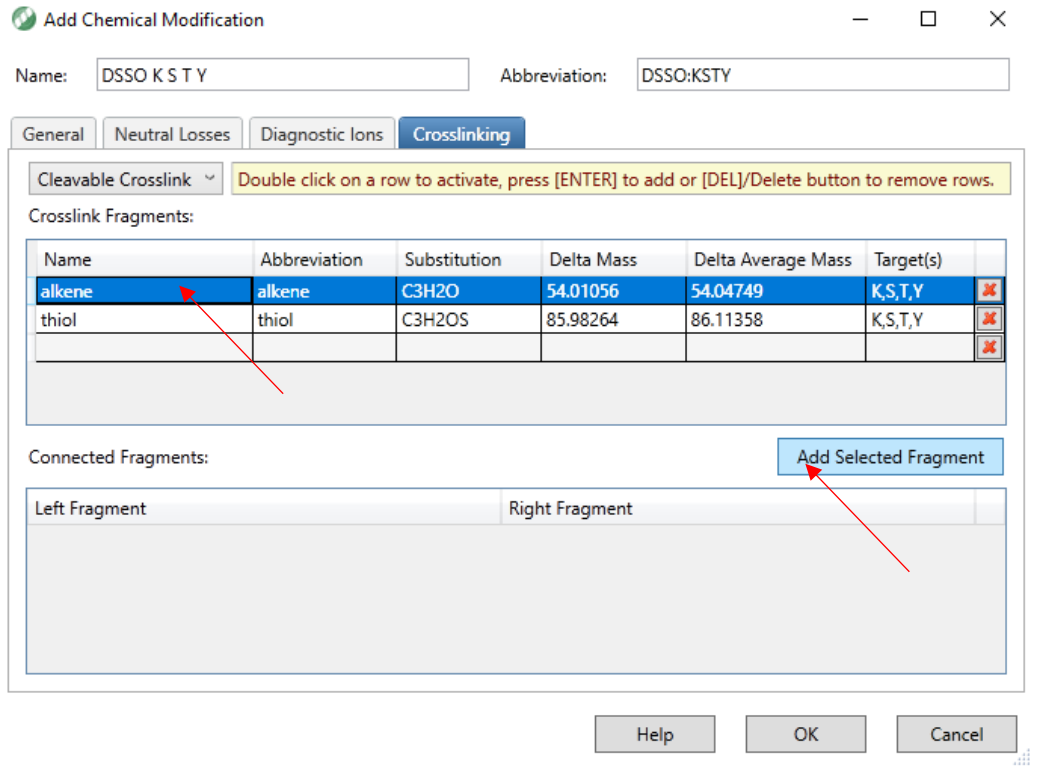

Change to the “Crosslinking” tab and select “Cleavable Crosslink”.



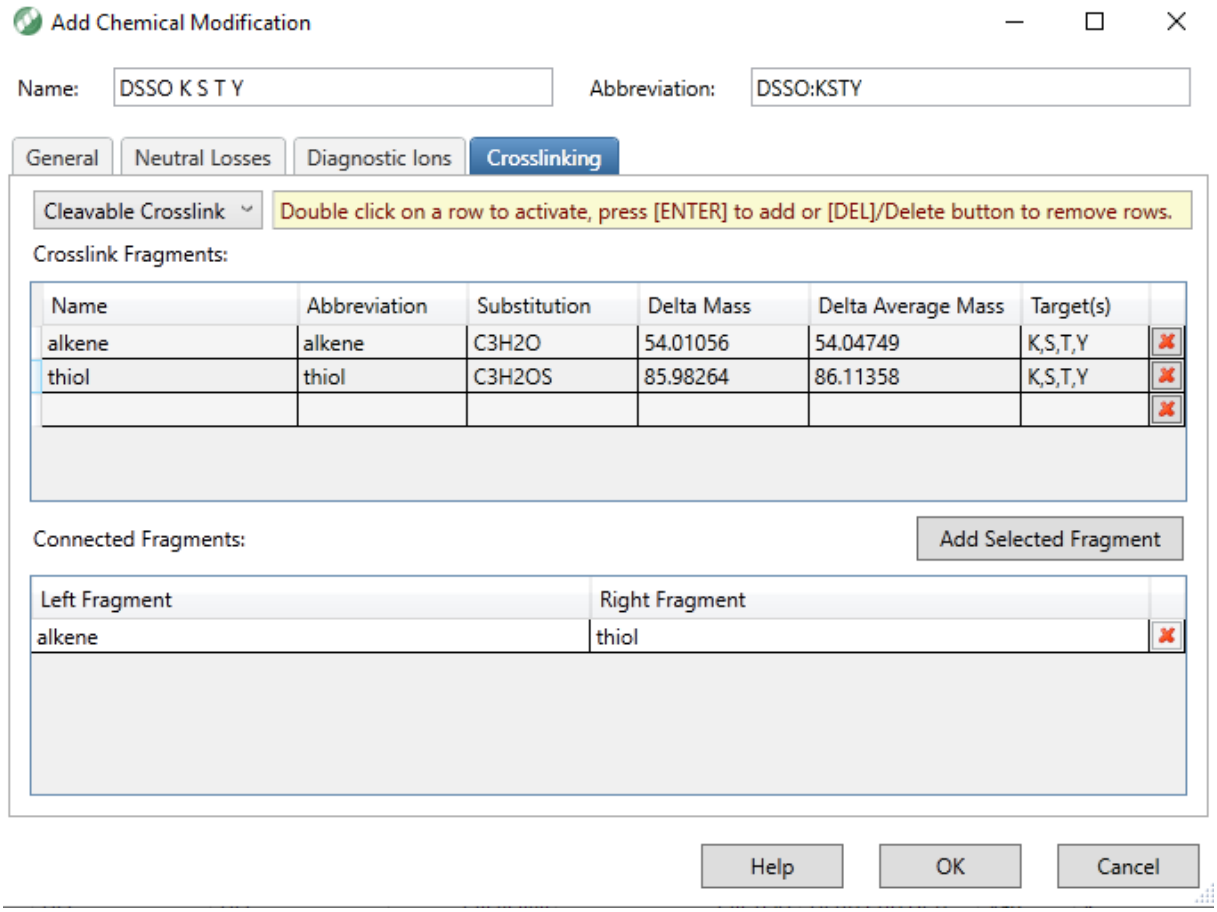
Now enter the two crosslink fragments that can form during cleavage. You only need to enter “Name”, “Abbreviation”, “Substitution” and “Target(s)”, the rest is automatically calculated by Proteome Discoverer.



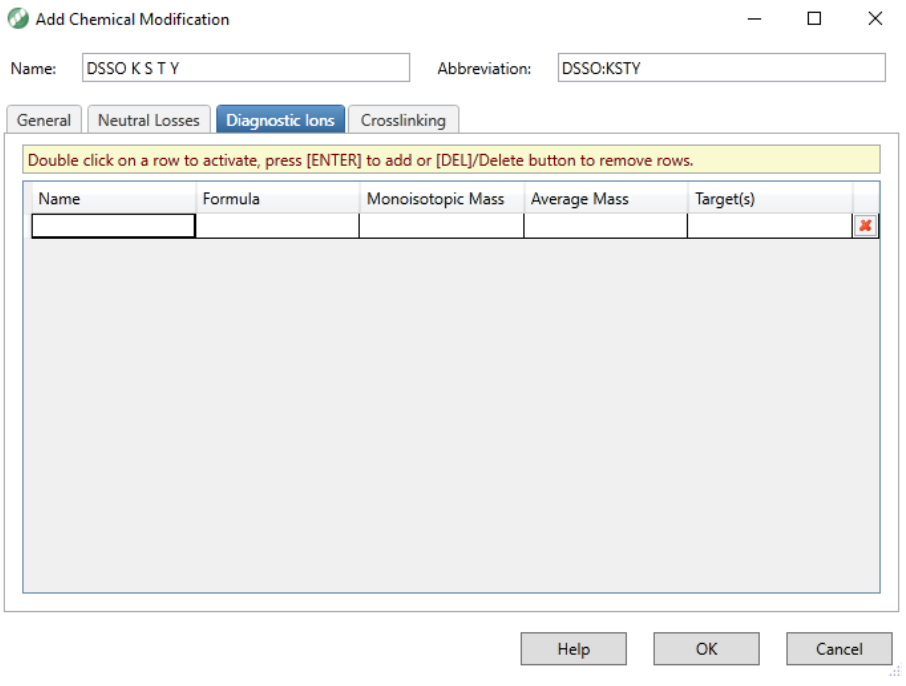

Now add the fragments to the “Connected Fragments” box by selecting first the lighter fragment and “Add Selected Fragment” and then the heavier one and “Add Selected Fragment”.



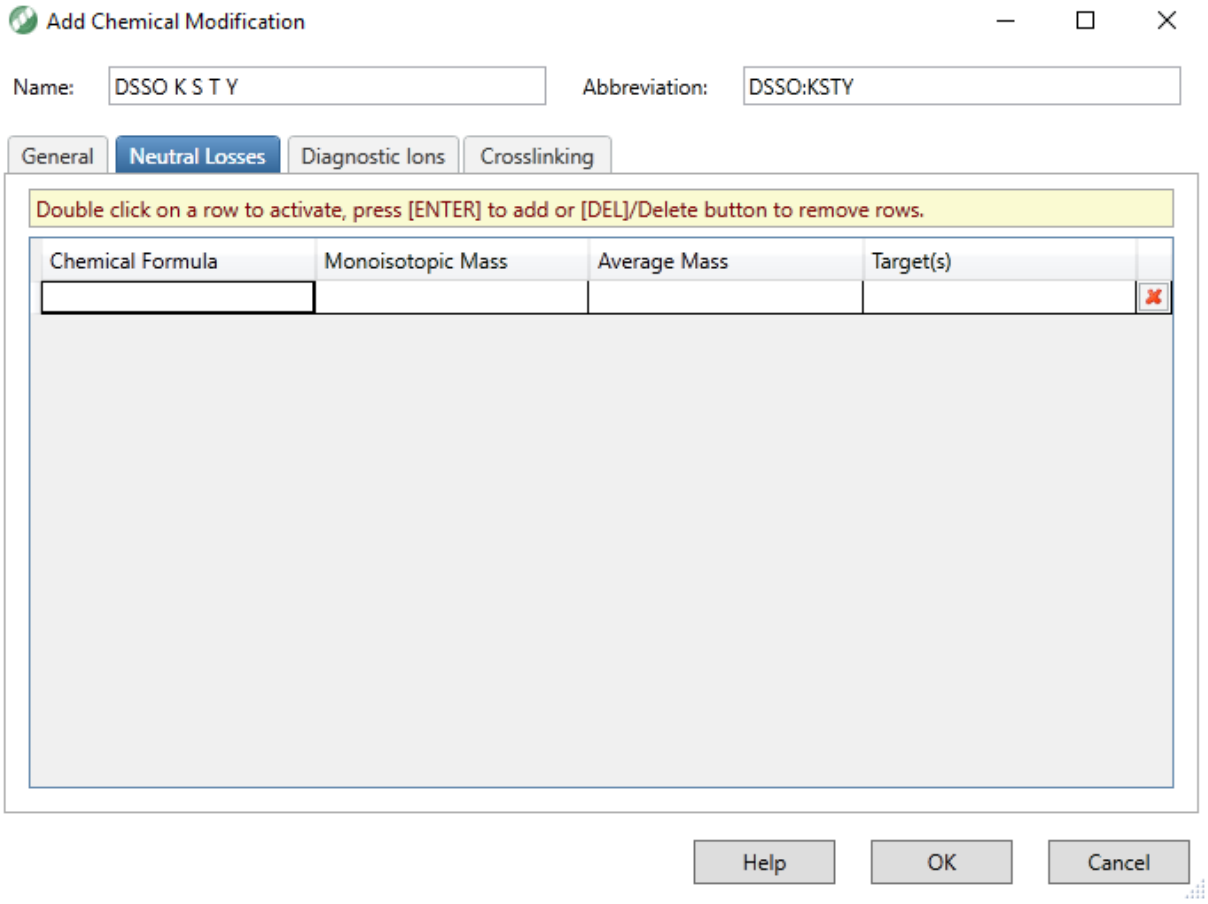
The result should look like this:




In the “Diagnostic Ions” tab any crosslinker specific diagnostic ions should be added. MS Annika 2.0 will use these to identify cross-linked spectra if no doublets are found. Alternatively, the diagnostic ions can also be set directly in the “MS Annika Detector” node.



If your crosslinker yields more than two fragments during cleavage, these should be specified in the "Neutral Losses" tab. Any additional fragment not specified in the "Crosslinking" tab should be specified here (if they are expected to be observed in the cross-linked spectra). Additionally, you also need to adjust the "Additional Crosslink Doublet Distances" setting to cover all possible doublet distances that might appear due to the additional fragments. MS Annika 2.0 can deal with any number of possible doublets as long as the light fragment stays constant. If you are synthesizing a new crosslinker that yields more than two fragments, please don't hesitate to reach out to us to make sure all the settings are set correctly.

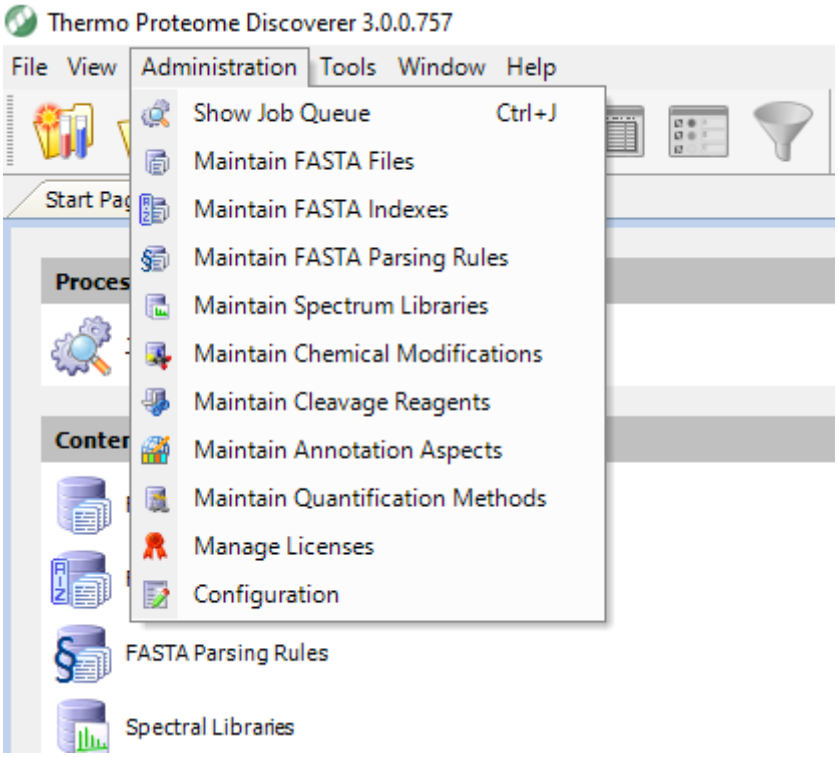



When everything is set, click "OK" in the bottom right and the new crosslinker will be added to the list of available crosslinkers in Proteome Discoverer.

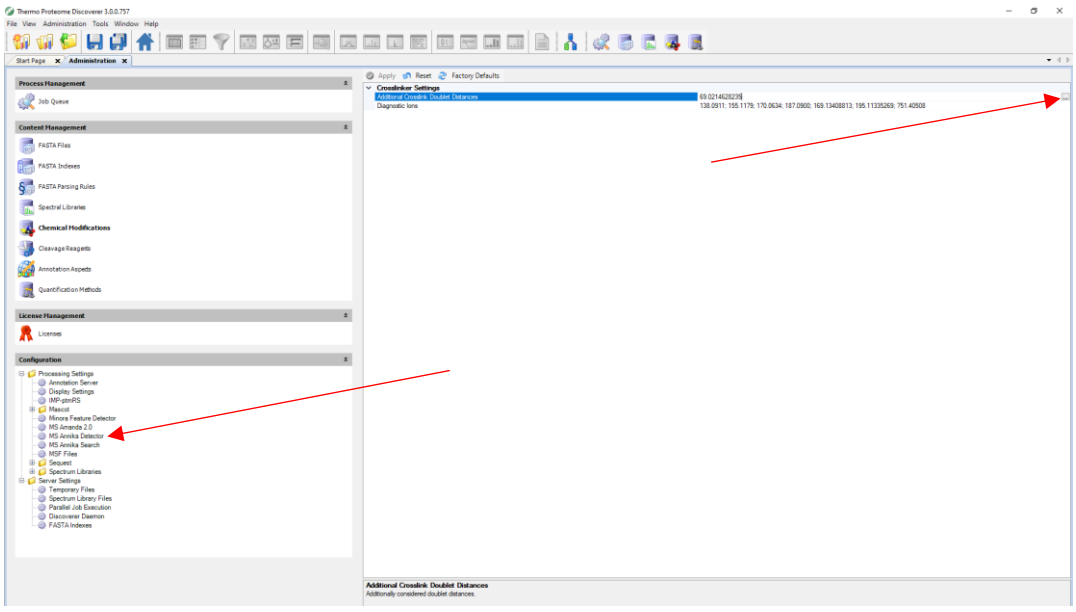
| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ☑ | DSSO | DSSO | 158.00376 | 158.17636 | H(6) C(6) O(3) S | Any | K |
| ☑ | DSSO Amidated | DSSO:NH3 | 175.03031 | 175.20692 | H(9) C(6) O(3) NS | Any | K |
| ☑ | DSSO Hydrolyzed | DSSO:H2O | 176.01433 | 176.19165 | H(8) C(6) O(4) S | Any | K |
| ☑ | DSSO K S T Y | DSSO:KSTY | 158.00376 | 158.17636 | H(6) C(6) O(3) S | Any | K,S,T,Y |

## Changing MS Annika 2.0 settings and Additional Crosslink Doublet Distances

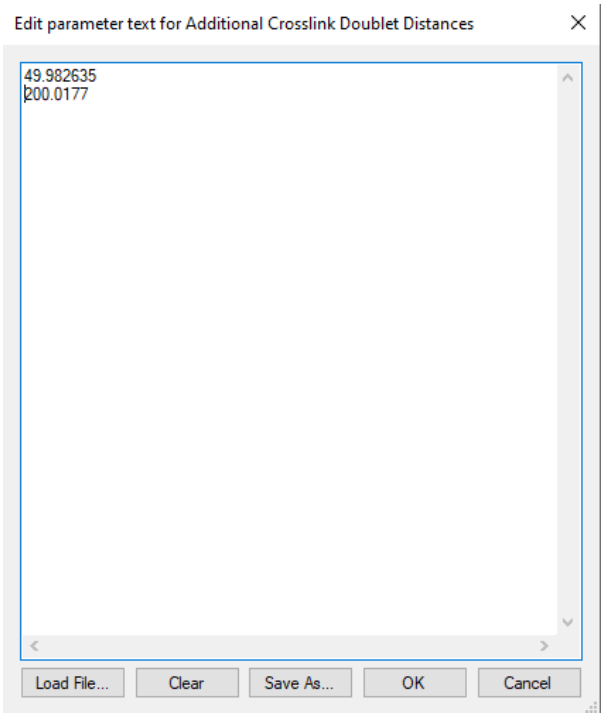
Certain menu items of MS Annika 2.0 like “Additional Crosslink Doublet Distances” and “Diagnostic Ions” can only be modified in the MS Annika 2.0 settings. For that go to “Administration” and “Configuration”.



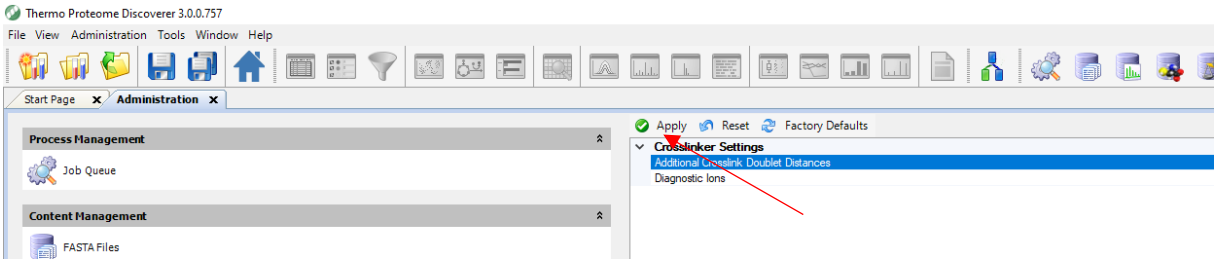

Then select the node that you want to adjust the settings for. For this example, we will add another crosslink doublet distance, more specifically that of DSBSO. Select the “MS Annika Detector” entry and then select “Additional Crosslink Doublet Distances” and click on the three … that appear in the right corner.

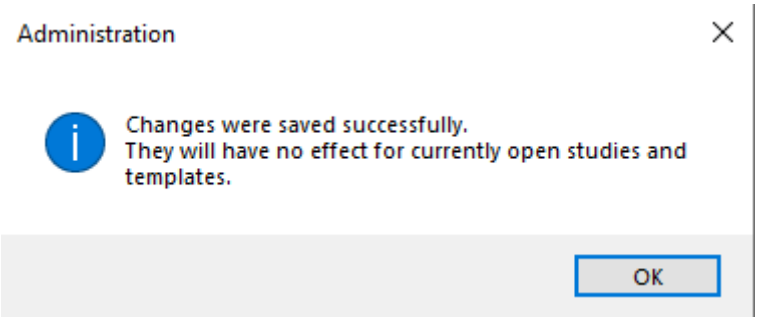


The “Edit parameter text for Additional Crosslink Doublet Distances” window should pop up. By default, a single entry is pre-defined: 49.982635 – the mass difference of the alkene/sulfenic acid doublet of DSSO. We are now going to add the mass difference of the alkene/sulfenic acid doublet of DSBSO which is 200.0177 Da. Begin a new line and enter “200.0177”, then click “OK” to confirm the change.




To apply the made change we additionally need to hit “Apply” in the top left corner of the settings window. The changes will not take effect until re-opening any open study.



## Additionally recommended Proteome Discoverer nodes

The MS2 search of MS Annika 2.0 can heavily benefit from the additional pre-processing steps carried out by the IMP MS2 Spectrum Processor node. This applies to all input formats whether it's Thermo RAW files, MGF files or Bruker timsTOF files and to all fragmentation methods whether it's CID, ETD, HCD or stepped HCD.

More information about the IMP MS2 Spectrum Processor node can be found here:

https://ms.imp.ac.at/?action=spectrum-processor

The appropriate version for your Proteome Discoverer version can be found in the table displayed here:

https://ms.imp.ac.at/?action=home

The version for Proteome Discoverer 3.0 can be directly downloaded from here:

https://ms.imp.ac.at/?file=spectrum-processor/ms2spectrumprocessor_3.0.zip

An exemplary workflow using the IMP MS2 Spectrum Processor node with MS Annika 2.0 can be found here:

https://github.com/hgb-bin-proteomics/MSAnnika#example-workflows

**Important note:** The IMP MS2 Spectrum Processor cannot be used in combination with the MS3 search of MS Annika 2.0 as the MS3 search algorithm relies on the isotope distribution found in the MS2 spectra. Using the IMP MS2 Spectrum Processor node removes this information from the spectra and breaks the MS3 search.

## Getting help

In case you run into problems, something isn't clear, you found a bug, or you have a suggestion, please reach out to us!

You can either open an issue on GitHub at https://github.com/hgb-bin-proteomics/MSAnnika/issues or contact us directly at:

- viktoria.dorfer@fh-hagenberg.at
- micha.birklbauer@fh-hagenberg.at

More from our research group:

- MS Amanda: https://ms.imp.ac.at/?goto=msamanda
- MS Ana: https://ms.imp.ac.at/?action=ms-ana
- https://bioinformatics.fh-hagenberg.at/