



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

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### 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,

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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).

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. Using the GUI is recommended for any workflow that does not involve automated processing of several experiments. 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 panels. The 'Files' 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 checkbox for 'Use existing .quant files when available'. The 'Library-free search' panel contains a checkbox for 'Use library-free search / generate spectral library', a 'Training Library' text field, an 'Output library' text field set to 'lib.tsv', a 'Protease' dropdown set to 'Trypsin/P', a 'Missed cleavages' spinner set to '0', and a 'Maximum number of variable modifications' spinner set to '0'. It also features checkboxes for 'N-term M excision', 'Q/N deamidation', 'C carbamidomethylation', and 'M oxidation', as well as 'Peptide length range' (7-30), 'Precursor m/z range' (300-1800), and 'Fragment ion m/z range' (300-1800). The 'Main Settings' panel includes a 'Spectral library' text field, 'Add FASTA' and 'Clear list' buttons, 'Full report' (quant.tsv) and 'Gene report' (gene\_quant.tsv) text fields, a 'DIA-NN exe' text field (diann.exe), 'Threads' (12), 'Log level' (1), 'Precursor FDR (%)' (1.0), and 'Protein FDR (%)' (100.0) spinners. An 'Additional options' text area is also present. The 'Algorithm' panel has 'Mass accuracy' (0.0) and 'MS1 accuracy' (0.0) spinners, a 'Scan window' spinner (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 an 'Implicit protein grouping' dropdown set to 'genes'. At the bottom of the 'Main Settings' panel are 'Run', 'Not started', and 'Stop' buttons. The rightmost panel is a large area for output display. At the bottom right of the window are 'Clear log' and 'Save log' buttons.

The GUI window features four panels used to specify the data and processing settings: **Files**, **Main Settings**, **Library-free search** and **Algorithm**. The panel located to the right is used to display the output of the command-line tool. Extra commands entered in the **Additional options** text box 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.

## Files

Thermo .raw, .mzML and .dia (native DIA-NN format) files are supported. Reading Thermo .raw files requires Thermo MS File Reader to be installed. 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 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 in the same location as the raw files.

During the analysis, DIA-NN generates a .quant file containing identification and quantification information for each run analysed. These .quant files are placed in the same location as the raw files. Thus it is not recommended to analyse the same files simultaneously with multiple instances of DIA-NN, 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.

## Main Settings

### 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. DIA-NN accepts spectral libraries in a plain text format (tab-separated or comma-separated, i.e. .tsv, .csv, .xls or .txt).

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 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. The headers should be specified in the following order:

- **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.
- **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. DIA-NN does not need to recognize modifications if decoys are provided in the library (see below).
- **Precursor charge.**
- **Precursor m/z.**
- **Reference retention time.** There are no restrictions on the retention time scale used here.
- **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:

- **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. DIA-NN calculates protein q-values using proteotypic peptides only. If the protein FDR filter setting is below 100%, proteotypicity definition also affects protein inference and grouping.
- **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 only if it is told to consider isotopologues of the peptides and fragments in the library (this is enabled by default).

## FASTA

One or several FASTA files can optionally be specified. These will be used for protein annotation (if in UniProt format). If library-free search is used, the FASTA files will be digested in silico to generate a spectral library.

Optionally, peptides considered 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 search space, speeding up the analysis and improving identification performance.

## 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 will be generated. If a spectral library is provided in addition to the FASTA file, it will be used 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. If a spectral library is provided, but there is no FASTA file, this library will be used to analyse the data files and a new spectral library will be generated using only identified precursor ions (at the specified precursor and protein FDR cutoff rates) and featuring refined spectra and retention times; the retention time scale used in the spectral library will be retained. A new spectral library is saved in an OpenMS-compatible format.

### 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 library 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. For efficient training of the predictors at least 10’000 precursors should be present in the training library.

### 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 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.

### Variable modifications

For now, only deamidation (QN) and oxidation (M) have been implemented. 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 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.

## Fragment ion mass range

It is desirable to restrict this to a range within that of the instrument, if known.

## 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.

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.

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`”.

## Output

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).

### 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 are omitted from the report if the protein FDR threshold is set below 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 3 method, i.e. the intensities of the top 3 precursor ions identified at 1% FDR are summed for each run separately. Normalised – the same quantities

with cross-run normalisation applied (on the precursor level). Normalisation is performed using the top 40% least variable (across runs) precursors identified at 1% FDR.

#### 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 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.