



# DIA-NN GUI manual<sup>1</sup>

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<sup>1</sup> Please email any comments, suggestions, questions or feedback to Vadim Demichev (vadim.demichev[at]gmail.com) or create a new issue on GitHub (github.com/vdemichev/diann).

## Introduction

DIA-NN is a fast and easy to use tool for processing data-independent acquisition (DIA or SWATH) proteomics data. DIA-NN is designed to do as much as possible automatically, eliminating the need to optimise the processing parameters for each experiment.

DIA-NN distribution contains DiaNN.exe, which is a command line tool, and DIA-NN.exe, a GUI wrapper for this tool. The GUI works by launching DiaNN.exe and displaying its output. A report (with precursor ion and protein identification and quantification information) is generated by the command line tool. Optionally, a spectral library can be generated. Any analysis that can be performed with the GUI can also be performed with the command line tool and vice versa. Here we provide detailed information on data formats supported by DIA-NN and consider the ways to fine-tune its performance.

## GUI window

The screenshot displays the DIA-NN GUI window, which is organized into several functional panels. The **Input** panel on the left includes buttons for 'Add raw data', 'Clear list', and 'Convert to .dia', along with a large text area for file paths. Below this is a 'Spectral library' section with an 'Add FASTA' button and a 'Clear list' button. The 'DIA-NN exe' field is set to 'diann.exe'. The **Library-free search** panel at the bottom left contains checkboxes for 'Use library-free search', 'N-term M excision', 'M oxidation', and 'C carbamidomethylation', along with dropdowns for 'Protease' (set to 'Trypsin/P') and 'Missed cleavages' (set to '1'). It also features range sliders for 'Maximum number of variable modifications' (0), 'Peptide length range' (7-30), 'Precursor m/z range' (300-1800), and 'Fragment ion m/z range' (200-1800). The **Output** panel in the top center has checkboxes for 'Use existing .quant files when available', 'Generate spectral library', 'Optimise for training', 'Generate Prosit input from FASTA or spectral library', 'Generate PDF report', and 'Log level' (set to 1). It includes text fields for 'Main output' (C:\DIA-NN\report.tsv), 'Temp/.dia dir', and 'Output library'. The **Algorithm** panel at the bottom center contains sliders for 'Mass accuracy' and 'MS1 accuracy' (both 0.0), a 'Scan window' (0), and checkboxes for 'Process in batches', 'Use neural networks', 'Unrelated runs', 'Use isotopologues', 'RT profiling', 'Remove likely interferences', and 'Protein inference'. It also has dropdowns for 'Implicit protein grouping' (genes) and 'Quantification strategy' (any LC (high accuracy)). On the right side, a pipeline editor shows 'Step 1' with a 'Pipeline' list. At the bottom right, there are buttons for 'Clear pipeline', 'Execute', and 'Abort pipeline'. A large text area at the very bottom is reserved for the command-line tool's output. The window title bar reads 'DIA-NN DIA-NN'.

The GUI window features four panels used to specify the data and processing settings: **Input**, **Output**, **Library-free search** and **Algorithm**. To the right, there is a pipeline editor at the top and a panel used to display the output of the command-line tool at the bottom. Extra commands entered in the **Additional options** text box (**Output** panel) will also be passed to the command line tool; quotes can be used when passing options but are not required unless file names featuring multiple adjacent spaces need to be specified. See the project description on the GitHub for the information on DIA-NN's command line syntax and some useful commands. Most GUI elements feature tooltips with a short description of their function, activated by hovering a mouse cursor over the element.

## Input

Sciex .wiff, Thermo .raw, .mzML and .dia (native DIA-NN format) files are supported. Reading Sciex .wiff files requires the DIA-NN.Wiff.dll file (included in the DIA-NN distribution) as well as the Sciex DLL libraries to be present in the same folder as DiaNN.exe. For example, one can first install ProteoWizard (which includes the Sciex DLLs) and then install DIA-NN in the same folder using the installer provided (DIA-NN-Setup.msi). Reading Thermo .raw files requires Thermo MS File Reader to be installed. Please use specifically the version MS File Reader 3.0 SP3\*. MSX-DIA .raw files are currently not supported. It is highly recommended that .mzML files are centroided, preferably using the vendor centroiding algorithm for both MS1 and MS2 spectra. DIA-NN has been tested on .mzML files produced from Sciex .wiff and Thermo .raw by MSConvert GUI (part of the ProteoWizard project) with 32-bit binary precision, vendor centroiding enabled and all other options except “Write index” disabled.

DIA-NN can convert raw files to its own .dia format. This format allows very quick loading, essentially limited only by the hard drive speed. Converted files are placed either in the same location as the raw files, or to a folder specified using the “Temp/.dia dir” option in the Output panel.

## Spectral library

Used to specify the spectral library. The library is required unless a library-free search against a FASTA database is to be performed. In that case, if specified, the spectral library will be used to enhance and speed up library-free search (experimental). DIA-NN accepts spectral libraries in a plain text format (tab-separated or comma-separated, i.e. .tsv, .csv, .xls or .txt) as well as in its own compact binary format (.speclib).

Libraries in the PeakView format as well as libraries produced by TargetedFileConverter (part of OpenMS), exported from Spectronaut (Biognosys) in the .xls format or generated by DIA-NN itself are supported “as is”. For libraries generated with FragPipe, “--library-headers \*,\*,\*,\*,\*,\*,Protein ID,Entry Name” should be added to the “Additional options” text box.

For libraries generated by other means, DIA-NN needs to be told the header names (separated by commas) (for the columns it requires) using the --library-headers command. Use the \* symbol instead of the name of a header to keep its recognition automatic. See below the descriptions of the respective columns (in the order the headers need to be specified).

- **Modified peptide sequences.** Outside parentheses, all symbols apart from letters are ignored. Modifications are specified within parentheses (“()” or “[ ]”) after the modified amino acid or (e.g. “[Acetyl (Protein N-term)]”) before the first amino acid. Recognition of some common modifications is built in. Custom modifications can be specified using the --mod command, e.g. “--mod UniMod:5,43.005814” will add carbamylation (KR) to the list of modifications recognized by DIA-NN. Specifying “label” after the modification mass will tell DIA-NN that the modification in question is an isotopic label, e.g. “--mod UniMod:259,8.014199,label” would correspond to Lysine +8 SILAC label (SILAC recognition is built-in though, so no need to add it separately). DIA-NN does not need to recognise modifications if fragment charge and type (either y-series or b-series) information is provided (this is usually the case) or if decoys are provided in the library (see below). The --clear-mods command makes DIA-NN forget all the built-in modifications (spectral library-based search only), so that the output would contain modification names as specified in the spectral library, whereas by default DIA-NN produces output with modification names in UniMod format.
- **Precursor charge.**
- **Precursor m/z.**
- **Reference retention time.** There are no restrictions on the retention time scale used here.

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\* MS File Reader 3.0 SP3 download link:

<https://thermo.flexnetoperations.com/control/thmo/login?nextURL=%2Fcontrol%2Fthmo%2Fdownload%3Felement%3D6306677>

- **Fragment ion m/z.**
- **Relative intensity of the fragment ion.** There are no restrictions on the scale used here.

The following optional headers can also be specified:

- **UniProt identifiers** of the proteins matched to the peptide (separated by semicolon). These will be used for peptide annotation as well as protein quantification. If a FASTA file is specified, UniProt identifiers will be searched against this file and annotated with protein and gene names. If the library contains no column specifying which peptides are proteotypic (see below), DIA-NN will consider any peptide matched to a single UniProt identifier proteotypic. DIA-NN does not check the validity of the UniProt identifiers provided, i.e. arbitrary text can be used instead.
- **Protein names.** These will be used for annotation if a FASTA file is not provided.
- **Gene names.** These will be used for annotation if a FASTA file is not provided.
- **Proteotypicity.** The value should be 1 if the peptide is to be considered proteotypic (i.e. there is only a single protein from which it can originate). DIA-NN calculates protein q-values using proteotypic peptides only.
- **Decoy.** Indicates whether the peptide is a decoy. If there are decoy peptides in the library, DIA-NN uses these and does not generate its own decoys.
- **Fragment ion charge.** If this column is not provided, DIA-NN infers the charges itself, although this inference relies on recognition of all peptide modifications and is not guaranteed to be correct in all the cases. However, DIA-NN needs to know fragment ion charges if (1) the library contains modifications DIA-NN does not recognise, and these have not been added with the `--mod` command, and decoys are not provided in the library or (2) DIA-NN is told to consider isotopologues of the peptides and fragments in the library (this is enabled by default).
- **Fragment ion type.** Should be 'y' or 'b'. DIA-NN needs to know the fragment ion type if the library contains modifications DIA-NN does not recognize, and these have not been added with the `--mod` command, and decoys are not provided in the library.
- **Fragment series number, Fragment loss type, Q-value.**
- **Elution Group.** This column specifies a unique string identifying the elution group, e.g. the set of all precursor ions which are expected to have exactly the same retention time. If not provided, DIA-NN infers elution groups automatically, assuming 'co-elution' of precursors corresponding to the same peptide with different charge states or isotopic labels (relying on the built-in SILAC recognition and on label information provided when supplying the `--mod` command – see above). The (experimental) `--peak-translation` command will make DIA-NN force the same retention time for precursors in an elution group, improving identification performance. Heavy/light ratios (equivalent to absolute quantities when used with spike-in quantification standards, e.g. PlasmaDive or PQ500) will also be calculated (more information in the description of the Label.Ratio output column, see below).

Thus, `--library-headers *,*,Precursor_Mz,*,Fragment_Mz,*,Protein_Ids`, for example, would instruct DIA-NN to recognise “Precursor\_Mz” and “Fragment\_Mz” as the headers for the columns containing precursor and fragment ion m/z values, and “Protein\_Ids” as the header for the column containing UniProt identifiers. Importantly, rows describing fragment ions corresponding to the same precursor must be grouped together (this is the case with all software-generated libraries).

After loading a library in a text format, DIA-NN always automatically saves it in the compact .speclib format to the same folder. Any text/.speclib library can also be exported to an OpenMS-compatible text format by selecting the “Generate spectral library” option in the Output panel and running DIA-NN without specifying any raw data files (as otherwise DIA-NN would only save precursors identified in these files and would refine the spectra and

retention times based on these identifications – see below). Only fragments annotated with the fragment type and charge information or fragments recognized by DIA-NN automatically (i.e. y/b-series fragments, potentially with a single H<sub>2</sub>O, NH<sub>3</sub> or CO neutral loss) will be exported. Commands `--min-fr` and `--max-fr` allow to specify the minimum and maximum numbers of fragments per precursor in the exported library. For example, `--export-library --min-fr 6 --max-fr 20` will instruct DIA-NN to discard all precursors with less than 6 fragments annotated and retain only top 20 fragments, based on their reference intensities. Such filtering might be useful e.g. to reduce the size of libraries produced by Prosit.

## FASTA

One or several FASTA files can optionally be specified. These will be used for protein annotation. If the library does contain information on protein IDs associated with each peptide, these should match the protein sequence IDs in the FASTA database, e.g. if the FASTA database is in the UniProt format, the library should also contain UniProt IDs. If the library does not contain protein information (e.g. Prosit output library), protein information will be extracted from the FASTA database by digesting the latter in silico (using the currently specified protease, as in library-free search, see below) and matching library peptides to the digest.

If library-free search is used, the FASTA files will be digested in silico to generate a spectral library, which DIA-NN will then use to analyse the acquisitions.

Optionally, peptides considered when loading FASTA databases can be filtered using the `--fasta-filter` command, e.g. `--fasta-filter C:/human_peptides.fasta`. Peptides can be provided as a fasta file (e.g. DIA-NN recognises the format of peptide lists from PeptideAtlas builds) or as a simple list of stripped sequences (one per line); all lines starting with ‘>’ are ignored. Filtering allows to dramatically reduce the library-free search space, speeding up the analysis and improving identification performance.

## Output

During the analysis, DIA-NN generates a .quant file containing identification and quantification information for each run analysed. By default, these .quant files are placed to the same location as the raw files. Thus, it is not recommended to analyse the same raw data files simultaneously with multiple instances of DIA-NN (unless .quant files are saved to a separate location or stored in memory – see below), although multiple instances of DIA-NN can be used to analyse different experiments. The size of the .quant files is roughly proportional to the number of precursors identified at the given FDR setting and is negligible unless a library-free search without any FDR filtering is used (not recommended). The .quant files can be reused to speed up reanalysis of the data, but only if the spectral library and FASTA files specified as well as the FDR filtering settings are exactly the same as the settings that were used to generate these .quant files.

An alternative location for .quant files can be provided by specifying the “Temp/.dia dir” in the Output panel. The `--no-quant-files` command instructs DIA-NN to store .quant files in memory, without saving to disk. These options allow to analyse raw data files stored in a write-protected folder, e.g. in a network location.

## Main output

DIA-NN saves its report as a simple table in the tab-separated format (.tsv). Default output header names can be replaced (in the full report only) with those specified with the `--output-headers` command, analogous to `--library-headers` (see above). Along with the main report, DIA-NN produces a file [main report name].genes.tsv containing only gene-level information. The `--compact-report` command makes DIA-NN retain only the most important columns in the report, thus reducing the file size.

## Protein.Group

DIA-NN aims to reduce the number of proteins matched to each precursor ion. Maximal parsimony approach is implemented using a greedy set cover algorithm. Furthermore, TrEMBL proteins can be omitted, if the precursor in question is also matched to at least one SwissProt protein (this can be disabled with `--no-swissprot`).

## Protein.Names and Genes

Protein names and genes corresponding to proteins listed in the Protein.Group column.

### Protein.Q.Value

The best q-value across all proteins matched to the precursor ion. Protein q-values are calculated separately for all protein isoform names, protein names or genes (not protein groups or gene groups) – depending on the implicit protein grouping setting. Only proteotypic peptides are used to calculate q-values. Thus, when DIA-NN reports the number of proteins identified at 1% FDR (for each run separately, once it is analysed), it underestimates the number of protein groups that are actually detected. Importantly, proteins with no proteotypic peptides found always have q-value equal to 1.0 (= 100%).

### Quantities

PG.Quantity – protein group (i.e. the set of proteins listed in Protein.Group), Gene.Group.Quantity – gene group (i.e. the set of genes listed in Genes), Gene.Quantity.Unique – the gene quantified using only peptides that are specific to it. Quantification is performed using the top N method (with N = 1 by default), i.e. the intensities of the top N precursor ions (matched to the protein/gene group) identified at 1% FDR are summed for each run separately. The `--top` command allows to specify N, e.g. `--top 3` would set N to 3. Normalised – the same quantities with cross-run normalisation applied (on the precursor level). The first normalisation step is performed globally using the top 40% least variable (across runs) precursors identified at 1% FDR. These figures can be adjusted, e.g. `--norm-fraction 0.3 --norm-qvalue 0.001`. Subsequently, local (in terms of RT; can be turned off with `--global-norm`) normalisation is performed.

### Label.Ratio

Experimental. Only calculated when using `--peak-translation`. An estimate of the ratio between the quantity of the precursor and the sum of quantities of all other precursors in the same elution group and with the same charge (i.e. the only difference between the precursors is the isotopic label). DIA-NN aims to estimate the ratio only with the use of fragments that are (i) not shared by the respective differentially labelled peptides (ii) least affected by interferences. Label.Ratio can be used for improved relative quantification using SILAC or for absolute quantification using spike-in quantification standards. For LabelRatio to be calculated with high precision, the labelled and unlabelled precursors should have the same spectra (i.e. if the light peptide has fragment y7-H<sub>2</sub>O annotated with reference intensity 0.6, the heavy one should also feature y7-H<sub>2</sub>O with reference intensity 0.6 – and vice versa: this is logical, as one would expect identical fragmentation of precursors which differ only in the presence of heavy isotopes).

### Extra information

CScore and Decoy.CScore columns provide information on the scores for each precursor ion and the respective decoy, which were used to calculate the q-values. Fragment.Quant.Raw, Fragment.Quant.Corrected and Fragment.Correlations columns list raw and interference-corrected fragment intensities and correlation scores (higher = better) (the fragments being ordered by their library intensities from highest to lowest). This information allows to easily direct DIA-NN output to scripts that perform custom protein inference, quantification, etc.

### Quality control

Along with the main report, DIA-NN produces a file [main report name].stats.tsv with some quality control information for all the samples in the experiment. For example, it contains such information as the precursor and protein identification numbers, total MS1 and MS2 signal, total quantity (sum of all precursor quantities), average full width at half maximum (FWHM) for chromatographic peaks, expressed both as the number of SWATH scans and as time in minutes, mass accuracy correction data as well as retention time prediction accuracy, average precursor length, charge and the number of missed tryptic cleavages. The generation of this file can be turned off with the `--no-stats` command.

### Generating a spectral library

Precursor ions identified at the specified FDR threshold will be used to generate a new spectral library; the retention time scale used in the spectral library/training library (see below) will be retained. A new spectral library is saved in an OpenMS-compatible format. This feature allows to (i) generate spectral libraries directly from DIA data using library-free search; (ii) optimise the spectra and retention times in a spectral library for the specific LC-MS setup: this can significantly decrease the numbers of missing values. The “Optimise for training” option



instructs DIA-NN to strictly filter the fragment ions that are being saved to the spectral library. Use of this option is recommended only for generating libraries that will be used to train the peptide fragmentation predictor (see below).

### Generating Prosit input

This option allows to generate a .csv file to be used as input for the Prosit online tool (currently available at <https://www.proteomicsdb.org/prosit/>) from either a FASTA database (digested in silico using the settings specified in the Library-free search panel) or a spectral library. Prosit analysis should be exported in Spectronaut-compatible format (this produces a comma separated file but without the .csv extension, so just rename myPrositLib.spectronaut to e.g. myPrositLib.csv).

### Generating PDF report

This will instruct the GUI to launch a script (dia-nn-plotter.exe) to produce a PDF report (visualising various quality control metrics) from the DIA-NN output, once the analysis is finished. Condition and replicate IDs are inferred from the raw file names using the following procedure: after removing the common prefix and suffix, the last integer number is excised from the file names, with what remains serving as the condition identifier. For example, when analyzing C:/Raw/A1.wiff, C:/Raw/A2.wiff, C:/Raw/B1.wiff and C:/Raw/B2.wiff, DIA-NN will identify two conditions: A and B. Performance metrics, including CV values, are calculated and visualized for all the conditions separately.

## Library-free search

### Use library-free search / generate spectral library

This will instruct DIA-NN to in silico generate a spectral library from the FASTA file provided and use it to analyse the data files. In addition to the analysis report, a new spectral library can be generated (“Generate spectral library” option in the Output panel). If a spectral library is provided in addition to the FASTA file, it will be used (experimental) to enhance and speed up the library-free search. In this mode, q-values for precursor ions that belong to the library will be calculated separately.

### Training library

Specifying a training library allows DIA-NN to in silico predict peptide fragmentation patterns (only charge one y-series fragments without neutral losses are considered) using an algorithm similar to that of MS Simulator. In addition, a linear retention time predictor is trained. It is essential that if a spectral library is also specified (in the Main Settings panel), its retention time scale should be the same (e.g. both the training and the spectral libraries may use the iRT scale). It is desirable but not essential that the training library is generated on the same LC-MS setup. If no suitable training library is available, one can first run DIA-NN without it (identification performance will be somewhat worse) and then run it again using the newly generated library as the training library. Training library must include fragment charge, type (y or b) and series number information. For efficient training of the predictors at least 10'000 precursors should be present in the training library. Successful training would lead median y-series fragments Pearson correlation over 0.7-0.8 (median Pearson correlation is reported by DIA-NN). Note that using a training library is likely to bias the identification towards peptides without variable modifications, so it is not recommended to use it when looking specifically for PTMs.

### Protease

Several built-in options are available for the in silico digest. Alternatively, one can specify custom cleavage specificity, e.g. “--cut-after KR --no-cut-before P” corresponds to a canonical tryptic digest. Multiple proteases are not supported, however DIA-NN can be provided with an in silico digest in the FASTA format instead of the FASTA database.

### Modifications

In general, it is not recommended to enable variable modifications, as this slows down analysis considerably and expands the search space, potentially negatively affecting identification performance. It is essential that the removal of likely interferences is enabled (see the Algorithm settings below), if e.g. deamidation is enabled and

the runs have been acquired on an instrument that does not allow to effectively distinguish between deamidated ions and heavy isotopologues.

Arbitrary user-defined modifications can be added using the `--fixed-mod` and `--var-mod` commands, e.g. `--var-mod UniMod:5,43.005814,KR` will enable lysine and arginine carbamylation as a variable modification. Use lower-case letters for the amino acids to restrict the modification to N-terminus.

### Precursor ion mass range

This allows to restrict the range of precursors being considered, e.g. to the range covered by the SWATH cycle of the instrument. Allows to slightly reduce memory consumption.

### Fragment ion mass range

This allows to restrict the range of fragment ion masses being considered.

## Algorithm

Mass accuracies can be determined automatically (when set to the default 0.0) or specified by the user. Initially, DIA-NN performs a preliminary search with mass accuracy set to 100ppm, followed by mass correction. If the masses reported by the instrument are guaranteed not to be that much off (e.g. the instrument is regularly calibrated), this setting can be reduced, e.g. to 30ppm with `--mass-acc-cal 30`. This can produce a noticeable speed up for library-free searches.

DIA-NN can use an ensemble of neural networks to calculate q-values. By default, these are trained for a single epoch, minimizing the chance of overfitting. It is not recommended to disable the removal of likely interferences when using neural networks. The number of epochs can be increased by setting `--nn-epochs`, and the number of neural networks in the ensemble – by setting `--nn-bagging` (more = better and slower; default = 12). To almost completely eliminate any potential overfitting and allow for an increased number of epochs, neural networks can be used in a “cross-validation mode”, when each network is only used to score peptide-spectrum matches that have not been used for its training. However, in this case it is desirable to also increase the number of networks, slowing down the analysis. For example, `--nn-cross-val --nn-bagging 96 --nn-epochs 5`.

The implicit protein grouping option allows to specify the proteotypicity definition that will be used by DIA-NN. For example, if set to the default “genes”, DIA-NN will consider a peptide proteotypic, if all of its associated proteins correspond (according to the FASTA files provided) to a single gene. Proteotypicity definition affects protein q-value calculation, as the latter relies on proteotypic peptides only, and, if the protein FDR filter setting is below 100%, protein inference and grouping.

If it is known that no chromatographic peak broadening has occurred during the course of the experiment (this can be checked by looking at the peak width data in the .stats.tsv quality control report or the PDF report - these have been described above), it might be beneficial to switch the quantification strategy to “robust LC”, to somewhat improve the quantification precision. The “high accuracy” mode enables interference removal from fragment elution curves, thus making quantities more reliable (i.e. giving more confidence that what is being quantified is indeed the peptide of interest and not the peptide of interest plus some unknown interfering peptide). However, this introduces some extra noise, so if the goal is to obtain low CV values, the alternative “high precision” mode would be more suitable.

## Pipelines

Pipeline support allows to set up automatic analysis of a series of experiments, using a separate set of settings for each. The “Add to pipeline” button adds the current set of settings (including all input and output file names) to the pipeline. Selecting one of the pipeline steps (with a mouse) loads the respective set of settings. The “Update step” button overwrites the selected pipeline step with the current set of settings. Buttons with pictograms to the right from the “Clear pipeline” button can be used to open and save .pipeline files. Note that the .pipeline format is not guaranteed to retain compatibility across different versions of DIA-NN GUI.