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# Data-Independent Acquisition (DIA) Data Processing using Spectronaut/directDIA (Biognosys): Secretome Analysis

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## ABSTRACT

Proteolytic peptide measurement of secretome samples using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Acquisition by Data-Independent Acquisition (DIA) on an Orbitrap Eclipse Tribrid Mass Spectrometer for peptide/protein identification and quantification by processing DIA data using Spectronaut software.

## MATERIALS

- Spectronaut software (version 17.6.230428.55965; Biognosys)

**Protocol status:** Working
We use this protocol and it's working

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**Keywords:** LC-MS/MS, DIA, Orbitrap Eclipse, Proteomics, Mass Spectrometry, Spectronaut, Human, Secretome, SASP, Conditioned Media, Ovary

1 Search the DIA data with Spectronaut using the spectral library-free directDIA algorithm against a Human UniProt-SwissProt proteome database (https://www.uniprot.org/proteomes), applying the settings described in **Table 1**.

| Pulsar Search | | |
|---|---|---|
| **Peptides** | Enzymes/Cleavage Rules | Trypsin/P |
| | Digest Type | Specific |
| | Max Peptide Length | 52 |
| | Min Peptide Length | 7 |
| | Missed Cleavages | 2 |
| | Toggle N-terminal N | True |
| **Labeling** | Channel 1 | False |
| | Channel 2 | False |
| | Channel 3 | False |
| **Modifications** | Max Variable Modifications | 5 |
| | Fixed Modifications | Carbamidomethyl (C) |
| | Variable Modifications | Acetyl (Protein N-term)<br>Oxidation (M) |
| **Speed-Up** | Use Dynamic IM Peak Filter | True |
| | Target TIC Fraction | 0.9 |
| | MS2 Index | Automatic |
| **Identification** | PSM FDR | 0.01 |
| | Peptide FDR | 0.01 |
| | Protein Group FDR | 0.01 |
| | directDIA Workflow | directDIA+ (Deep) |
| | PTM Localization Filter | False |
| **Tolerances** | Thermo Orbitrap | |
| | Calibration Search | Dynamic |
| | MS1 Correction Factor | 1 |
| | MS2 Correction Factor | 1 |
| | Main Search | Dynamic |
| | MS1 Correction Factor | 1 |
| | MS2 Correction Factor | 1 |
| **Workflow** | Fragment Ion Selection Strategy | Intensity Based |
| | In-Silico Generate Missing Channels | False |
| | Use DNN Predicted Ion Mobility | Auto |
| **Result Filters** | Fragment Ions | |
| | Ion AA Length (min) | N = 3 |
| | Ion Charge | False |
| | Ion Loss Type | False |
| | Ion Type | False |
| | m/z | Min = 300; Max = 1,800 |
| | Relative Intensity | Min = 5 |
| | Precursors | |
| | Amino Acids | False |
| | Best N Fragments per Peptide | Min = 3; Max = 6 |
| | Best N Peptides per Protein Group | False |
| | Channel Count | False |
| | FASTA Matched | False |
| | Missed Cleavage | False |
| | Modifications | None |
| | Peptide Charge | False |
| | Proteotypicity | False |

| DIA Analysis | | |
|---|---|---|
| **XIC Extraction** | XIC IM Extraction Window | Dynamic |
| | Correction Factor | 1 |
| | XIC RT Extraction Window | Dynamic |
| | Correction Factor | 1 |
| | MS1 Mass Tolerance Strategy | Dynamic |
| | Correction Factor | 1 |
| | MS2 Mass Tolerance Strategy | Dynamic |
| | Correction Factor | 1 |
| **Calibration** | MZ Extraction Strategy | Maximum Intensity |
| | Allow source-specific iRT Calibration | True |
| | Precision iRT | True |
| | Exclude De-amidated Peptides | True |
| | iRT <-> RT Regression Type | Local (Non-Linear) Regression |
| | MS1 Mass Tolerance Strategy | System Default |
| | MS2 Mass Tolerance Strategy | System Default |
| **Identification** | Precursor Qvalue Cutoff | 0.01 |
| | Precursor PEP Cutoff | 0.2 |
| | Protein Qvalue Cutoff (Experiment) | 0.01 |
| | Protein Qvalue Cutoff (Run) | 0.05 |
| | Protein PEP Cutoff | 0.75 |
| | Single Hit Definition | By Stripped Sequence |
| | Exclude Single Hit Proteins | False |
| | Exclude Duplicate Assays | True |
| | Exclude Predicted Fragment Scores | False |
| | Generate Decoys | True |
| | Decoy Generation Method | Mutated |
| | Preferred Fragment Source | NN Predicted Fragments |
| | Decoy Limit Strategy | Dynamic |
| | Library Size Fraction | 0.1 |
| | Pvalue Estimator | Kernel Density Estimator |
| **Quantification** | Precursor Filtering | Identified (QValue) |
| | Imputation Strategy | Use Background Signal |
| | Proteotypicity Filter | None |
| | Protein LFQ Method | Automatic |
| | Quantify MS Level | MS2 |
| | Quantify Type | Area |
| | Cross-Run Normalization | False |
| | Interference Correction | True |
| | Only Identified Peptides | True |
| | Exclude All Multi-Channel Interferences | True |
| | MS1 Min | 2 |
| | MS2 Min | 3 |
| | Major (Protein) Grouping | by Protein Group Id |
| | Minor (Peptide) Grouping | by Stripped Sequence |
| | Major Group Quantity | Sum peptide quantity |
| | Major Group Top N | Min = 1; Max = 7 |
| | Minor Group Quantity | Sum precursor quantity |
| | Minor Group Top N | Min = 1; Max = 10 |
| **PTM Workflow** | PTM Localization | False |

| | | |
|---|---|---|
| **Workflow** | Method Evaluation | False |
| | MS2 DeMultiplexing | Automatic |
| | Profiling Strategy | iRT Profiling |
| | Carry-over exact Peak Boundaries | None |
| | Profiling Row Selection | Minimum QValue Row Selection |
| | Qvalue Threshold | 0.01 |
| | Profiling Target Selection | Automatic Selection |
| | Run Limit for directDIA Library | -1 |
| | Unify Peptide Peaks Strategy | None |
| **Protein Inference** | Protein Interference Workflow | Automatic |
| | Inference Algorithm | IDPicker |
| **Post Analysis** | Differential Abundance Testing | Unpaired t-test |
| | Assume Equal Variance | False |
| | Group-Wise Testing Correction | False |
| | Difference Abundance Grouping | Major Group (Quantification Settings) |
| | Smallest Quantitative Unit | Precursor Ion (Quantification Settings) |
| | Use All MS-Level Quantities | False |
| | Calculate Explained TIC | None |
| | Calculate Sample Correlation Matrix | False |
| | Hierarchical Clustering | True |
| | Distance Metric | Manhattan Distance |
| | Linkage Strategy | Ward's Method |
| | Order Runs by Clustering | True |
| | Z-score Transformation | False |

**Table 1.** Settings for the data-independent acquisition data processing with Spectronaut/directDIA