User Manual

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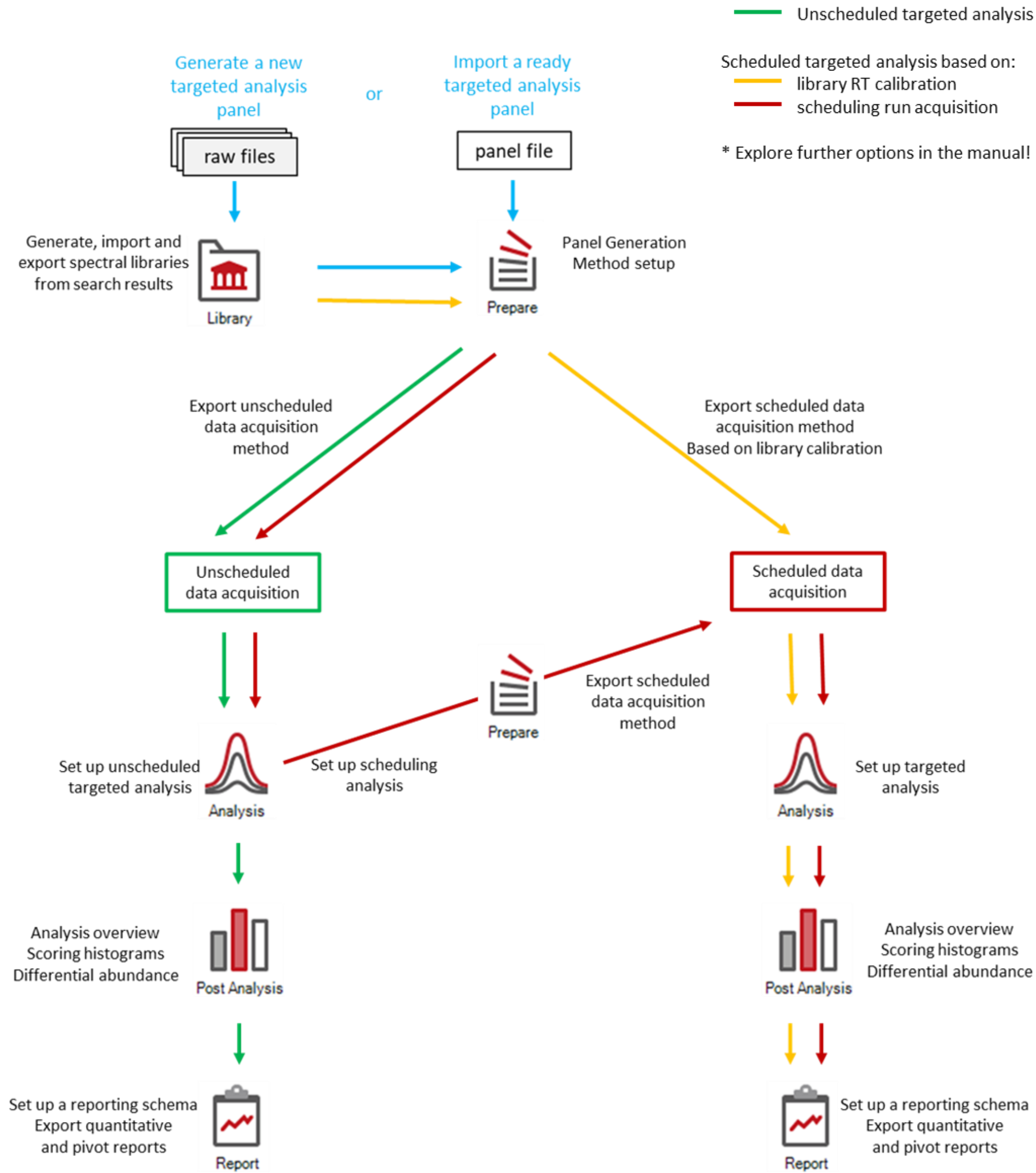
2 SpectroDive at a glance



3 General Information

3.3 Scope of SpectroDive™

SpectroDive™ is the Biognosys' proprietary software for automated MS acquisition method set-up, ion signal processing and data analysis for targeted proteomics such as PRM and MRM experiments. It features:

- High Precision iRT Calibration
- Powerful XIC peak picking
- Immediate and intuitive data visualization
- Customized data reporting
- Fully automated quality control

3.4 SpectroDive™ 11 Release Features

New Absolute Quantification Workflow

- ❖ Creation and visualization of calibration curves
- ❖ Determination of limits of detection (LOD) and of quantification (LLOQ, ULOQ)
- ❖ Quantification of samples using calibration curves

Ion Mobility Support

- ❖ FAIMS-PRM: analysis and method export
- ❖ prm-PASEF: analysis and method export

Support for New Acquisition Methods

- ❖ Hybrid-DIA: analysis and method export for targeted peptides

Improved Performance

- ❖ Improved peak picking
- ❖ Improved Pulsar search engine for panel generation

Improved User Experience and Visualization

- ❖ Improved XIC Alignment plots with support for unlimited number of runs
- ❖ Detachable perspective and plots for side-by-side visualization
- ❖ Improved XIC grid overview plots
- ❖ One-click tree navigation in plots
- ❖ Improved plot and tree filter selection
- ❖ Quick-Action bar for analysis perspective

- Improved UI for analysis log
- New prm-PASEF mobilogram plot
- Streamlined settings for clarity with new advanced category
- Switched to a 2-color system for identification status annotation

3.5 Operating System Requirements

SpectroDive™ is only available for Windows operating systems. Command line operation is also supported (see section 5.11.5). The system specifications are:

Specifications	Minimum	Recommended
Operating System	Windows 10, x64	Windows 10 or higher, x64
CPU	Intel® Core™ CPU, 2.7 GHz (quad core) or similar	Intel® Core™ i7 4770, 3.4 GHz (octa core) or similar Intel or AMD CPU with 4 or more cores
Hard drive	500 GB free space	500 GB free space, solid state drive (SSD)
RAM	16 GB	64 GB or more
Software	.NET Framework 4.7	.NET Framework 4.7 or higher

3.6 Before you start Post installation recommendations

Post installation recommendation for performance improvement:

1. **Directories**: SpectroDive™ will set all directories in the *C:* drive by default. However, it is likely that the *C:* drive has a limited storage capacity. Thus, we **strongly recommend** changing the Temporary Directory and the Local Search Archives directories to a local destination with enough available memory. To do that, go to the *Settings Perspective* → *Global* → *Directories*.

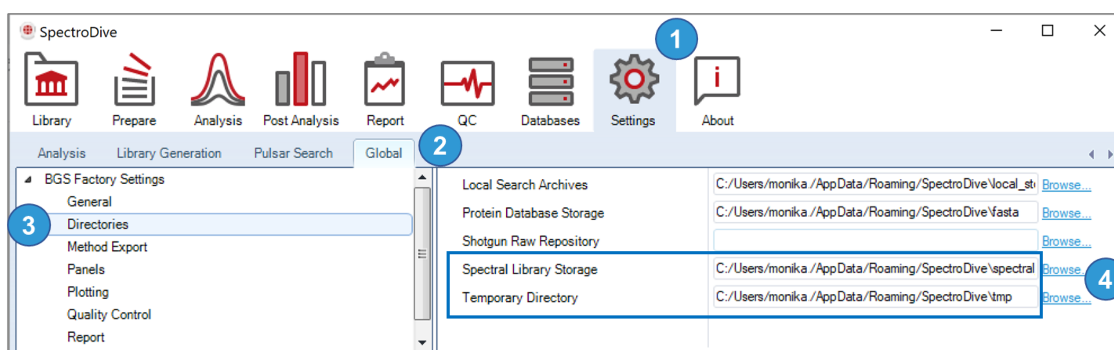


Figure 1. Change the default location for temporary files and Search Archives.

2. **Network drives and virtual machines**: We recommend running SpectroDive locally, i.e., having the resources (especially raw files, search archives and temporary directories) on a local drive. The use of virtual machines for SpectroDive is feasible but not advised. A failure in the connection to any network drive can cause SpectroDive to abort the process due to third-party library dependencies.

3.7 Supported Mass Spectrometers

SpectroDive™ supports mass spectrometers from Thermo Scientific™, SCIEX, Agilent and Waters. The specific supported models are:

MRM (triple quadrupole -QQQ- and hybrid quadrupole-linear ion trap -QTrap- MS)

- Thermo Scientific™ TSQ Series I and II
- SCIEX API Series™
- SCIEX Triple Quad™
- SCIEX QTRAP Series
- Waters Xevo TQ-S

PRM (hybrid quadrupole-Orbitrap -Q-OT- and quadrupole time-of-flight -Q-TOF- MS)

- Thermo Scientific™ Q Exactive™ Series
- Thermo Scientific™ Orbitrap Fusion™ Series
- Thermo Scientific™ Orbitrap Exploris™ Series
- SCIEX TripleTOF® Series
- Bruker timsTOF™ Pro, timsTOF™ Pro 2, timsTOF™ SCP, timsTOF™ HT, timsTOF™ Ultra and timsTOF™ Flex operated in the LC-ESI mode.

SureQuant and HybridDIA

- Thermo Scientific™ Orbitrap Fusion™ Series
- Thermo Scientific™ Orbitrap Exploris™ 480

3.8 Supported Data Acquisition Methods

SpectroDive™ analyzes targeted proteomics data at fragment ion level (MS2). Minimum requirements are a reversed phase chromatography with either a linear or nonlinear gradient. SpectroDive supports both MRM, PRM, SureQuant and hybridDIA data acquisition methods. An introduction to these acquisition methods can be found in the following Biognosys Webinar. Moreover, SpectroDive supports analysis of PRM experiments acquired with ion mobility dimension data like prm-PASEF and FAIMS PRM. In case you experience technical problems with the software or if you have feature suggestions please contact support@biognosys.com.

4 Getting Started

4.3 Installing SpectroDive™

The SpectroDive™ software licenses can be requested through our webpage. We also provide free trial licenses upon request. After requesting a trial license, you will get an email with a **link to our installer** and an **activation key** for the software.

NB! *The activation keys are bound to a single individual computer. If you need to install SpectroDive on more than one computer, please contact us at support@biognosys.com.*

4.4 SpectroDive™ Activation

When you install and start SpectroDive™ for the first time, you will be asked to activate your software by pasting your activation key into the SpectroDive activation dialogue. If your computer can reach our servers online, the activation will be automatic. If your SpectroDive computer does not have access to our servers, you can also activate your SpectroDive copy offline. The respective instructions will appear after a few seconds if online activation was not successful. Please save the registration information file on your computer and send this file to support@biognosys.com. In general, you will receive an activation file within one or two working days. To activate SpectroDive using an activation file, click on the "Browse License File…" button in the SpectroDive Activation dialogue.

4.5 Demo Data

Our MRM and PRM demo data is available for download here.

4.6 Tips for Better Experience

SpectroDive is full of informative tool-tips throughout the software. They will appear as you hover over many of the elements. There are many functionalities accessible by right-clicking an element: experiment tab, plots, nodes, etc. (Figure 2).

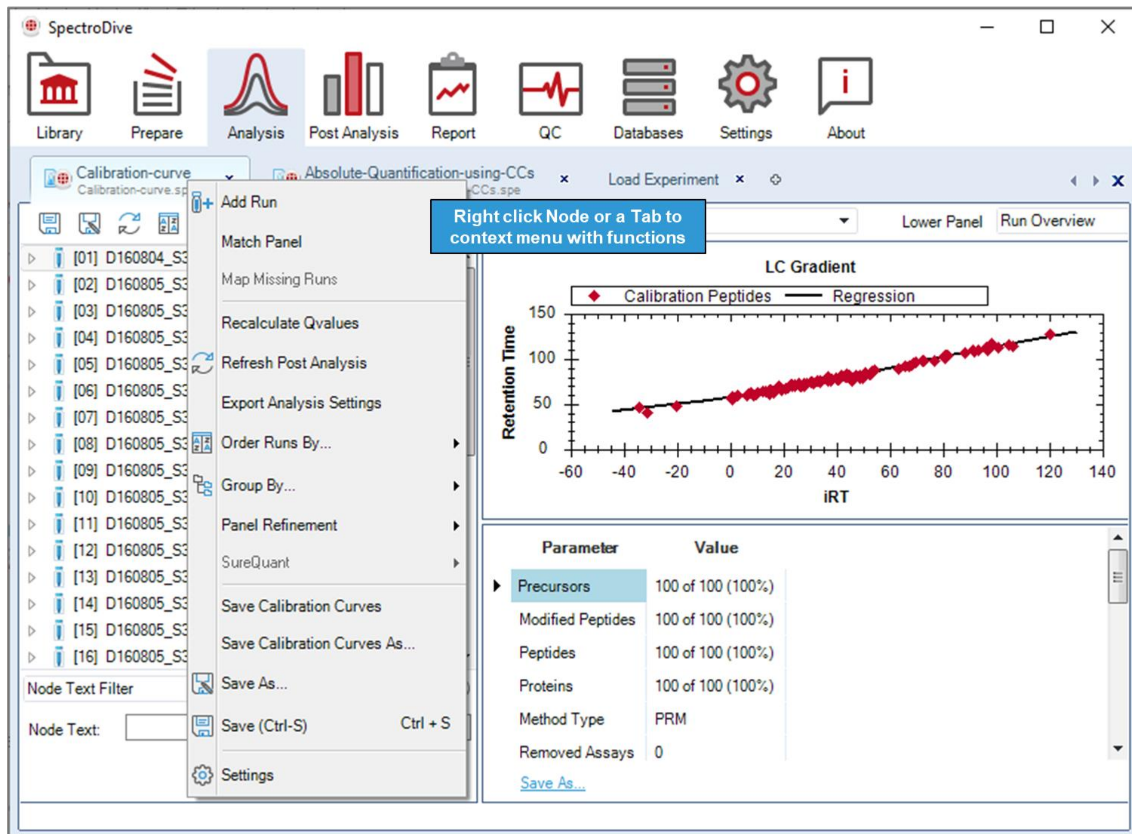


Figure 2. SpectroDive contains tips and menus when you right-click on many elements. The most common actions are available through intuitive icons in the experimental tab.

4.7 Ready-made Assay Panels

Multiplexed, ready-made assay panels can be imported when starting an experiment analysis by selecting a Biognosys' reference peptide kit file (*.*kit*) or importing an external assay panel file. Biognosys also offers high-quality commercial assay panels for MRM and PRM analysis. For example, for routine investigations of plasma samples Biognosys developed PQ500, a ready-made stable isotope standard peptides kit, for the targeted analysis of more than 500 proteins for human plasma profiling. You can read more about the PQ500 and other kits on https://www.biognosys.com/shop#mrmassaypanels. It is also possible to generate customized panels for your own needs by contacting us via support@biognosys.com.

5 SpectroDive™ Usage

5.3 Structure of SpectroDive™

5.3.1 Layout

SpectroDive™ is structured in different levels (Figure 3). The highest level is the Perspectives level. Within each Perspective, you can often find several pages separated into tabs. The layout of each page is normally structured into a left menu (tree) containing elements (nodes) and a right panel containing information related to the selected nodes (plots, repots and summaries).

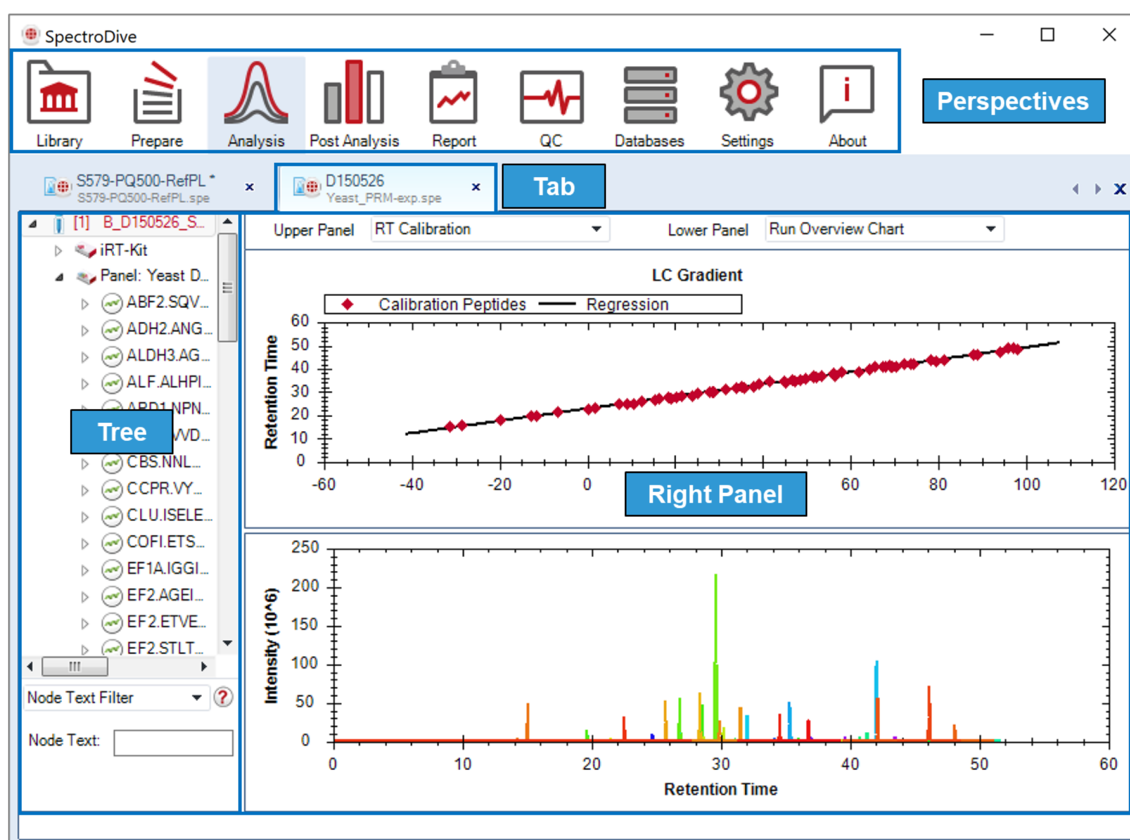


Figure 3. SpectroDive general layout structure. The display of Perspectives, Tab, Tree and Right panel is indicated by blue arrows and rectangles.

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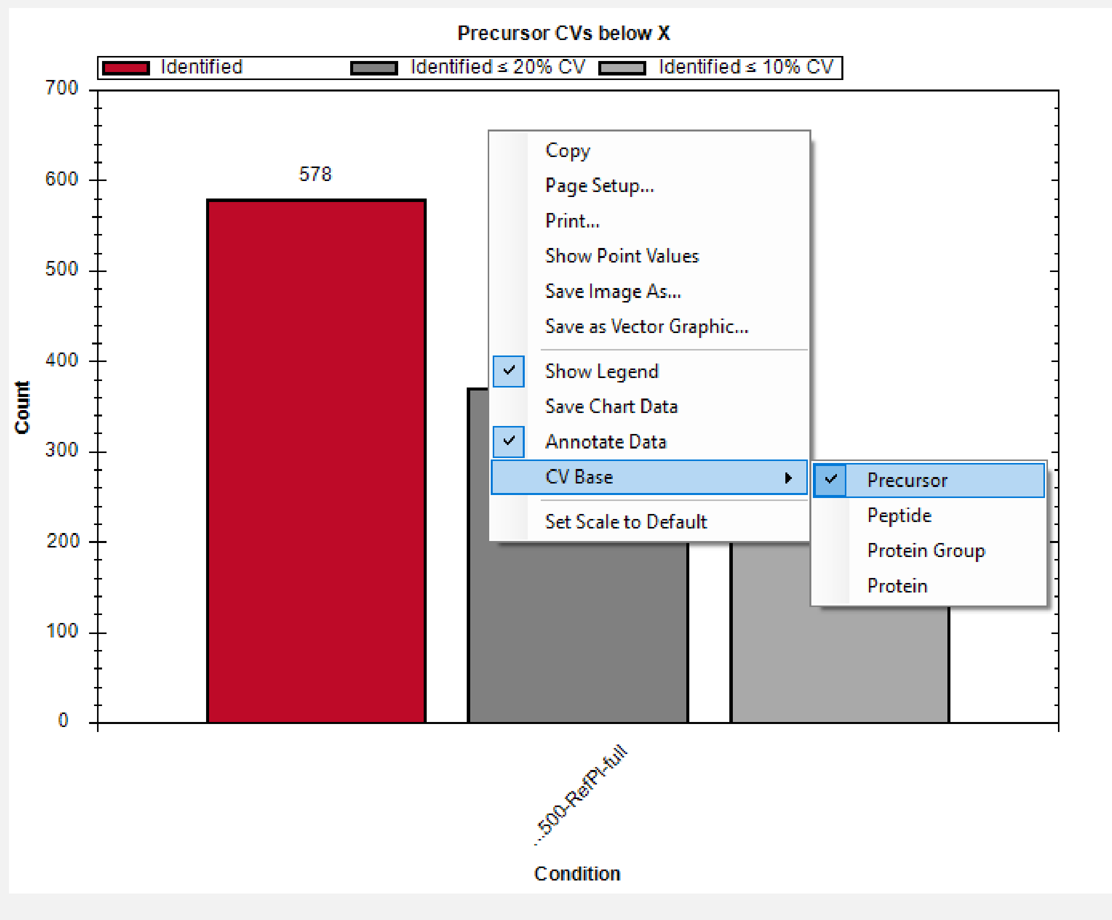
Box 1. Data visualization in SpectroDive: how to get the most out of it

SpectroDive provides, across all its perspectives, a comprenhensive number of plots of many types. Relevant details about your analysis, from panel generation, MS data analyis to post-analysis results as well as quality control, are shown in these plots. Most SpectroDive's plots are interactive and customizable to some extent. For example: zoom in on a plot by selecting the area you want to enlarge (find back to default scale by right-clicking on the plot). Drag or navigate a plot horinzontaly by Ctrl +click and drag. By right-clicking on a plot, you will find a contact menu with a large list of functinalities (see figure below). In this example, you can show or hide the legend, save the data used for the plot, choose the unit you want to show numbers for (protein, peptides), among many others.



5.4 Library Perspective

The Library Perspective in SpectroDive™ allows to build and manage spectral libraries which are used as assay pools for generating comprehensive MRM and PRM assay panels.

These are the main tasks you can perform in the Library Perspective of SpectroDive:

1. Generate a library with Pulsar, Biognosys' proprietary search engine (section 5.4.1).
2. Generate a library using search results from external search engines. Currently supported database search engines are MaxQuant (Cox *et al.*, 2011), Proteome Discoverer™, ProteinPilot™, Mascot, generic search result format mzIdentML (**.mzid*) and the BGS Generic Format (**.txt, *.tsv, *.csv*).
3. Import an external library (**.txt, *.tsv, *.csv, *.xls, *.kit*).

5.4.1 Library Generation from Pulsar

Pulsar is Biognosys' proprietary search engine, integrated into SpectroDive for library-based assay panel generation. Pulsar can search data-dependent acquisition (DDA), data-independent acquisition (DIA) and parallel reaction monitoring (PRM) data with full scan MS1 information. Both centroid and profile data can be processed. Pulsar is designed to be fast and to scale with the number of runs. Further, Pulsar can identify co-fragmented peptides in multiple search rounds by subtracting previously identified fragment ions from the spectra. False identifications are controlled by a false discovery rate (FDR) estimation at three levels: peptide-spectrum match (PSM), peptide, and protein group.

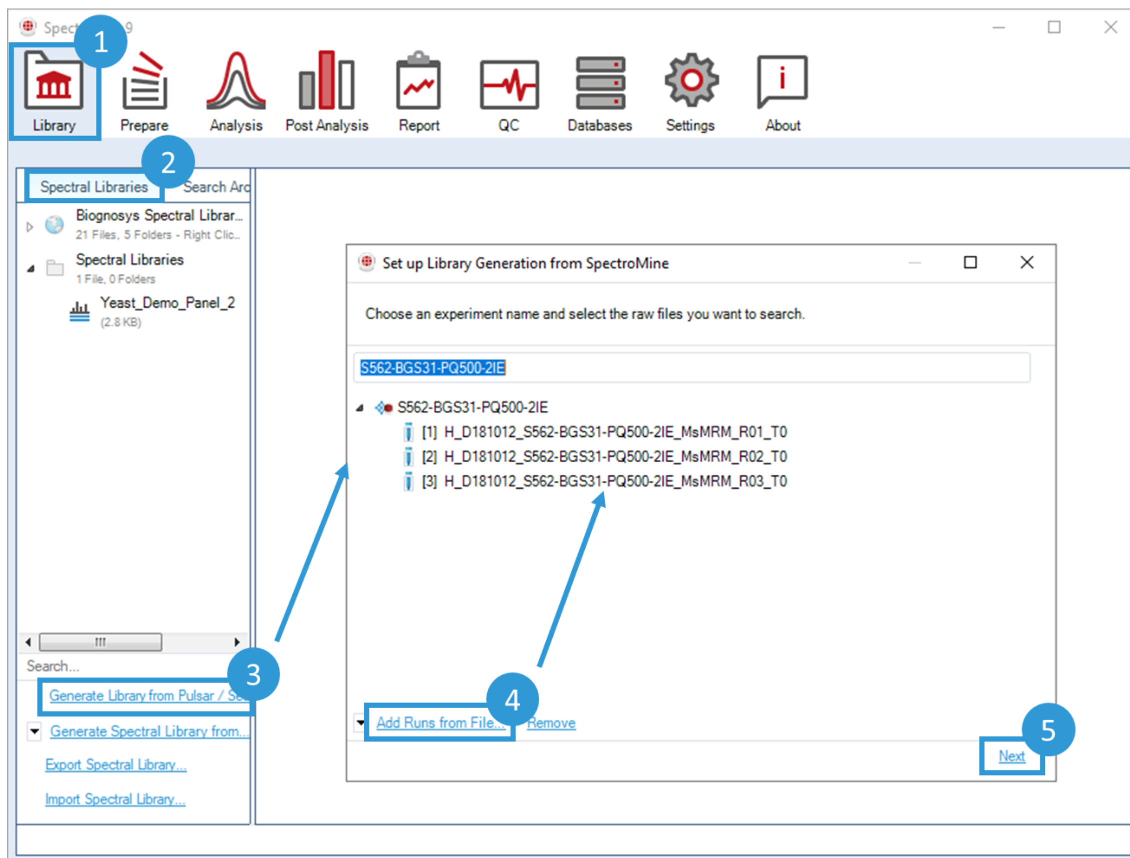


Figure 4. Library generation from the search engine Pulsar. In the Library Perspective and the Spectral Libraries tab, click "Generate Library from Pulsar / Search Archives…". Follow the wizard to complete the library generation process.

Every time a Pulsar search is performed, SpectroDive will save the results (PSMs) for each run as a Search Archive (Box 2). These Search Archives can be used to generate libraries without the need to search the runs from scratch again. Additional run files can be combined with existing Search Archives conveniently to generate new libraries. Refer to Table 1 to see a summary of the resources you will need in each of these cases.

To generate a library from Pulsar, go to the Library Perspective and click on "Generate Library from Pulsar / Search Archives…" in the bottom left corner. A wizard will appear to help you set up the experiment in sequential steps(see Figure 4 and Table 2). **Set up Library Generation from Pulsar**.

1. Firstly, choose an **Experiment name**. Then click on "Add Runs from File…" or "Add Runs from Folder…" and select the runs from which you want to create the library. You can mix runs acquired in different modes.
   NB! *If you want to generate a library from Search Archives only, skip this step.*

Table 1. SpectroDive can generate libraries using different input resources

Library based on	FASTA file
Run file(s) only	**required**
Search Archive(s) only	*not applicable*
Run file(s) and Search Archive(s) combined	**required**

2. **Choose FASTA File(s)** by clicking "Fasta File…". Protein databases can be assigned on a run basis. Multiple protein databases can be selected. FASTA files can be added at this step by clicking "Import…" in the bottom left corner.
   NB! *If you are doing a library from Search Archives only, skip this step.*
3. **Choose Pulsar Search Settings** by clicking "Search settings…" (for detailed explanations about each setting, see Appendix 2. Pulsar Search Settings, section 7.4).
4. Choose either the default **schema** which can be modified on the fly or a previously saved settings schema. Schemas can be assigned at either the experiment or run level. Only one settings schema can be set per run. When nothing is selected for a run, default settings will be applied.
   NB! *If you are doing a library from Search Archives only, skip this step.*
5. Next, you can add Search Archives (for more information see Box 2) to your library. Search Archives prevent you from having to research run files if you have already searched them in the past.
6. The next wizard page contains experiment-wide settings for library generation, such as PSM, peptide and protein FDR thresholds (for a detailed explanation about each setting, see Appendix 4. Library Generation Settings, section 7.5).
7. The last page shows an overview of the whole experiment set-up. Clicking "Finish" will start the experiment. By using the "View Live Log…" it is possible to follow the progress of the experiment. As soon as the library is generated, it will appear in the library tree. Libraries with FASTA files assigned are marked with a blue protein icon.

Box 2. Search Archives as a new concept in SpectroDive™.

Previously, already searched raw files had to be searched again from scratch in order to include them in a library with other runs and to maintain control of the FDR. This resulted in a great amount of time and computational resources which had to be reinvested.

With **Search Archives**, every time a library is generated using Pulsar, the search result is saved as a Search Archive and will appear in the Search Archive tab of the Library Perspective. Search Archives contain the information from a search before applying any FDR filter. This allows several Search Archives to be combined, or Search Archives with runs files, to generate libraries with a proper, library-wide control of the FDR.

There are two types of Search Archives:

1. Search Archives from run files (complete Search Archives). These archives contain all the information related to the Pulsar search of each individual run file.
2. Search Archives from other archives or from combinations of archives (meta-Search Archives). These archives contain the metainformation of:
    - A re-utilized complete archive
    - A combination of archives

When a library is generated by combining search archives and run files, a complete Search Archive will be stored for each run file and a meta-Search Archive will be generated containing the information of the combined analysis. When meta-Search Archives are used, the process will be directed to the corresponding complete Search Archives to retrieve the relevant information.

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Table 2. The sequential wizard steps required during library generation depending on the input

Library generation step	Run files only	Run files and Search Archives combined	Search Archives only
1. Choose an experiment name	**Required**	**required**	**Required**
2. Add runs	**Required**	**required**	*not applicable*
3. Select the protein databases (FASTA)	**Required**	**required**	*not applicable*
4. Choose Pulsar Search Settings	**required** *(with default)*	**required** *(with default)*	*not applicable*
5. Choose Search Archives	*not applicable*	**required**	**Required**
6. Choose library settings	**required** *(with default)*	**required** *(with default)*	**required** *(with default)*

5.4.2 Library Generation from External Search Engine Results

To generate a library from external search engine results, you will need:

1. The search result files or folders → **required**
2. The run files from which the search was done → **required**

SpectroDive supports search results from:

- MaxQuant (Cox *et al.* 2011)
- Proteome Discoverer™
- ProteinPilot™
- Mascot

Table 3 summarizes the type of files or folders needed for each search engine, and whether some actions are required for correct integration of the post-translational modification (PTM) annotations (Table 3).

In addition to the specific result formats above, SpectroDive also supports results in **mzIdentML** format (**.mzid*, the HUPO-PSI standard for protein/peptide identifications, containing only fragment ion information, not mass spectra). Finally, any search results can be reformatted into the **Biognosys (BGS) Generic Format**.

Table 3. Supported search engines and information required by SpectroDive when generating a library from search results

Search engine	Search result	Peptide modifications	
		Default	*Custom*
MaxQuant [a]	Folder with search result files	Included	Import (**.xml* file) from MaxQuant installation folder (\bin\conf\modifications.xml)
Proteome Discoverer	**.msf* for PD 1.4 **.pdResult* for PD > 2.0	Included with the search results	
Protein Pilot	**.xls* (MS Excel) with the suffix "_FDR"	Included	Import "*Unified Modification Catalog.xlsx*" file located in Program Files\ProteinPilot\Help folder
Mascot	**.dat* file	Included [b]	Add manually (see 5.10.2.2)
Any	**.mzid* file and the BGS Generic Format	Included	Add manually (see 4.8.2.2)

[a] Detailed information on how to setup a MaxQuant search for spectral library generation can be found in the Biognosys Science Hub at www.biognosys.com/science-hub.

To generate a library from search results:

1. Go to the Library Perspective → Spectral Libraries and click on "Generate Spectral Library from…" in the bottom left corner (Figure 5). Choose your search engine.
2. Navigate to the files or folders containing the search results (see Table 3). SpectroDive will try to map the run files automatically (see Box 3). If it fails to do so, you will have to manually link the files by clicking "Assign Shotgun Files…"
3. Choose your library settings in the Library Settings panel or run under default settings. (for a detailed explanation of each setting, see Appendix 4. Library Generation Settings, section 7.5).
4. Choose a FASTA file in the FASTA File tab. If your FASTA file is not yet in the tree, you can add it at this point by clicking "Import…" in the bottom left corner. By clicking "Load", SpectroDive will perform the library generation. Your new library will automatically appear in the Library Perspective upon completion.

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5.4.2.1 *Spectral Library Generation from Biognosys (BGS) Generic Format*

SpectroDive supports generating spectral libraries from the minimal BGS Generic Format. This allows end-users to use their favorite search engine with the aid of a basic script which would convert their search result into BGS Generic format. This is a plain-text format where each row represents a PSM. Table 4 shows the information required in this file.

Table 4. Information of the search results contained in the BGS generic format

Header	Information	
Raw File	The name of the DDA file in which this PSM was found. This column is used to map the DDA file to the PSM.	**required**
Stripped Sequence	The stripped sequence of the peptide that was found by the search engine for this PSM.	**required**
Precursor Charge	The charge that was associated with this PSM by the search engine.	**required**
Labeled Sequence	The sequence with encoded modification and label information. The only requirement is that the modification/label information should be enclosed in [modification/label] or (modification/label) brackets.	**required**
Retention Time	The retention time of the PSM. If available, retention time at apex intensity should be used.	**required**
Scan Number	The scan number of the PSM.	**required**
Scan Event	The scan event of the PSM. ***Note!*** *Only relevant for the SCIEX WIFF files.*	**required**
MS1 Intensity	The intensity of the PSM as reported by the search engine.	**recommended**
Protein Group Id	The protein group assigned by the search engine for this peptide. It is not necessary if using protein inference in SpectroDive.	**recommended**

Box 3. Mapping run files to search results

In the process of library generation, SpectroDive will try to map the run files automatically by name matching to the search results. First, it will look for raw files in your Shotgun Raw Repository (Settings Perspective → Global → Directories). If unsuccessful, it will look in the Search Archives location (the specified directory for "Local Search Archives"). If the automatic mapping fails, you will see a red cross. ✗

If this is the case, you will have to manually map the runs. Click on "Assign Shotgun Files…" to find the missing runs. You can either navigate to a common directory or browse for your runs individually. After the runs have been found, the red cross will change into a green tick mark. ✓

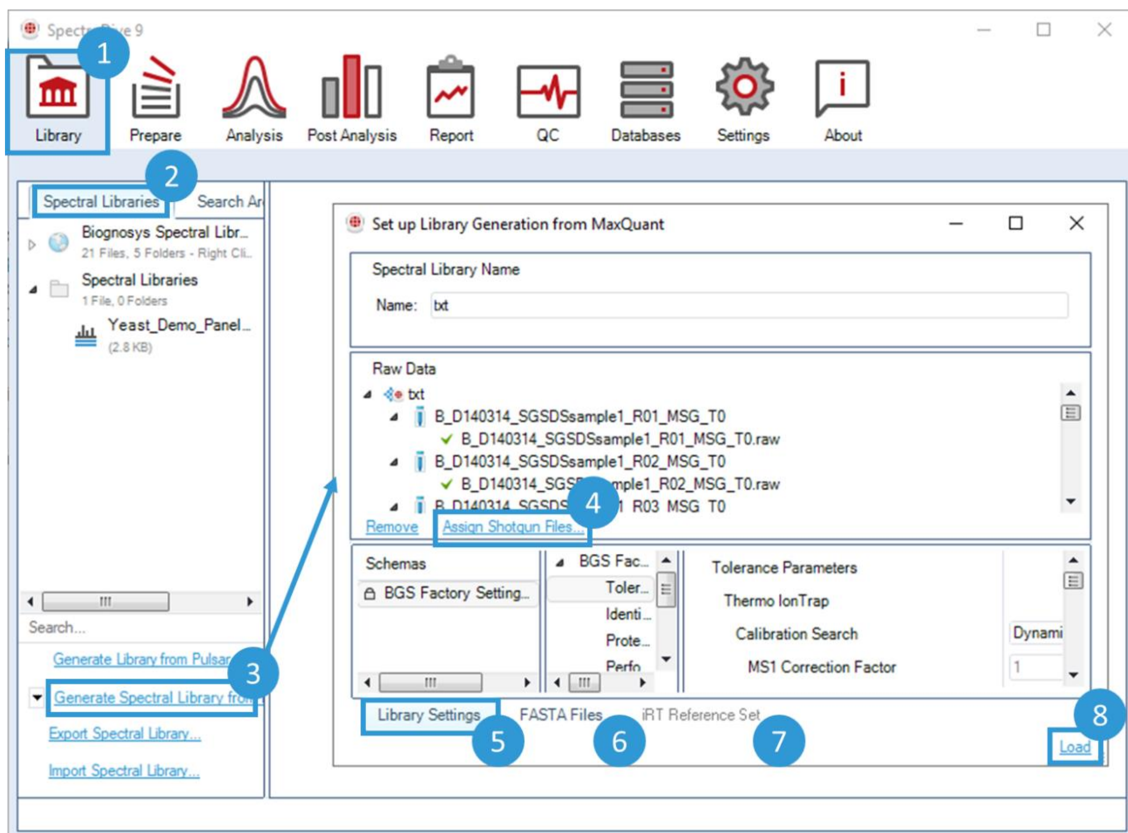

Figure 5. Library Generation from external search engines. In the Library Perspective, the Spectral Library tab, click "Generate Spectral Library from…" and choose the search engine. Load your search results and assign your shotgun raw data files (Box 3). Follow the wizard to complete the process.

5.4.3 Importing an External Spectral Library

To import an external library into SpectroDive, click on "Import Spectral Library…" in the bottom left corner of the Spectral Libraries tab in the Library Perspective (Figure 6).

There are two ways of importing a library into SpectroDive:

1. Importing a **.kit* library (BGS library format). In this case, no further action is required, and the library will be loaded automatically into the Library tree.
2. Importing a compatible table as a plain text, separated value format (**.txt*, **.csv*, **.tsv*, **.xls*) with headers. For a library in table format to be imported in SpectroDive, it must comply with the minimal required information. The mandatory headers are shown in Table 5.

Table 5. Required information for importing a Library file in SpectroDive

Header	Refers to
Q1	The *in silico* calculated *m/z* of the precursor ion, based on the peptide sequence. Do not round this number.
Q3	The *in silico* calculated *m/z* of the fragment ion, based on the peptide sequence. Do not round this number.
iRT	Indexed Retention Time - the peptide retention time in the reverse phase chromatography converted into a dimensionless value (Escher *et al.* 2012). If accurate iRTs are provided, the analysis will speed up significantly, and the quality of your results will increase (sensitivity, specificity, accuracy). If no iRTs are available, SpectroDive will predict an iRT for each peptide. This is less accurate than an empirically determined iRT value. To derive iRTs for your peptides, we recommend spiking Biognosys' iRT Kit peptides into your samples for shotgun runs. If the Library Perspective is used, iRT values will be automatically determined.
PrecursorCharge	The peptide precursor ion charge. This information is used to label your precursors in SpectroDive and to automatically generate a unique ID for your precursor if necessary.
RelativeFragment Intensity	The relative peptide fragment ion intensity expressed as a percentage of the most intense fragment ion. Please report the raw number without the percentage sign. This information improves the limit of detection.
FragmentCharge	The fragment precursor ion charge. This information is used for labeling and scoring your fragment ions in SpectroDive.
StrippedSequence	The stripped amino acid sequence of the peptide excluding any modifications. Please only use the single letter code for the 20 standard proteinogenic amino acids. This information is used for labeling and scoring your fragment ions in SpectroDive. Further, it can be included in the automatically generated unique ID for your precursor.
FragmentNumber	The peptide fragment ion number. This number should be between 1 and the length of your peptide in amino acids minus one. This information is used to label and score your fragment ions in SpectroDive.
FragmentType	The peptide fragment ion type. Usually, this is "y" or "b". This information is used to label and score your fragment ions in SpectroDive.

Header	Refers to
ModifiedSequence	If your peptide is modified use this column to specify the amino acid sequence including modifications. The modified sequence should be constant for one unique precursor. This information is used to label your precursors in SpectroDive and automatically generate a unique ID if necessary. SpectroDive will try to parse and map modifications from the provided sequences to the internal modification database. This field does not contain any label specific modifications.

The "Import Spectral Library…" dialogue (Figure 6) will try to auto-detect column names. If there are new column names, SpectroDive will ask whether you want to store them as a recognized synonym for the specific column name. This allows SpectroDive to automatically select these columns the next time you load a spectral library with a similar format. You can remove any user-defined column synonyms in the Databases Perspective under Table Import.

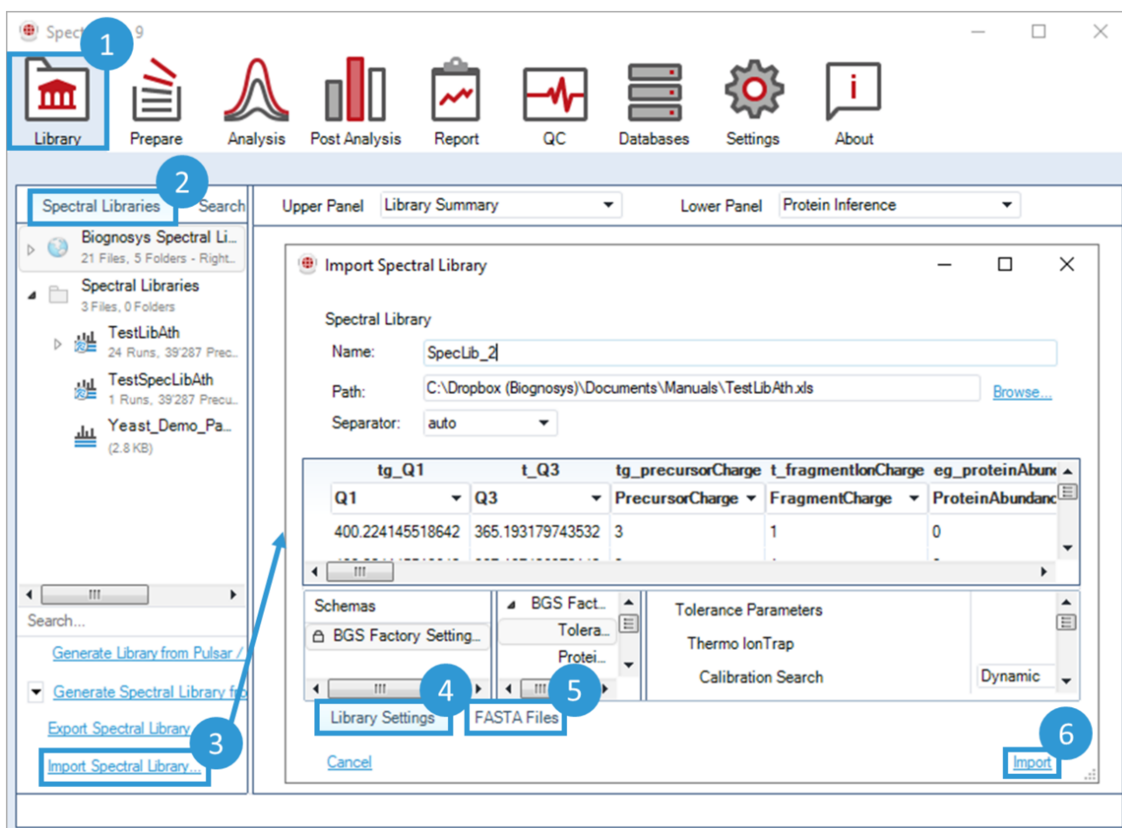

Figure 6. Importing an external library. The "Import Spectral Library…" dialog only applies to formats different than **.kit*. You can refine your library using the "Library Settings" and "FASTA Files" tabs.

The import function also allows you to refine your library. In the Library Settings panel, you can choose several options to be applied to your library (for details, see

Appendix 4. Library Generation Settings, in section 7.5). For example, you can perform protein inference again. To do this, go to the FASTA File panel and choose your protein sequence database. To view an example of a library, see our online SpectroDive Support Materials or export a library from the Library Perspective in **.xls* format.

5.4.4 Spectral Library Overview

SpectroDive provides several data visualization plots for obtaining an overview of your spectral library. You can access these plots by clicking on any spectral library in the tree and then selecting an appropriate plot type in the right panel (Figure 7).

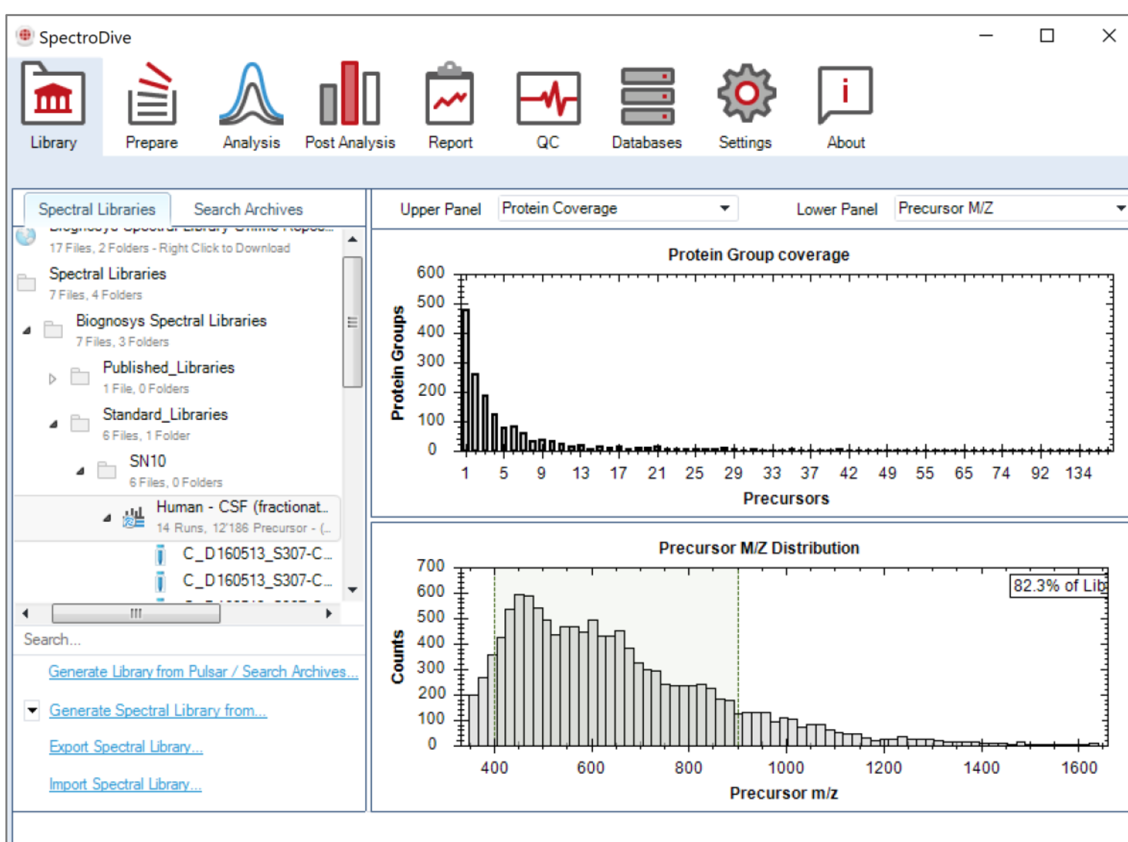

Figure 7. Spectral Libraries overview. Several plots can be selected from the drop-down menus. In this example, the top plot shows the Protein Group coverage, while the bottom plot shows the Precursor *m/z* distribution spread.

5.4.5 Making a Labeled or a Spike-in Library

A labeled or spike-in library can be generated through the library generation wizard. By selecting “Labeling” you can append heavy label channels or spike-in reference channel. You can select which labels should be applied by selecting from the list of modifications.

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5.5 Prepare Perspective

In MRM/PRM experiments (as well in SureQuant and hybridDIA), an assay panel represents a detailed transition list that is necessary to acquire and analyze the data. The Prepare perspective allows you to generate and edit such assay panels. It is organized in terms of sequential steps: 1) Generating or selecting an assay panel, 2) Specify a calibration run to be used for scheduled analysis (optional), and 3) Selecting your parameters for and exporting your scheduled or unscheduled instrument-specific acquisition method. The organization of these steps is presented in Figure 8.

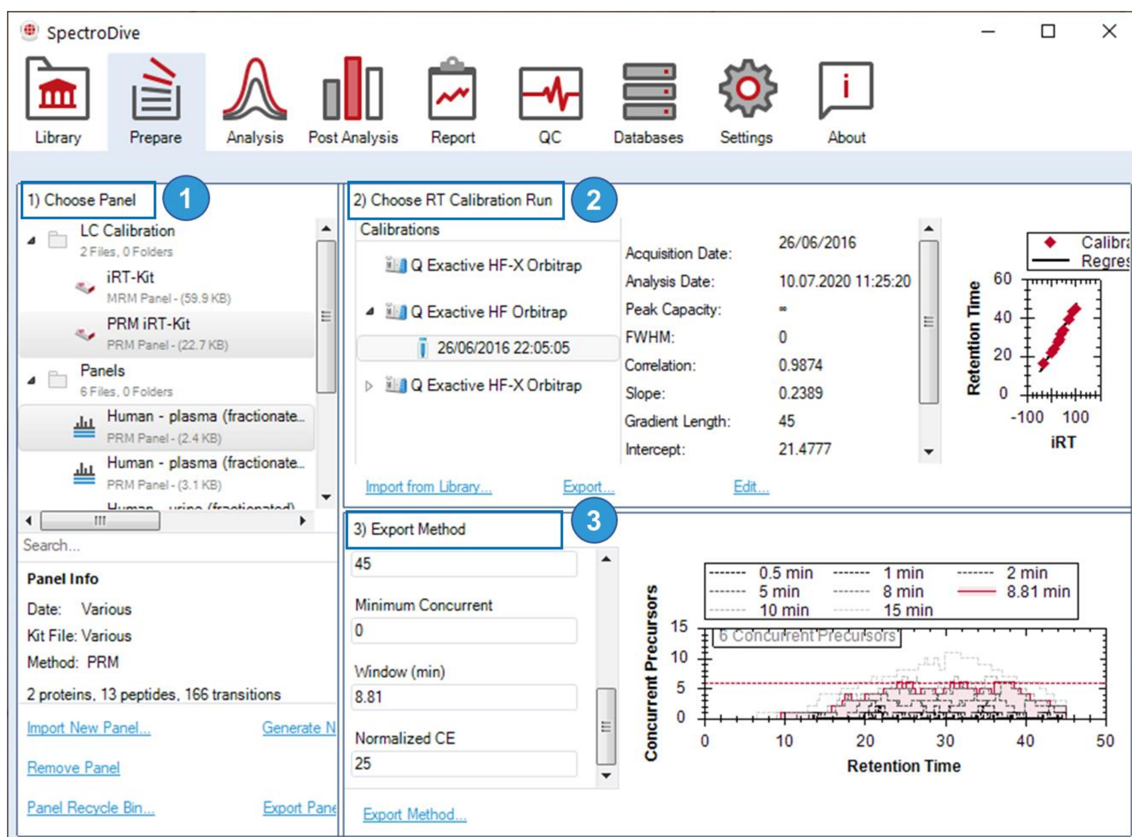

Figure 8. The Prepare Perspective consists of panel generation space (1), RT calibration space (2) and export of instrumental method space (3).

5.5.1 Generating Panels from Spectral Libraries

You can generate new targeted panels directly from a spectral library. In order to do that you first need to have the library loaded into the Library Perspective in SpectroDive, either generated with Pulsar (recommended), or from third party search results, or imported. Once you are set up, click on “Generate New Panel…” in the Prepare Perspective of SpectroDive to start the panel generation wizard. The wizard will guide

you through the process step by step. First you will be asked to select the library that you will use for the generation of the new MRM/PRM panel.

While creating the new panel, it is very convenient to search for the protein of the interest in the library by using search window (Figure 9). Secondly, you can preselect precursors based on a chosen criterion, like precursor m/z range or peptide length. Moreover, you can specify selection for fragment ions by choosing for example specific ion type or allowing specific neutral losses. You will also be able to modify your panel by defining new label channel for your precursors.

Figure 9 Generation of MRM/PRM panel directly from spectral library. A wizard will guide you through multiple filters and options that will allow you to select presursors and transitions for your panel.

NB! If you have used fractionated samples to generate a spectral library (for example Off-gel IEF, or high pH RP), this will have an impact on the peptide ranking within the library and such ranking should not be used in the panel generation process.

5.5.2 Importing Assay Panels

You can import assay panels into SpectroDive by selecting the "Import New Panel…" link and choosing either a Biognosys' KIT file (**.kit*) or a compatible spreadsheet in a plain text format. The panel imported from a spreadsheet must follow few formatting rules:

- Plain text, in a separated value format (**.tsv* or **.csv*)
- The column headers need to match to the SpectroDive specific column requirements

If a text-based file is used, a KIT Import Manager will be displayed which will try to auto detect the column names (Figure 10). If no automatic association is suggested, you can manually assign and store a new synonym for the column. User defined synonyms are managed in the Databases Perspective → Table Import tab.

Import Panel

Source

Path C:\Dropbox (Biognosys)\Documents\Manuals\TestLibAth.xls Browse…

Separator \t

Data

tg_Q1	t_Q3	tg_precursorCharge	t_fragmentIonCharge	eg_proteinAbundanceRank	eg_aminoAcidSequence	eg_
Q1	Q3	PrecursorCharge	FragmentCharge	ProteinAbundanceRank	StrippedSequence	Moc
400.224145518642	365.193179743532	3	1	0	IHNFGLIQEK	_IH
400.224145518642	387.187426372142	3	1	0	IHNFGLIQEK	_IH
400.224145518642	404.213974757762	3	1	0	IHNFGLIQEK	_IH
400.224145518642	517.298038734892	3	1	0	IHNFGLIQEK	_IH
400.224145518642	569.283057377092	3	1	0	IHNFGLIQEK	_IH
400.242869587582	445.251757248812	2	1	0	ALLGGNVR	_AL
400.242869587582	485.246672583762	2	1	0	ALLGGNVR	_AL
400.242869587582	502.273220969382	2	1	0	ALLGGNVR	_AL
400.242869587582	598.330736560892	2	1	0	ALLGGNVR	_AL
400.242869587582	615.357284946512	2	1	0	ALLGGNVR	_AL
400.765646192627	347.228896545912	2	1	0	VAGILTVK	_VA
400.765646192627	460.312960523042	2	1	0	VAGILTVK	_VA
400.765646192627	537.339509503675	2	1	0	VAGILTVK	_VA
400.765646192627	630.418488220742	2	1	0	VAGILTVK	_VA
400.765646192627	701.455602005452	2	1	0	VAGILTVK	_VA

Next

Figure 10. The Import Panel Manager allows you to import your text-based assay panel information into SpectroDive by matching the column headers to the SpectroDive specific column requirements.

SpectroDive will warn you if a mandatory or recommended column is missing. Once the panel is successfully imported, it will be displayed in the Panels list. You can organize your panels by creating new folders by right-clicking.

5.5.2.1 *Panel information*

A SpectroDive panel represents a detailed MRM/PRM transition list. Please use the following information in Table 6. to achieve the best possible results. In addition to these required columns, SpectroDive can also take few additional columns that will enable absolute quantification (see Table 7).

Table 6. Required information for importing a panel in SpectroDive

Header	Refers to
Q1	The *in silico* calculated *m/z* of the peptide precursor ion. Do not round this number.
Q3	The *in silico* calculated *m/z* of the peptide fragment ion. Do not round this number.
iRT	The peptide retention time in the reverse phase chromatography converted into iRT space (Escher *et al.* 2012). If accurate iRTs are provided, the analysis will speed up significantly, and the quality of your results will increase (sensitivity, specificity, accuracy). If no iRTs are available, SpectroDive will predict an iRT for each peptide. This is less accurate than an empirically determined iRT value. To derive iRTs for your peptides, we recommend spiking the Biognosys' iRT Kit into your shotgun runs. If the Library Perspective of SpectroDive is used, iRT values will be automatically determined for your library.
RelativeFragment Intensity	The relative peptide fragment ion intensity expressed as a percentage of the most intense fragment ion. Please report the raw number without the percentage sign. This information improves the limit of detection.
StrippedSequence	The stripped amino acid sequence of the peptide excluding any modifications. Please only use the single letter code for the 20 standard proteinogenic amino acids. This information is used for labeling and scoring your fragment ions in SpectroDive. Further, it can be included in the automatically generated unique ID for your precursor, if necessary.
PrecursorCharge	The peptide precursor ion charge. This information is used to label your precursors in SpectroDive and to automatically generate a unique ID for your precursor if necessary.
FragmentType	The peptide fragment ion type. Usually, this is "y" or "b". This information is used for labeling and scoring your fragment ions in SpectroDive.
FragmentNumber	The peptide fragment ion number. This number should be between 1 and the length of your peptide in amino acids minus one. This information is used for labeling and scoring your fragment ions in SpectroDive.

Header	Refers to
FragmentCharge	The fragment precursor ion charge. This information is used for labeling and scoring your fragment ions in SpectroDive.
ModifiedSequence	If your peptide is modified use this column to specify the amino acid sequence including modifications. The modified sequence should be constant for one unique precursor. This information is used to label your precursors in SpectroDive and automatically generate a unique ID if necessary. SpectroDive will try to parse and map modifications from the provided sequences to the internal modification database. This field does not contain any label specific modifications.
ProteinId	The ID of the protein, the peptide is derived from. This information is used to label your peptides. SpectroDive provides filtering capabilities in the Analysis Perspective including filtering for the protein ID.
Compensation Voltage	Compensation voltage value applied for acquisition of a given precursor during FAIMS-PRM acquisition (optional information)
IonMobility	Ion Mobility value associated with a given precursor in prm-PASEF experiments (optional information).

Table 7. Additional required information for enable absolute quantification in SpectroDive

Header	Refers to
PeptideResponseFactor	Specifies the amount SIS on column by protocol. SpectroDive will multiply the target/reference ratio of a peptide with its response factor.
PeptideAbsoluteAmount Unit	only used as a label in the report and plots (not compulsory).
ProteinScalingFactor	Specified for each peptide and can be derived based on (***PeptideResponseFactor* Protein MW)/Amount of Sample injected***. SpectroDive will then calculate a protein response based on each of its peptide by multiplying that peptides target/reference ratio with protein scaling factor. If a protein has more than one peptide, an average quantity will be reported.
ProteinAbsoluteAmount Unit	only used as a label in the report and plots (not compulsory).

5.5.2.2 *Splitting Panels for method generation*

If a panel is too large to be measured in a single run, SpectroDive allows you to generate appropriate methods to measure blocks of the panel. This is done with the panel-assisted method export:

1. Split the larger panel into smaller panels by right-clicking on it and selecting “Split…”. This will open a “Split Panel” form where you can specify the number of transitions per split. If your panel contains decoys, they will be carried over into each split. SpectroDive will distribute the assays evenly over the iRT range across the splits.

2. Export your methods based on the split panels and acquired your sample using such methods.

When analyzing the data acquired using the split panels, you can use the full parent panel for ease of use.

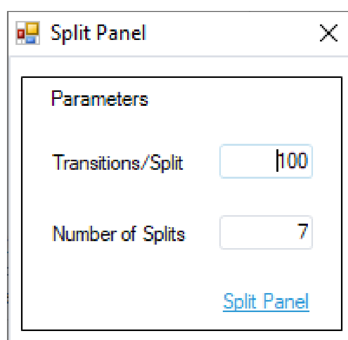

Figure 11. The Split Panel allows you to systematically split a large panel into smaller panels that can then be measured independently by creating an MS acquisition method file for each.

5.5.2.3 *Generating Modified Panels*

A modified panel can be generated from an existing panel by right-clicking on a panel node and selecting "Generate Modified Panel". This will open a wizard in which you will be able to select specific precursors and/or transition groups for creating a new modified panel (Figure 12).

Generally, generating modified panel allows you to make selection of precursors and fragment ions from existing panel, as well as define new labelled channels, similarly like during generation of new panel directly from spectral library (see section 5.5.1).

If using a Biognosys MRM Assay Panel (such as the PQ500), the selection is only allowed at the precursor level.

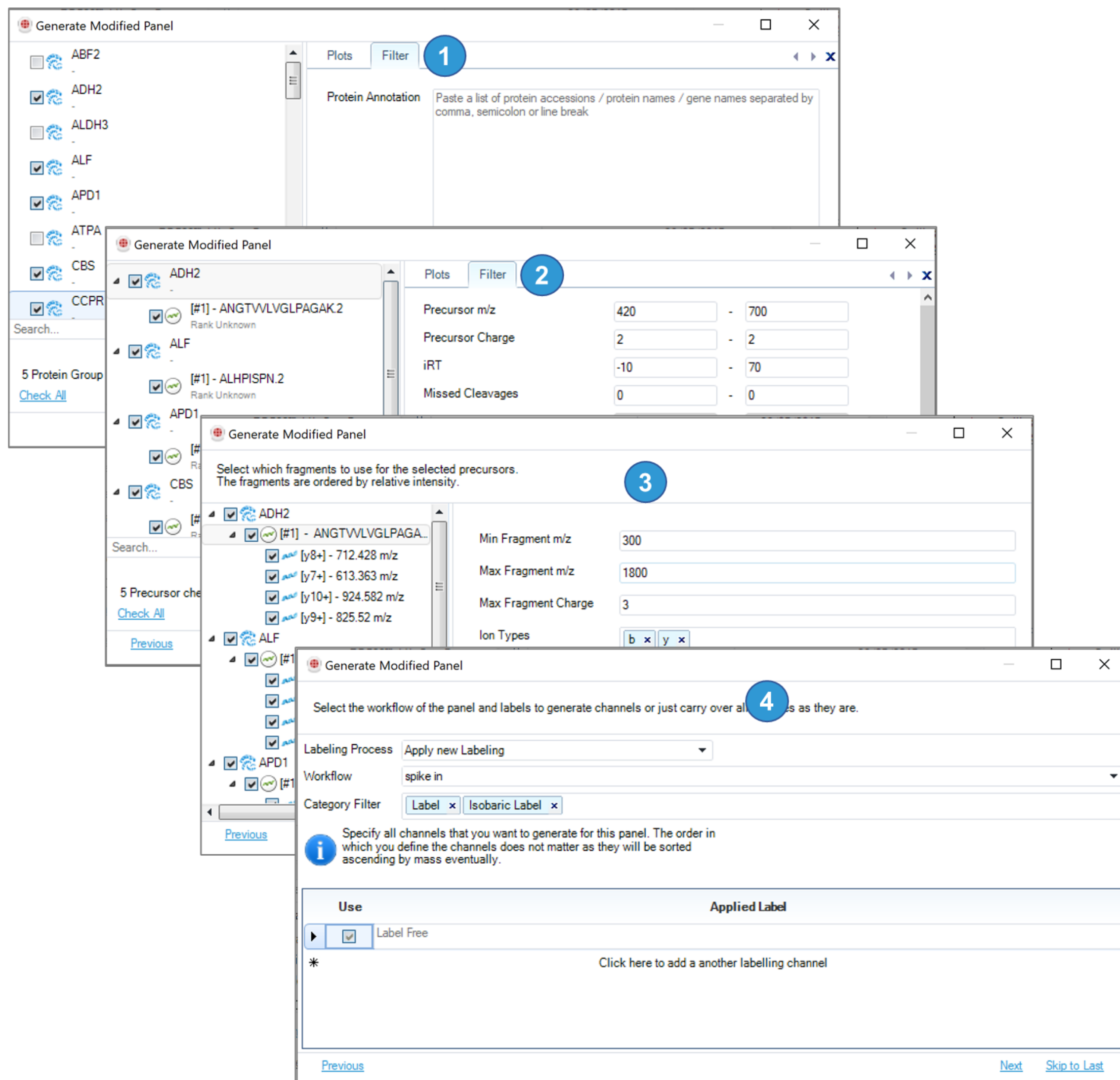

Figure 12. Generating modified panel gives you a finer control over creating a new panel from the existing source panel.

5.5.3 RT Calibration

There are different workflows for creating an RT Calibration Run:

1) By using one unscheduled MRM/PRM run with iRT kit spiked in. This allows for linear RT calibration.
2) By using a wider window panel-based MRM/PRM run for linear or non-linear calibration.
3) By using a DIA run with spiked-in panel peptides for non-linear calibration. This allows for calibration of non-linear LC gradients.
4) Finally, RT Calibration could be imported directly from the DDA or DIA Spectral Library. This allows you to bypass the need to acquire unscheduled runs to

schedule your targeted method, assuming that the library was generated from samples acquired with the same chromatography.

SpectroDive will generate an RT calibration run within the Analysis Perspective (see section 5.6). Select the "Set up Scheduled Analysis from File…" option and upload a raw file that contains the MS data for the target peptides of interest and proceed with the analysis wizard as done for a conventional targeted analysis. This will generate an RT calibration run that can be viewed in the Prepare Perspective. The RT Calibration Run will be used for calibrating the iRT values of your assays to generate a scheduled targeted acquisition method. If needed, although highly unlikely, you can edit a specific RT Calibration Run in order to select which of the iRT peptides are to be used for calibration by double-clicking on the run name in the RT Calibration Run panel (Figure 13). Notably, iRT calibration is supported for both linear and non-linear gradients.

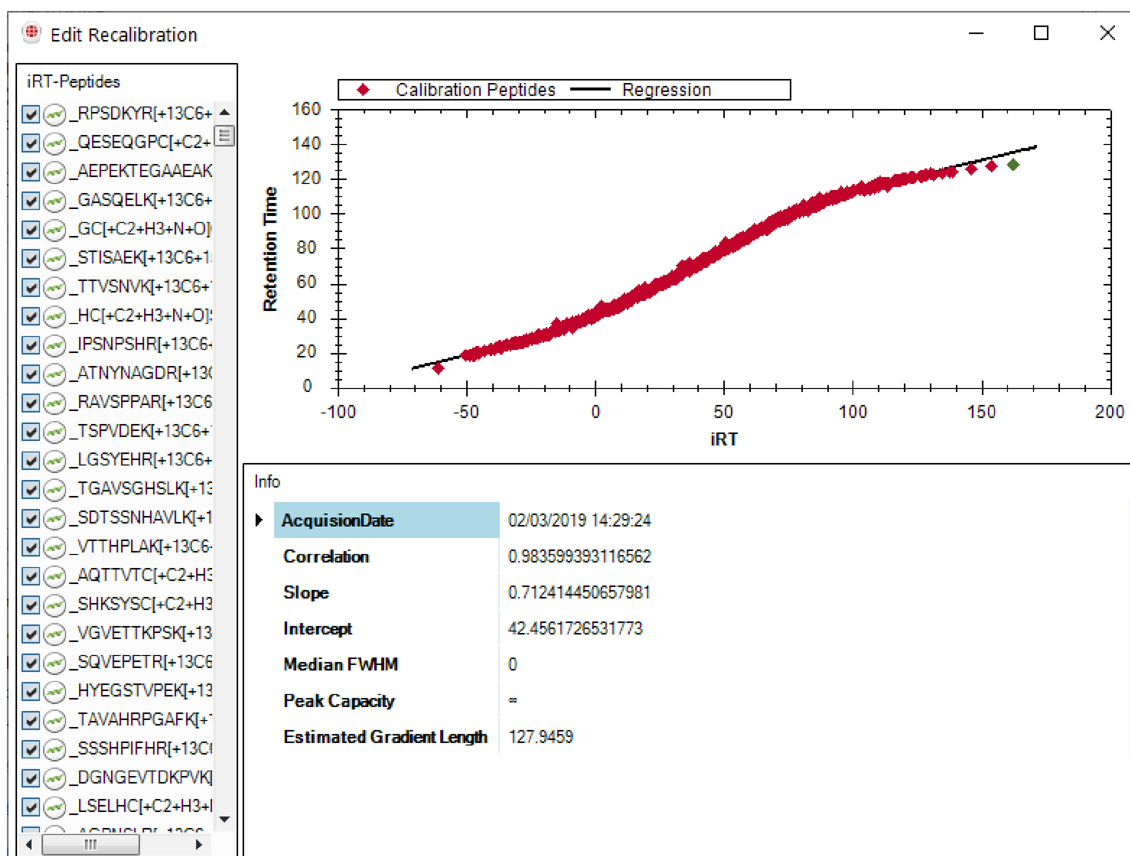

Figure 13 Edit calibration allows you to reject specific iRT peptides and/or review information specific to the calibration curve.

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5.5.4 Exporting Method Files

You can export a method file for your panel by selecting the correct MS instrument. If making a scheduled experiment, SpectroDive will automatically schedule the assays based on the specified calibration file and window size parameters. RT Calibration could be done based on a calibration run acquired specially for that purpose (see Box 4) or based on the information obtained from the spectral library (Figure 14).

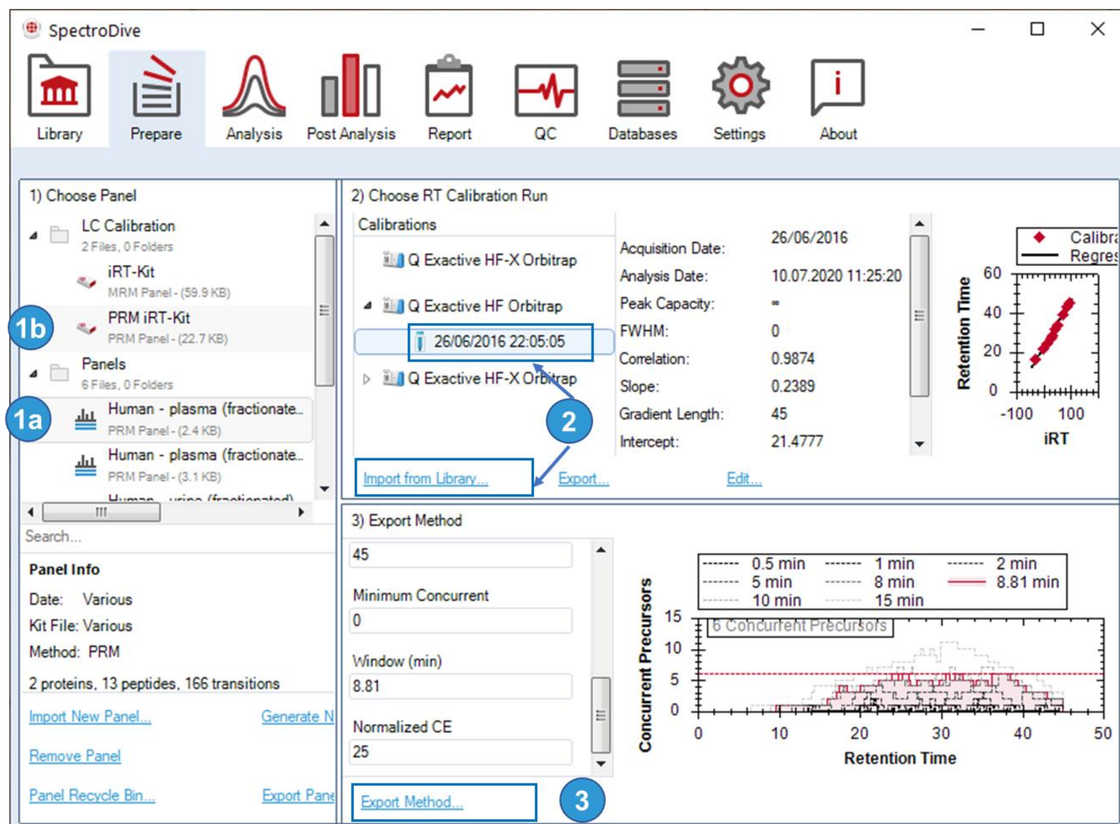

Figure 14 In order to export scheduled method, select your panel, choose RT calibration run (you can also choose RT calibration from the library) select option «Export Method».

The concurrent transitions plot (Figure 15) gives you an overview of the scheduling of your panel. Adjust the retention time window for scheduling in the field 'Window (min)' in order to prevent exceeding a maximum number of concurrent transitions to avoid unfavorable signal-to-noise ratios in the data (e.g. for MRM acquisition, assuming a constant cycle time of 2.5s, one should not exceed maximal 250 concurrent transitions since this may lead to a dwell time of lower than 10ms). One of the features introduced since SpectroDive 9.0 is the option to export method files compatible with the MaxQuant.Live, allowing for real-time control the data acquisition, currently only available for the Thermo Q-Exactive HF/HF-X instruments.

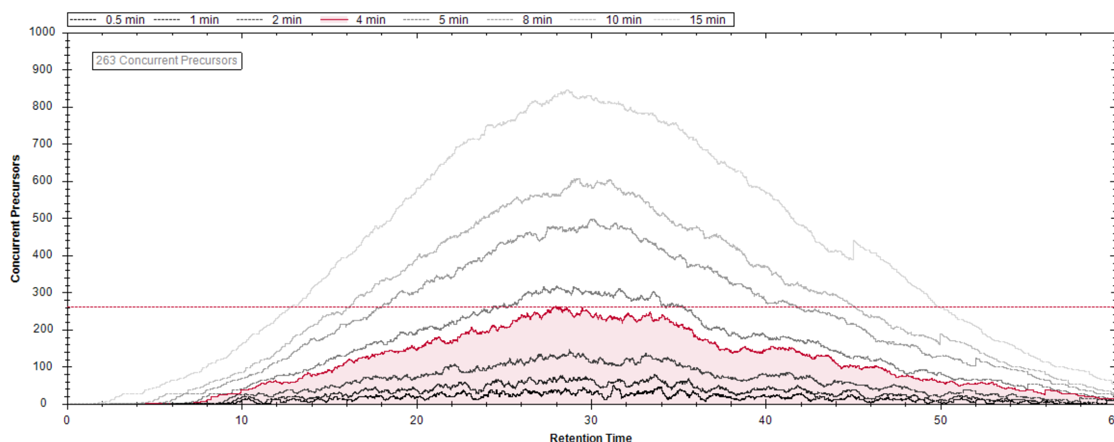

Figure 15. The concurrent transitions plot gives you an overview of the scheduling of your panel.

Box 4. Generating a scheduled acquisition method – step by step guide

1. Generate an assay panel for the analysis, either by importing a ready-made panel or creating a new one from a spectral library, as described in section 5.4Library Perspective.
2. Export unsheduled MS acquisition method in the Prepare Perspective by going through two steps: 1) Choose Panel and 3) Export Method Selecting – under Run Type select ”Analysis”, select the instrument-specific parameters for the acquisition method, under Scheduling specify “Unscheduled”, and click on Export Method…
3. Perform unscheduled data acquisition on your MS instrument.
4. In the Analysis Perspecive, select the option “Set up Analysis for Method Scheduling…” and upload the unsheduled raw data that you just acquired to generate an RT calibration. This will prompt the calibration process which creates an RT calibration run in the Prepare Perspective → 2) Choose RT Calibration Run (see section 5.5.3).
5. In the Prepare Perspective go through the three steps: 1) Choose Panel, 2) Choose RT Calibration Run – select the RT calibration that you just generated, and 3) Export Method – under Run Type select ”Analysis”, select the instrument-specific parameters for the acquisition method, under Scheduling specify “Scheduled”, and click on Export Method…

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Box 5. Generating an Absolute quantification method – step by step guide

1. Generate an assay panel for the analysis that contains the stabel isotope labeled standard (SIS) peptides.
   NB! *Have in mind to inculde the required columns. PeptideResponseFactor and ProteinScalingFactor. These columns will be auto detected if named exactly same.*
2. Export the MS acquisistion method in the Prepare Perspective and click on Export Method…
3. Perform data acquisition on your MS instrument.
4. Review your analysis. The peptide absolute quantity will be automatically calculated and dilspayed in the Analysis Perspective → MS2 XIC charts.
5. Report Absolute Quantities. In the Report Perspective, you can get the peptide absolute quantity by selecting the EG.AbsoluteAmount and protein absolute quantity by selecting the EG.ProteinAbsoluteAmount.

5.5.5 SureQuant – introduction

SureQuant workflow in its entirety can be broken down into 3 main steps (Figure 16). Firstly, defining a survey run based on your assay panel. Secondly, acquiring and analyzing the survey run to set up SureQuant acquisition (this mainly involves specifying the intensity thresholds to be used for triggering a high-resolution MS2 scan for each target), and finally, acquiring your samples with the customized SureQuant acquisition and analyzing it with SpectroDive. Many of these steps can be skipped if you use one of the pre-defined SureQuant panels that comes with the instrument software such as our PQ500 plasma panel.

If you are measuring a custom panel, then SpectroDive makes it extremely easy to perform these steps.

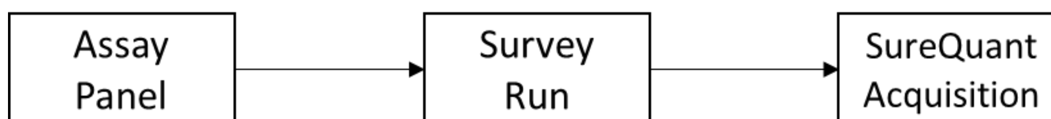

Figure 16 Three step concept of SureQuant workflow.

5.5.5.1 *Generating Survey Run*

Generating Survey Run requires you to already have a custom PRM panel in the Prepare Perspective. Please refer to section 5.5.1 for more information on panel generation. Once you have your panel, it is very easy to create a survey method that can be directly imported into the instrument software: 1) Select your panel in the Prepare Perspective.

Make sure that the panel is flagged as a PRM panel to get the choice of SureQuant compatible instruments. 2) You can skip choosing a RT calibration run as there is no scheduling involved. 3) In Export method section select Thermo for Vendor, secondly select a SureQuant compatible instrument (Fusion Series or Exploris series), and lastly select SureQuant Survey for Software Version. 4) Click "Export Method…" link to export your survey method which should be ready to be imported into your instrument software (Figure 17).

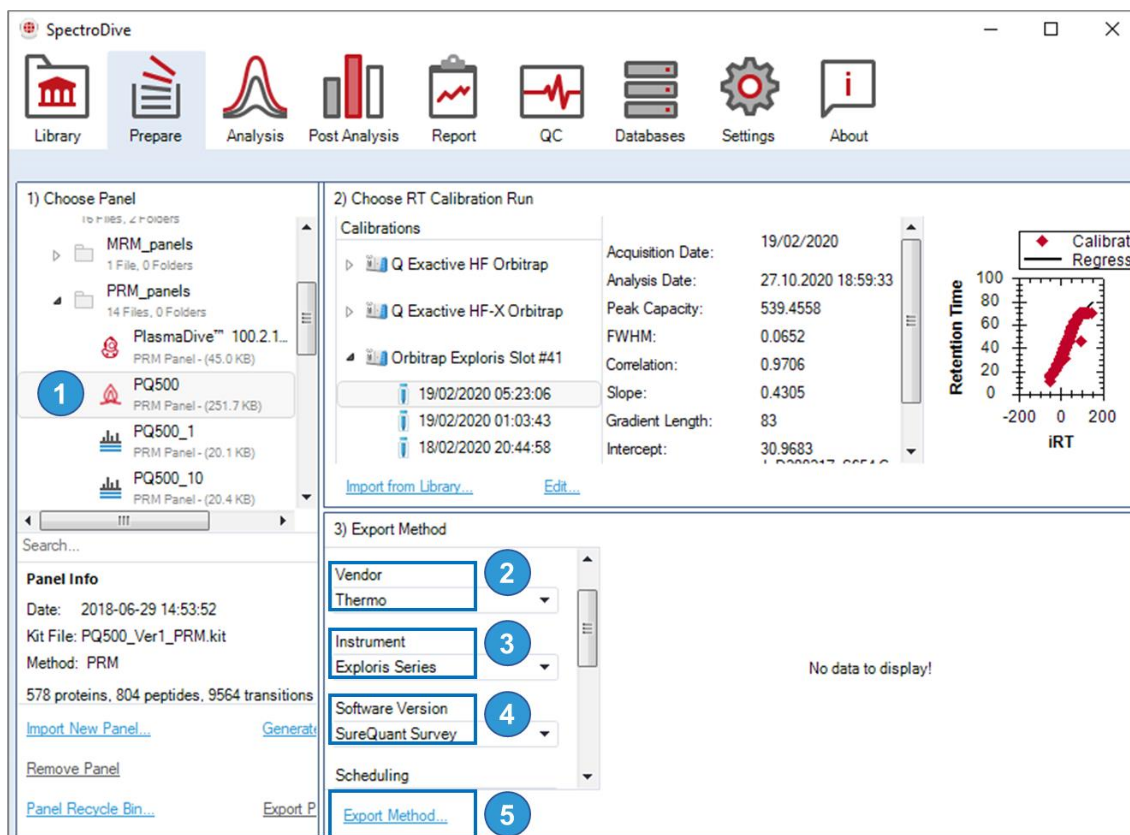

Figure 17 Generating of Survey Run method in Prepare Perspective.

5.5.5.2 *Generating SureQuant Method from Survey Run*

Once you have acquired the survey run, it is time to use it to setup the SureQuant acquisition method. First go to the Analysis Perspective in SpectroDive and set up a new experiment by clicking on "Set up a Targeted Analysis…" link. In the experiment setup, select your survey run and specify the corresponding panel from the Prepare Perspective (see section 5.6.1). Once the run is processed by SpectroDive, you simply right-click on the experiment tab and select SureQuant → Export SureQuant Method (Figure 18).

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SpectroDive will create a method folder with all the relevant files you will need for setting up your SureQuant acquisition. The files are created so that they can be easily imported into the instrument software. Within the method folder, you will find

- A TargetMass folder which includes peptides with their corresponding intensity thresholds. The peptides are divided by the corresponding label and charge state.
- A TargetMassTrigger folder which includes the fragment ions to be used for triggering.
- A ReadMe file that summarizes all the pertinent information like the parameters which were used, etc.

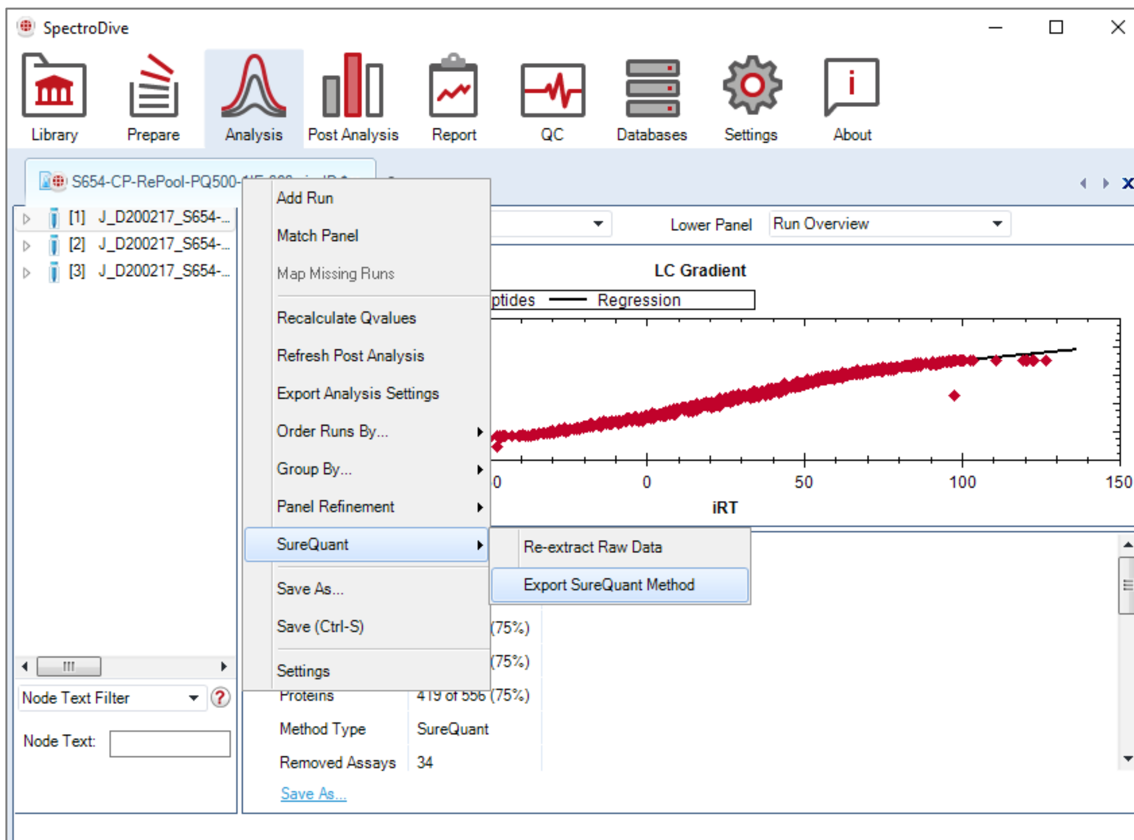

Figure 18 Exporting SureQunat method after analysisng Survey run.

SpectroDive uses certain settings which define how the method is setup. These settings are specified in Settings → Global → SureQuant. Use the handy hover text to look up the exact definition of each of the parameter or refer to the Appendix 6.4. The most important parameter is the Peak Fraction which defines the percentage of the mono-isotopic peak height that is used for specifying the triggering intensity threshold for each target. A good default is 1% but depending on the size of the panel there this setting might be further optimized.

5.6 Analysis Perspective

The Analysis Perspective allows you to manually review your data. Here you can set up an targeted data analysis, set up a scheduling analysis and set up a panel iRT refinement analysis. You can also upload and review saved SpectroDive experiments.

5.6.1 Set Up Targeted Analysis

To start setting up an analysis, you can select either individual files by clicking on the option "Set Up Targeted Analysis from File…" or specify a folder where all the necessary raw files are stored by using "Set Up Targeted Analysis from Folder…". The analysis setup dialogue will appear (Figure 19). First, load one or several run files. You can then assign the assay panel manually. While setting up your experiment, you can select an analysis schema for your experiment. You can generate new customized schemas in the Settings Perspective (see 7.3 Appendix 1). The analysis schema allows you to specify a certain set of parameters that you want to use for your analysis. The BGS Factory Settings suits most experiments.

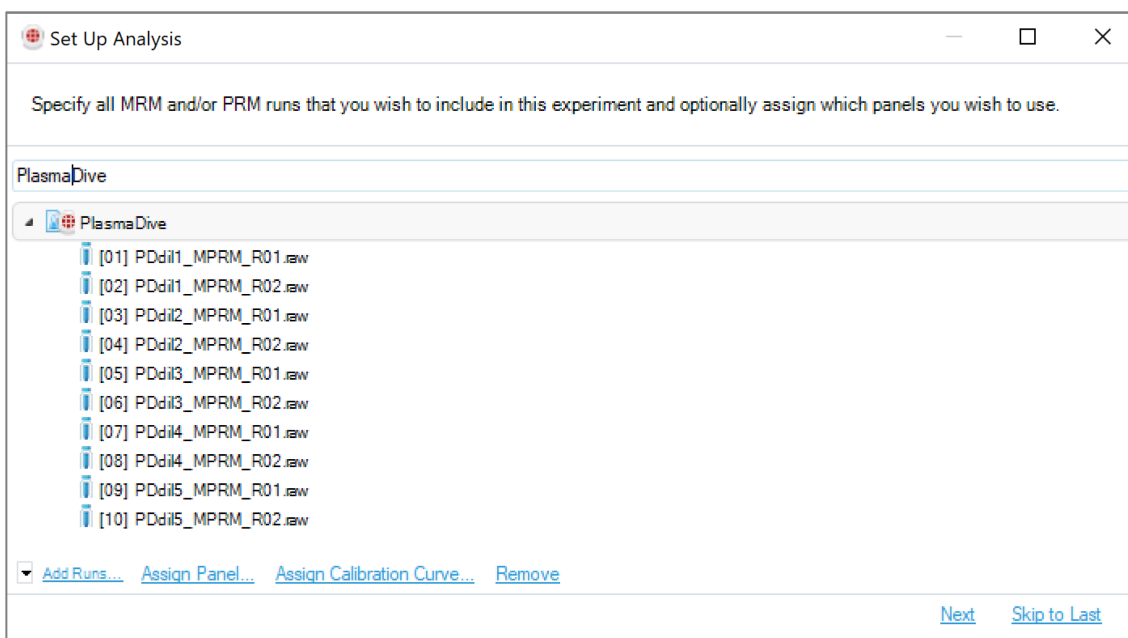

Figure 19. The Experiment setup dialogue of SpectroDive. A raw file is required.

To let SpectroDive perform the differential abundance tests and other condition-wise metrics, you need to specify your experimental setup during the configuration of your analysis. SpectroDive will ask you to annotate your runs and specify which condition, biological replicate, and fraction (if applicable) they belong to. Each condition in

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SpectroDive will get a color assigned during the setup which will be used for post-analysis labeling. The Conditions Set Up panel contains several columns:

- The "**Run Label**" column is used for plotting purposes.
- You can specify which condition should be considered as the reference. "**Is Reference**" is used only for plotting purposes (in the Post Analysys Perspective → "Diferential Abundance" when right-clicking on one of the profile plots under "Candidates" there is the option to choose "Normalize by Reference". This will divide every condition by the defined reference condition).
- You also have the option to enter quantity correction factors for each sample. SpectroDive will multiply the final quantities by this factor. One application example would be if quantities should be expressed per initial sample volume (for instance, in blood plasma).

Set Up Analysis

Specify conditions in order to perform statistical tests during post analysis.

#	Is Reference	Run Label	Condition	Fraction	Replicate	Quantity Correcti...	Label	Color	File Name
1	☑	sample1_R01	1	NA	1	1	1	Color [A=25...	B_D140314_SG...
2	☑	sample1_R02	1	NA	2	1	1	Color [A=25...	B_D140314_SG...
3	☑	sample1_R03	1	NA	3	1	1	Color [A=25...	B_D140314_SG...
4	☐	sample2_R01	2	NA	1	1	2	Color [A=25...	B_D140314_SG...
5	☐	sample2_R02	2	NA	2	1	2	Color [A=25...	B_D140314_SG...
6	☐	sample2_R03	2	NA	3	1	2	Color [A=25...	B_D140314_SG...
7	☐	sample3_R01	3	NA	1	1	3	Color [A=25...	B_D140314_SG...
8	☐	sample3_R02	3	NA	2	1	3	Color [A=25...	B_D140314_SG...
9	☐	sample3_R03	3	NA	3	1	3	Color [A=25...	B_D140314_SG...

Import Condition Setup... Export Condition Setup...

Previous Next Skip to Last

Figure 20. Conditions Setup panel during the Analysis set-up. You can manually adjust your conditions on the panel or proceed with Import Conditions Setup from a text file.

Unless actively disabled in the Analysis Settings, SpectroDive will perform a pairwise comparison (Student's t-test) of all conditions specified in the Condition Setup panel. The results are reported in the Post Analysis Perspective. There are several ways to introduce the annotation information into the Condition Setup panel:

1. If you maintain a file-name structure which is self-annotating, you can define a parsing rule to automatically parse the conditions and replicates (Settings → Global → General → File Name Parsing Schema; see section 5.11.4.1).
2. The Condition Setup table is editable: you can directly write in any of the fields to enter your information (Figure 20). The table will recognize your changes and adapt them to the rest of the fields automatically. Be aware that the conditions editor is space and case sensitive.

3. Import your annotation from an external text file. The easiest way to do this is by exporting the current setup, modify it and save it as a text file that can be imported.

After you click "Finish" SpectroDive will show the Analysis Perspective and start the analysis. First, SpectroDive will automatically try to detect the iRT Kit and calibrate analysis parameters for each run based on your specific data. The evaluation of signals from the iRT Kit allows for fast data processing and easy setup of scheduled methods, it improves the specificity, and enables automated quality control. As such, it is highly recommended to spike in the iRT Kit in all the samples. Browsing your data will be possible a few seconds after the initial calibration process is finished (Figure 21). At the bottom left, you can see a progress bar informing you about the overall status of the analysis.

5.6.2 Absolute quantification using calibration curves

A calibration curve is a valuable method to determine the concentration of an analyte in an unknown sample, by comparing the unknown sample to a set of standard samples with known concentrations. It can also be used to determine the sensitivity of a method, reported by the limit of detection (LOD). The precise relationship between the measured signal and the peptide quantity can be assessed with a calibration curve constructed from serially diluted standards. A measured signal is precisely quantified if it falls in the linear quantification range, namely between the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ). We recommend watching our video tutorial that guides you through the analysis set-up for the absolute quantification using calibration curves.

1. Generation of the calibration curves file. Select the link to set up a targeted analysis. In the first window of the wizard, choose the calibrant runs and assign the panel of proteins for the targeted analysis.
2. Choose the parameters for calibration curve generation. In the Workflow node define the experiment type. In example, an experiment where the light endogenous peptides are present at constant concentrations should be analyzed using an inverted spike-in workflow, with the light peptide serving as the reference channel for identification.
3. In the calibration curve node select "Process as Calibration Curve". An important setting to consider is the "Concentration variation on peptide type". By default, it is set to "Heavy peptides", which is correct for the above experiment type. Switch to "Light peptides" if the light endogenous peptides are used for calibration curve

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generation. By expanding the option "Determine Quantification Range" you will calculate the linear range and visualize LLOQ and ULOQ values on the Calibration Curve plot.

4. Input the peptide information necessary to generate the calibration curve. We recommend editing the peptide information in this window and exporting it as a *.ccs binary file format for future analysis. If you use a Biognosys panel, the starting quantities are automatically fetched.
5. Define the condition setup and export it to a text file that can be used and edited for future experiments. It is important to define the dilution factors of the calibrant runs. For instance, set the blanks to 0, the undiluted samples to 1, and all dilutions above 1.
6. Once the analysis is complete, it can be revised in the Analysis perspective (see Section 5.6.8). Save the calibration curves file (*.ccs) by clicking on the Ellipses icon (…) under the experiment tab.
7. Start the quantitative experiment. Select the run files to quantify, assign the same protein panel used above for the calibration curve, and the calibration curves file that was just generated. In the Workflow settings set up the experiment type. However, skip the Calibration Curve node for the quantitative analysis.
8. Define the experiment setup and start the quantitative analysis.

5.6.3 Reviewing an analysis

The Analysis Perspective is organized as a data tree on the left and plots, reports, and summaries are on the right side (Figure 21). By default, the hierarchy of the data tree is:

> *Run*
  > *Panel*
    > *Elution group*
      > *Precursor*
        > *Transition*

In this data tree, you can right-click on any of the elements to find new functions which can be applied to that element. For example, you can apply different functions such as accept, reject, exclude, hide, and delete depending on the level in the tree. If an elution group is accepted, then it will be considered as identified regardless of the actual empirical q-value. Conversely, if an elution group is rejected, it will be treated as not identified. If an elution group or transition group is excluded, then it will not be shown in the report. If a transition is excluded, then it will not be used for quantification nor scoring.

If you hide something, it will simply not be used in a plot. Deleting something will remove it completely from the panel, as if it were not in the panel in the first place. The right side is divided into two panels (Upper and Lower) and you can display two different plots simultaneously. These plots will change based on what is selected in the data tree. Different plots are applicable to different levels. To know which plots are available for each level, see Appendix 4. Analysis Perspective Plots (section 7.7).

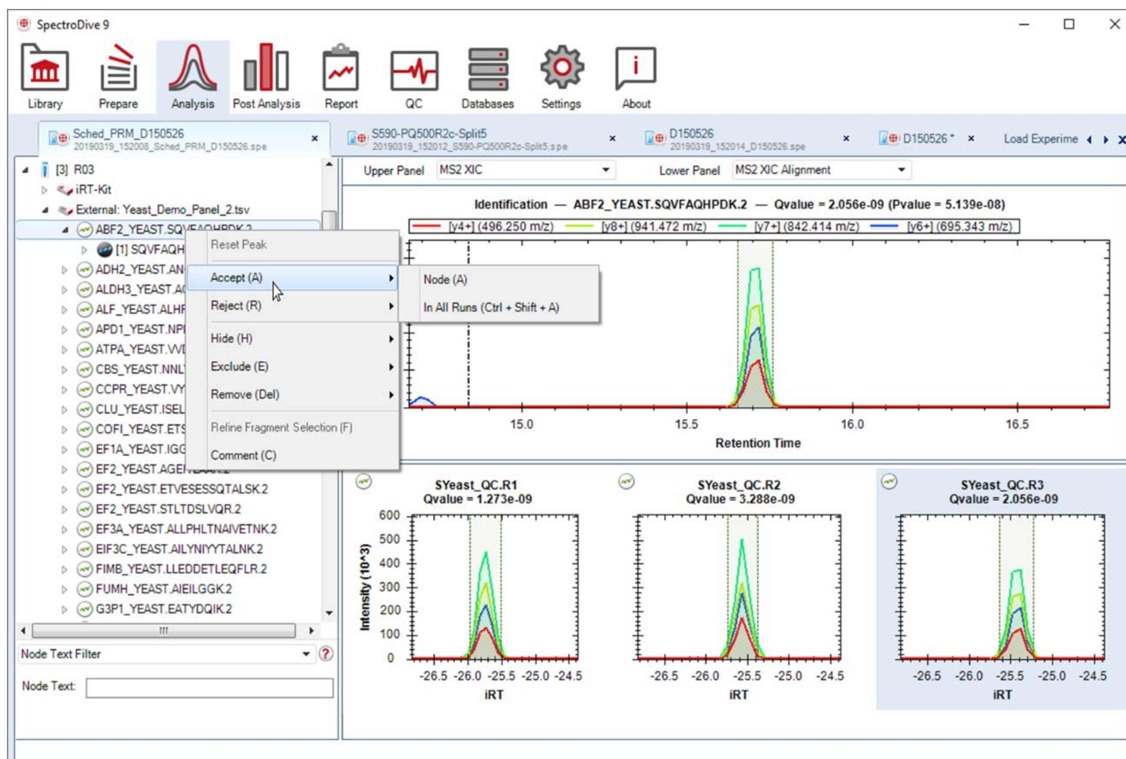

Figure 21. The Analysis Perspective of SpectroDive. The lower right panel shows the correlation of relative fragment ion intensities between measurement and the values provided in the panel.

5.6.4 Tree Filtering

Using the filtering system implemented in SpectroDive, you can apply one or several filters on the Analysis data tree. These filters only influence what is shown in the Analysis Perspective but not, for instance, in the Post Analysis Perspective. Select a filter from the dropdown menu and set the filter criteria. The filter is now marked as selected within the dropdown menu. To combine filters, select a different filter and define the value that should be applied. A precursor must apply to all selected filter in order to be shown in the review tree.

***NB!** Sometimes it is not obvious that a filter is applied. Make sure you check the filter list before reviewing your analysis further.*

Figure 22. The Tree Filtering options in the Analysis Perspective. Check the box for a filter and give the corresponding value. An example of filtering for a peptide sequence is shown on this figure.

5.6.5 Tree Grouping

You can apply different grouping strategies on the data tree by right-clicking on the experiment tab. There are two grouping possibilities:

- **Protein:** Allows a grouping of the precursors on protein ID level. The parent node shows overview information about this group.
- **Panel (default):** Shows all precursor according to their associated assay panel.

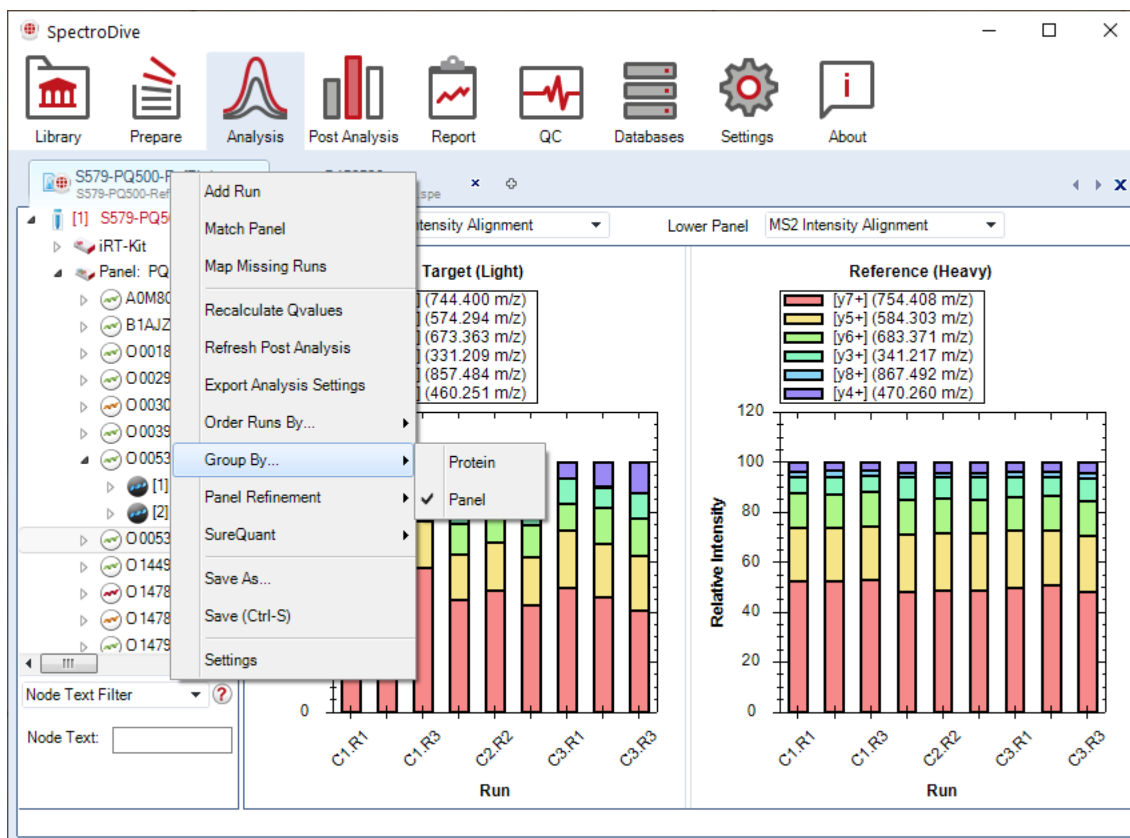

Figure 23. The "Group By…" option in the experiment tab.

5.6.6 Panel Refinement

In SpectroDive you can perform refinement of the iRT values as well as refinement of fragment selection in a given panel based on specific runs. This can be useful at an early stage of panel development or if migrating the panel to a very different chromatographic setup (iRT refinement).

Once the panel is refined, it will be updated in the Prepare Perspective. Refinement can be reverted by right-clicking on refined panel in the Prepare Perspective and selecting “Reset to Factory Default”. Notably, panel refinement is available for all supported workflows: MRM and PRM, including SureQuant.

5.6.6.1 Fragment Selection Refinement

You can review fragment ions measured in your analysis and change their selection for the refined panel. It is a convenient way of the MRM or PRM method optimization where the best of the tested transitions could be chosen for the target quantitation. Moreover, for the PRM acquisition, not only library present fragments but also theoretical ones are available for the selection (Figure 24).

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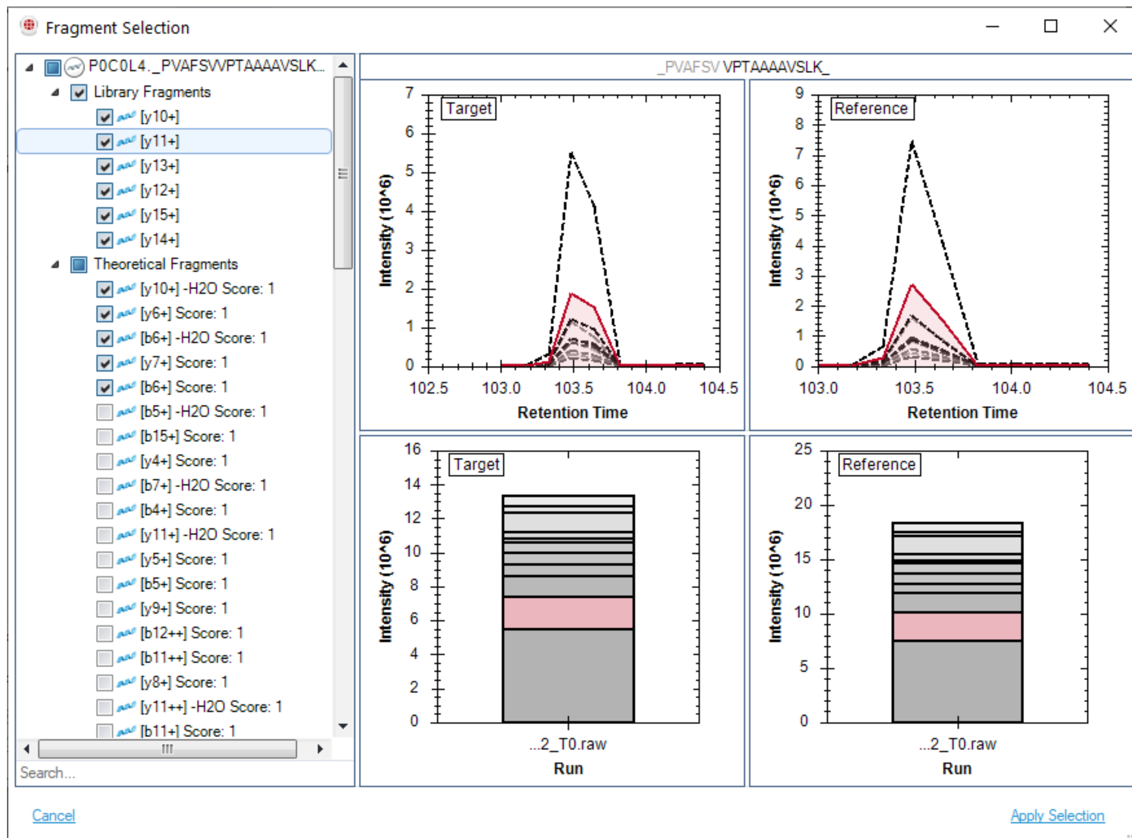

Figure 24 Fragment Selection Refinment performed for the precursor acquired with PRM method. Fragment chosen in selection tree on the left is highlighted with red color in panels on the right.

In order to execute refinement of the panel, the changes need to be committed in the analysis perspective. You can do this by right-clicking on the experiment tab and selecting “Refine Assay Transitions” on the “Panel Refinement” function (Figure 25).

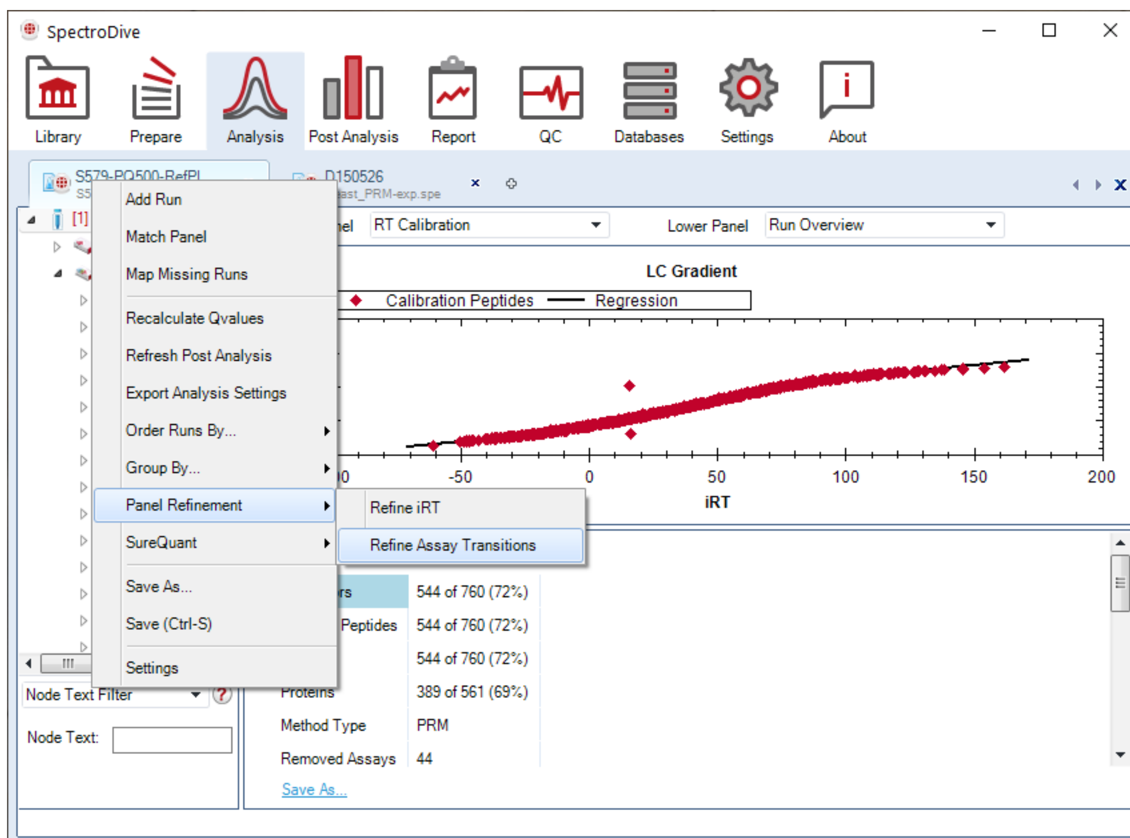

Figure 25 Execution of fragment selection changes is possible by choosing «Refine Assay Transition» option in the Analysis Perspective.

5.6.6.2 *iRT Refinement*

iRT Refinement requires to firstly acquire your PRM or MRM data with a specific method, designed for that process. To do so, you must have your panel imported into the panel repository in the Prepare Perspective. When you are ready to export a method file for the panel, select "**iRT Refinement**" in the field "Run Type" instead of “Analysis” (Figure 26). This will create a special method file for acquiring runs meant for refining your panel. SpectroDive will automatically use a wider window by default when exporting a method meant to be used for iRT refinement. To accommodate this, it will use only two transitions per peptide. Once you have acquired the runs using this method file, load them in the Analysis Perspective → “Set up Panel iRT Refinement Analysis…” and follow the wizard. At this point you can right-click on the experiment tab and select “Refine iRT” which will open the iRT Refinement dialog.

NB! *The iRT refinement will only be done for peptides which are manually accepted in the Analysis Perspective. You also need to commit panel changes by right-clicking on the experiment tab.*

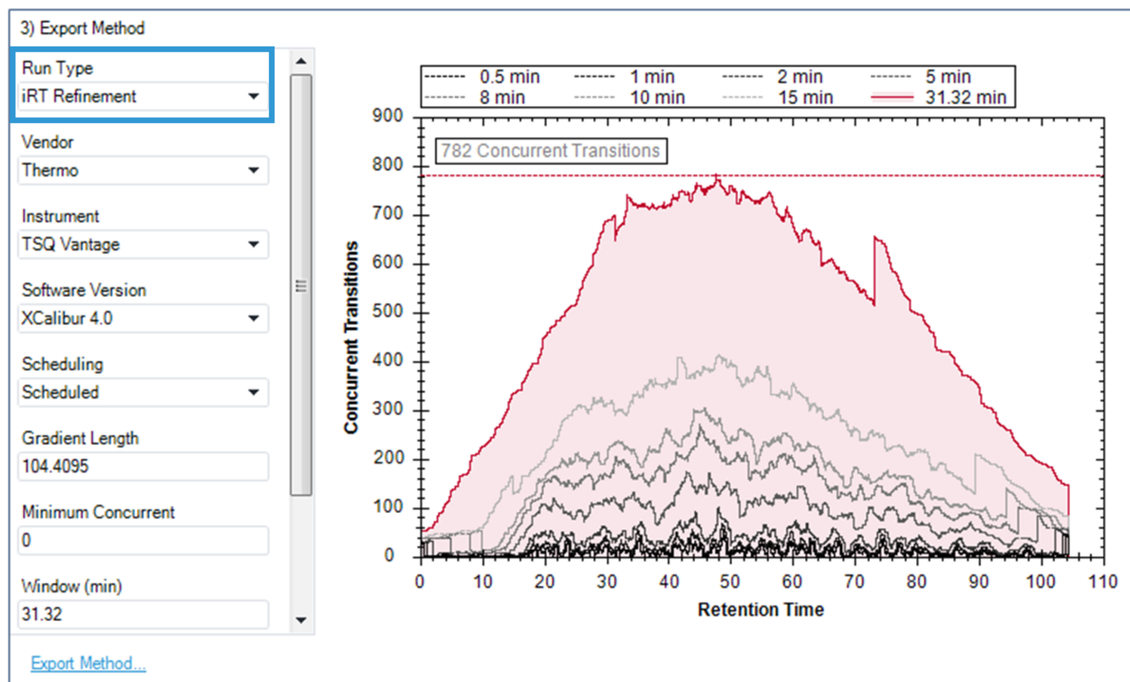

Figure 26 The iRT Refinement (blue rectangle) allows you to refine the iRT values in your panel.Saving and Loading Experiments.

SpectroDive supports saving and loading of a complete analysis, termed “Experiment”. Saving an experiment will also store manually integrated peaks, comments, excluded transitions, and information on whether a peak was manually accepted or rejected. To save an experiment, right-click on the experiment tab and select “Save As…” in the context menu. The most recently loaded or saved experiments are shown in the “Recent Experiments” list of a new Page in the Analysis Perspective. A saved experiment in SpectroDive will contain the full analysis information and can get very large in size, but no longer requires the raw file or panel to be available. SpectroDive uses the file extension SPE for its saved experiment files.

5.6.7 Quantitative workflows supported in SpectroDive

SpectroDive supports labeled and label-free workflows, and specific scoring methods are developed for each approach.

- **Label-free**: Default workflow for all channel experiments. Peak detection, scoring and identification are applied as usual.
- **Labeled**: Peak detection and scoring will be applied to all channels. Quantification in Post Analysis will be performed on the light to heavy ratio.

- **Spike-in**: Peak detection will be performed on only the reference (heavy) channel. Scoring and identification will be performed on the target (light) and reference (heavy) channels. The heavy channel is expected to be easily detectable and considered a peak-picking aid in this experiment. Quantification in Post Analysis will be performed on the target to reference ratio.
- **Inverted spike-in**: Similar to spike-in but the light channel is considered as the reference.

For more details about how to set these workflows, see Appendix 1. Analysis Settings (section 7.3).

5.6.8 Calibration Curves Revision

Calibration curves can be visualized for all peptide precursors (Figure 27). The green diamonds represent a confidently identified peptide for a given calibrant run. On the x-axis we can read the nominal concentration calculated from the starting quantity and dilution factor, while the light blue box is the actual quantified value. A calibrant intensity that falls below the Limit of Detection (LOD) is below the noise floor, depicted as an orange box. The red dashed line represents the mean intensity of the blanks.

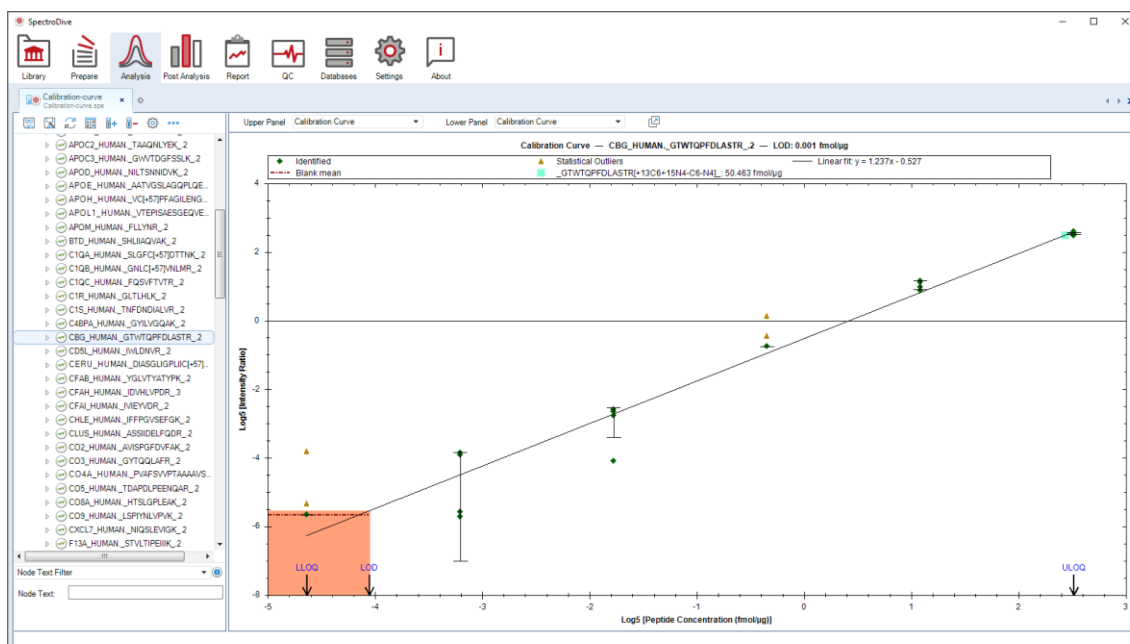

Figure 27 Calibration curve of an identified precursor across runs, showing the linear quantification range between LLOQ and ULOQ, and the limit of detection (LOD).

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If replicates were defined in the condition setup, the plot shows statistical outliers as triangles. The vertical error bars are based on coefficient variation of intensities. By hovering over a calibrant level error bar, you can see the precision of the quantification.

The runs that fall in the linear fit between the LLOQ and the ULOQ can be quantified with confidence. The calibration curve plot for all peptides should look the same in the first (calibration curve generation) and second part (quantification using the calibration curve) of the analysis. The light blue square represents the actual quantified value on the x-axis based on the calibration curve's intensity ratios displayed on the y-axis.

5.6.9 Saving experiments

SpectroDive supports saving and loading of a complete analysis, termed "Experiment". Saving an experiment will also store manually integrated peaks, comments, excluded transitions, and information on whether a peak was manually accepted or rejected. To save an experiment, right-click on the experiment tab and select "Save As…" in the context menu. The most recently loaded or saved experiments are shown in the "Recent Experiments" list of a new Page in the Analysis Perspective. A saved experiment in SpectroDive will contain the full analysis information and can get very large in size, but no longer requires the raw file or panel to be available to view data. SpectroDive uses the file extension SPE for its saved experiment files.

Due to improved external framework changes, it is possible that the following information might be lost for experiments performed with SpectroDive 10.5 or earlier. For each run: custom label/name, comments, original run order, and color code for graphing, total ion current, and MS1 and MS2 empirical resolutions. In order to recover this information, we recommend loading the saved experiment files (SPE) in version 10.6 of SpectroDive, which will automatically ask to update the file. For this to work, the "Ask to Upgrade Experiments" global setting should be checked in the settings perspective.

5.7 Post Analysis Perspective

The Post Analysis Perspective in SpectroDive™ reports summary information about the Analysis, Scoring Histograms and the results of the Differential Abundance (Figure 28).

Figure 28. Post Analysis Perspective. Several summaries, tables and plots are available as you navigate through the nodes in the tree. On the figure a view of the scoring histograms for the spike-in peptides is displayed.

5.7.1 Analysis Overview

Here you will find some experiment-wide information that will give you a rough idea about dataset characteristics. Under Overview, you will see a summary with the number of proteins and peptides identified (by conditions), miss-cleavages, library recovery, and other metrics that can help you judge the experimental outcome. To support this overview, you will find several plots related to the number of identifications, the data completeness, the coefficient of variation, and the normalization. On each of these plots, you can change many settings by using the right-mouse click option. To see the full details of each plot, see Appendix 6. Post Analysis Perspective Plots (section 7.9). Learn more about plots in SpectroDive in Box 1.

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5.7.2 Scoring Histograms

Under this node, you will find plots related to the behavior of the target and the decoy distribution estimation. This behavior defines the discriminant scores (Cscores), *q*-values, and sensitivity on the precursor level. Scoring histograms are shown for each workflow in the current experiment depending on your experimental set-up. Find all the details about these plots in Appendix 6. Post Analysis Perspective Plots (section 7.9).

5.7.3 Differential Abundance

5.7.3.1 *Candidates table*

Results of differential abundance testing will show up under this node. The Candidates node, once selected, shows a table with the results, annotated by pair-wise comparison (Figure 28):

- The direction and the percentage of change are noted by color and color intensity, respectively; the level of significance is noted by the size of the circle.
- The fold changes are expressed as log-transformed ratios of averaged replicates (AVG Log2 Ratio).
- The table is, by default, filtered by a multiple testing corrected *q*-value of 0.05 and an absolute *log*2 ratio of 0.58. You can change these filters to your preferred cutoffs. The filters applied to this table will automatically apply to the Volcano Plot as well.
- You can add and hide columns in this table by right-clicking on any of the headers and selecting "Column Chooser". For example, you may want to add the *p*-value column.
- It is possible to search any character in the table with the Search field at the bottom of the table.

The candidates table can be exported as an Excel file by clicking on "Export Table…" at the bottom.

In addition to the table, the candidates will be shown as plots on the right side. These plots can be customized in several ways by right-clicking on it and choosing your preferred options.

5.7.3.2 *Differential Abundance Plots*

Under the Differential Abundance node, several plots related to the significance test are also generated. Please find detailed information on each of these plots in Appendix 6.

Post Analysis Perspective Plots (section 7.9). The most relevant are the Heatmap and the Volcano Plot:

1. The Heatmap will be clustered row and column wise according to the Post Analysis settings. The raw data of the Heatmap can be exported via right-click on the plot (Figure 29).

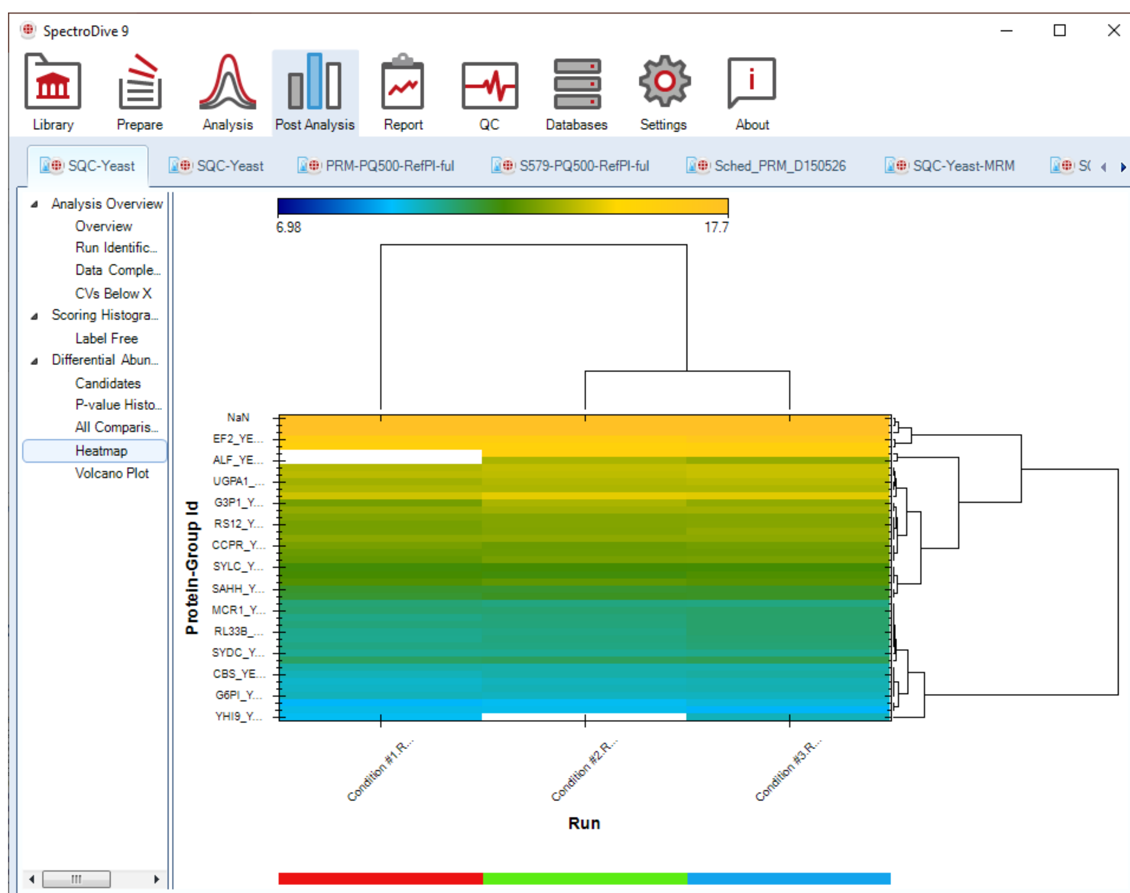

Figure 29. Heatmap with clustering in both rows and columns. The heatmap is built using the set of confidently identified datapoints. By right-clicking on the plot, you can perform several actions, such as save the Image As, export the data matrix or modify the scaling.

2. The Volcano Plot shows the results of the differential abundance test by plotting the peptides or proteins fold change against the significance level. The candidates will appear in red on the plot (Figure 30). If more than two conditions are investigated you can create separate volcano plot for all conditions analyzed together and separate for the conditions analyzed pairwise.

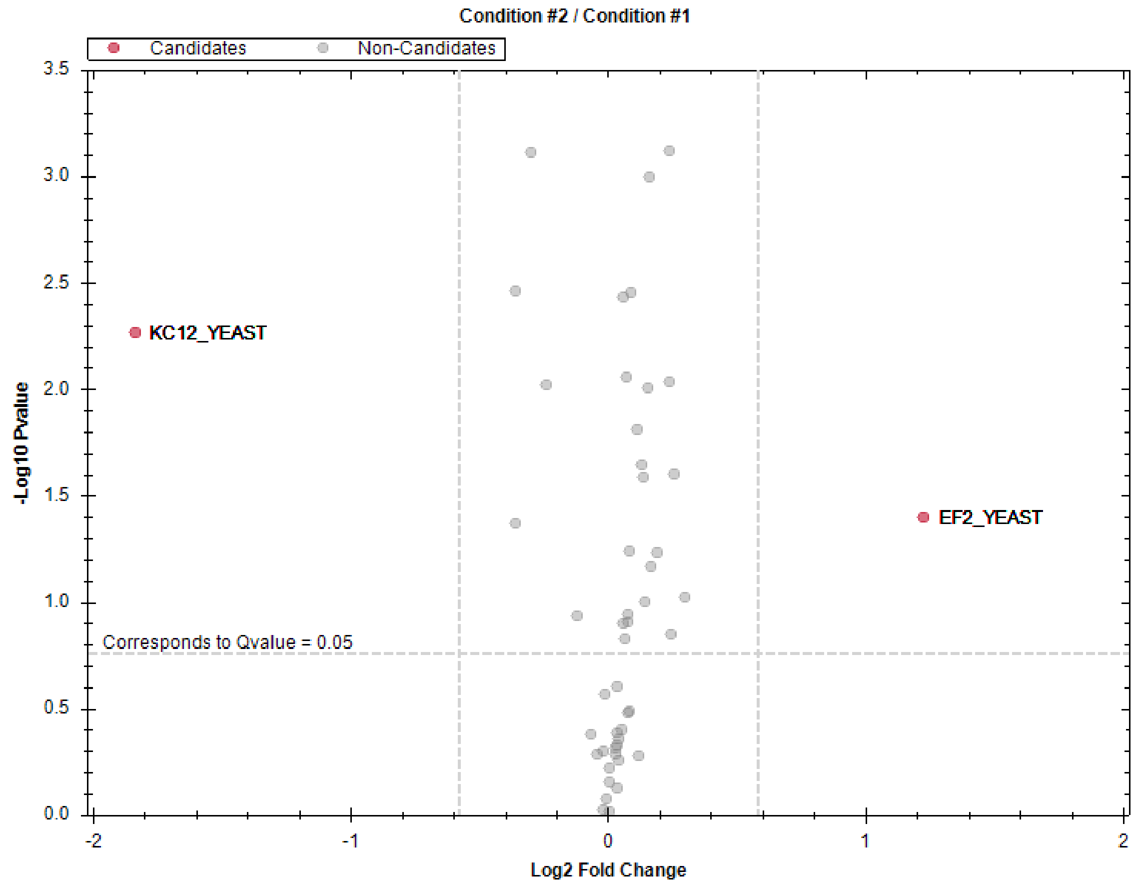

Figure 30. The Volcano Plot shows the candidates in red. This plot is updated when you modify the Candidates table. By right-clicking, you can choose several actions, such as deactivate the legend or annotate the candidates or not.

5.8 Report Perspective

SpectroDive™ has a very powerful reporting strategy. In the Report Perspective, you can design and customize your report to contain any information you may need about the analysis. Report schemas can be saved to be reused. You can also change the column names by double-clicking on the tree node for the corresponding column.

The Report Perspective contains four panels, from left to right:

1. The Schema tree: all different report building schemas. If you save a custom one, it will appear here.
2. Column chooser: all possible reportable elements with check boxes to add or remove them. Below this panel, there is a search field to help you navigate through the different fields.
3. Filters: selection criteria you have applied to your report.
4. Report preview: this one will only be visible if there is an analysis loaded. It will show a preview of how your report will look. This is very useful when you are modifying a schema. When you are happy with your report structure, you can export it by clicking on "Export Report…" in the bottom left corner to be able to see the whole matrix.

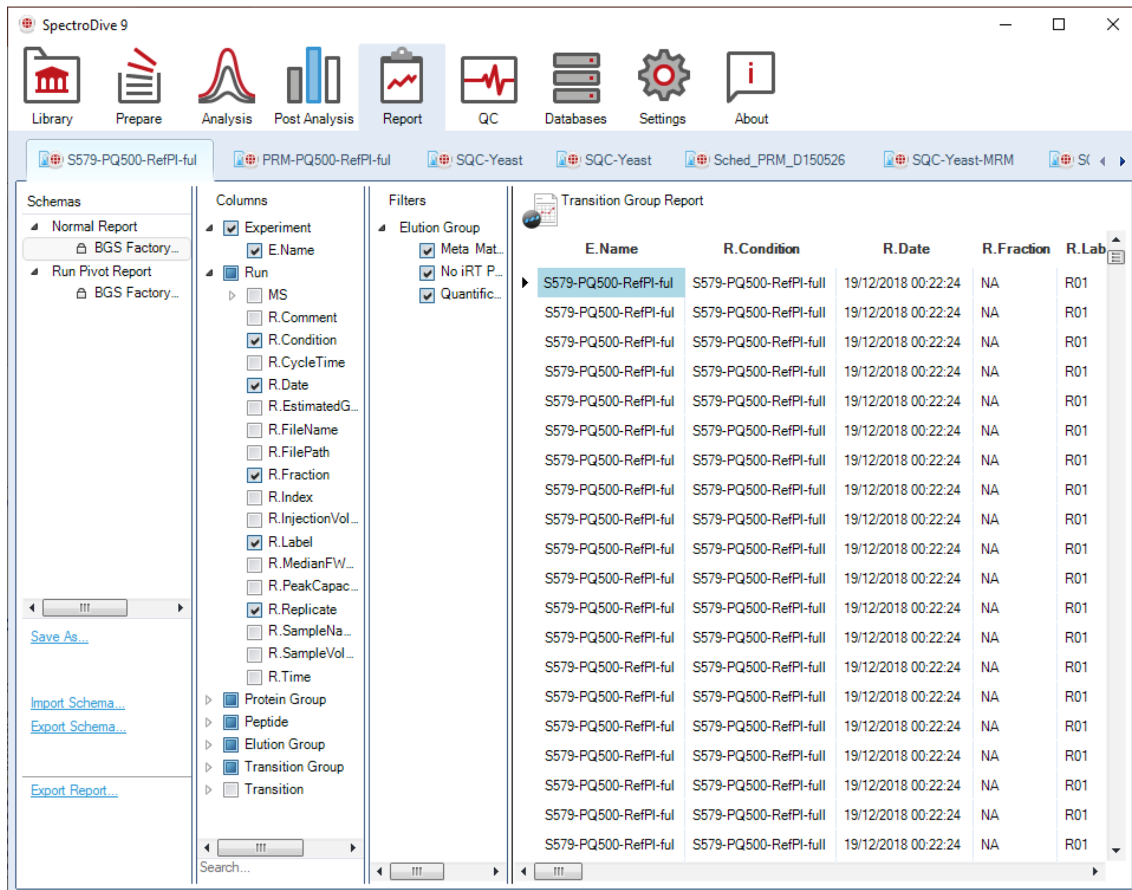

Figure 31.The Report Perspective. The figure shows how a Normal Report schema is modified (selecting the check boxes) and exported. Detailed explanation of the headers can be found by hovering over them as well as in Appendix 8. Most Relevant Report Headers (section 7.10)

5.8.1 Report Schemas

SpectroDive includes several preconfigured reporting schemas that may fit most frequent needs. If you want to design your own, you can use one of the included as a base to build your preferred report. Within the report schemas, there are two main formats you can export your data into: Normal Report and Run Pivot Report. You can find detailed information about each format below.

5.8.2 Normal Report

In a Normal Report (long format), you will find each reported event in a single row. A Normal Report will usually have many more rows than a Run Pivot Report. This format is the one allowing for the most comprehensive report of your data. To build your Normal Report, add or remove columns from the Columns panel by checking or

unchecking them (Figure 31). The Columns are organized by levels, from more general (Experiment) to more specific (Transition):

- >Experiment
  - >Run
    - >Protein Group
      - >Peptide
        - >Elution Group
          - >Transition Group
            - >Transition

Within each of these levels, the columns are again organized by categories (e.g., identification, quantification, scoring, etc.). The whole Columns tree is quite comprehensive and expanding/collapsing categories when looking for a column can be cumbersome. To make the search for columns easier, there is a search filed at the bottom of the Columns panel where you can type what you are looking for (Figure 31). Finally, to know which information a header contains, hover over it and you will see a text box popping up with a description. To see a detailed description of some of the most relevant columns, see Appendix 8. Most Relevant Report Headers (section 7.10).

5.8.3 Run Pivot Report

In a Run Pivot Report (wide format), each run (sample) is a header column. You can choose which elements you would like to be included by selecting them in the Columns panel under Row Labels (e.g., stripped peptide sequence) and which value you would like in the cells under Cell Values (e.g., quantitative value, Figure 32). If you choose more than one Row Label or Cell Value, the table will multiply its length column-wise. This report will probably have fewer rows than a Normal Report.

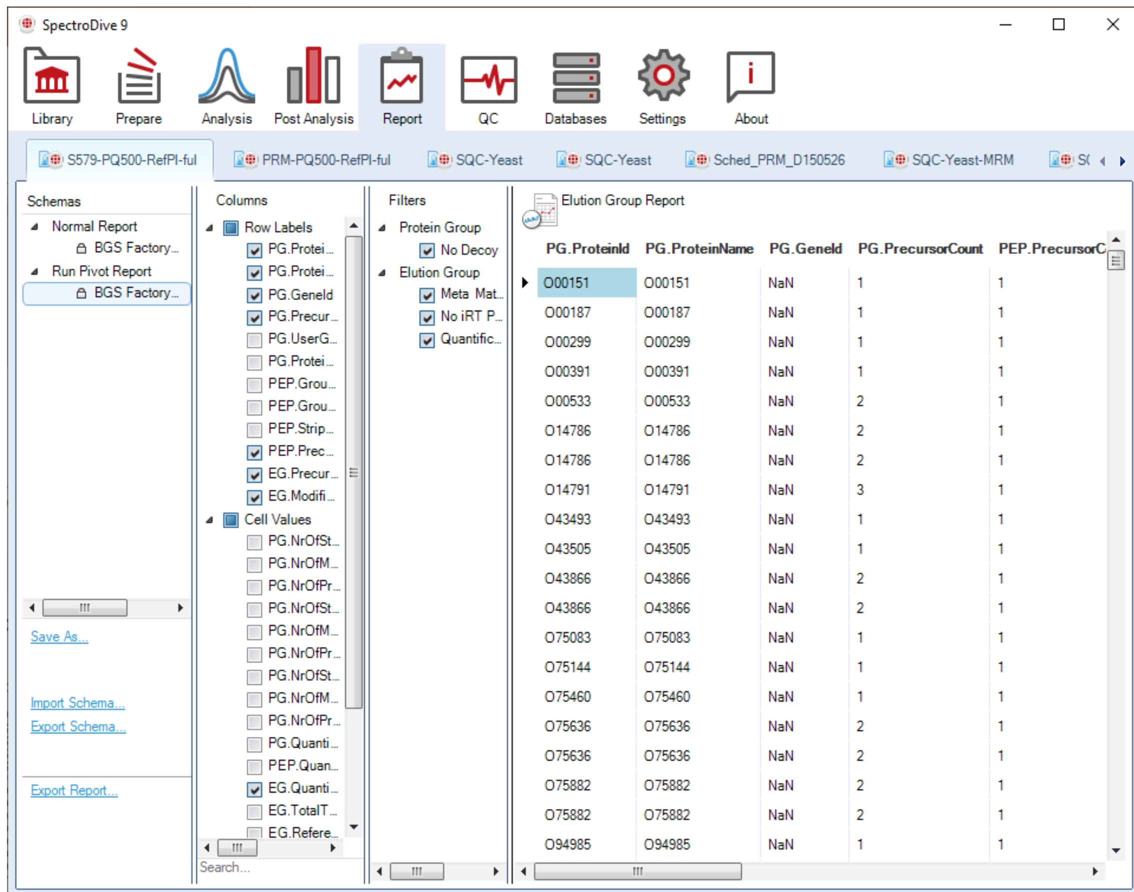

Figure 32. Run Pivot Report. This report is in wide format contains one column per run (sample).

5.9 Quality Control Perspective

The Quality Control Perspective of SpectroDive™ is based on the peptides in the Indexed Retention Time Reference Peptide Kit. Chromatography, mass spectrometer performance and analysis can be monitored over time using several performance indicators. Every successful analysis is stored in the Quality Control Perspective (Figure 33). SpectroDive automatically detects various instruments and will create a separate quality control history for each of them. If you have more than one instrument of the same type it might be useful to rename them manually. Additional folder structures can be made according to the established quality control testing performed in a specific laboratory. Only as many runs as specified in Settings → Global → General → QC Plot History Length are shown in the plots.

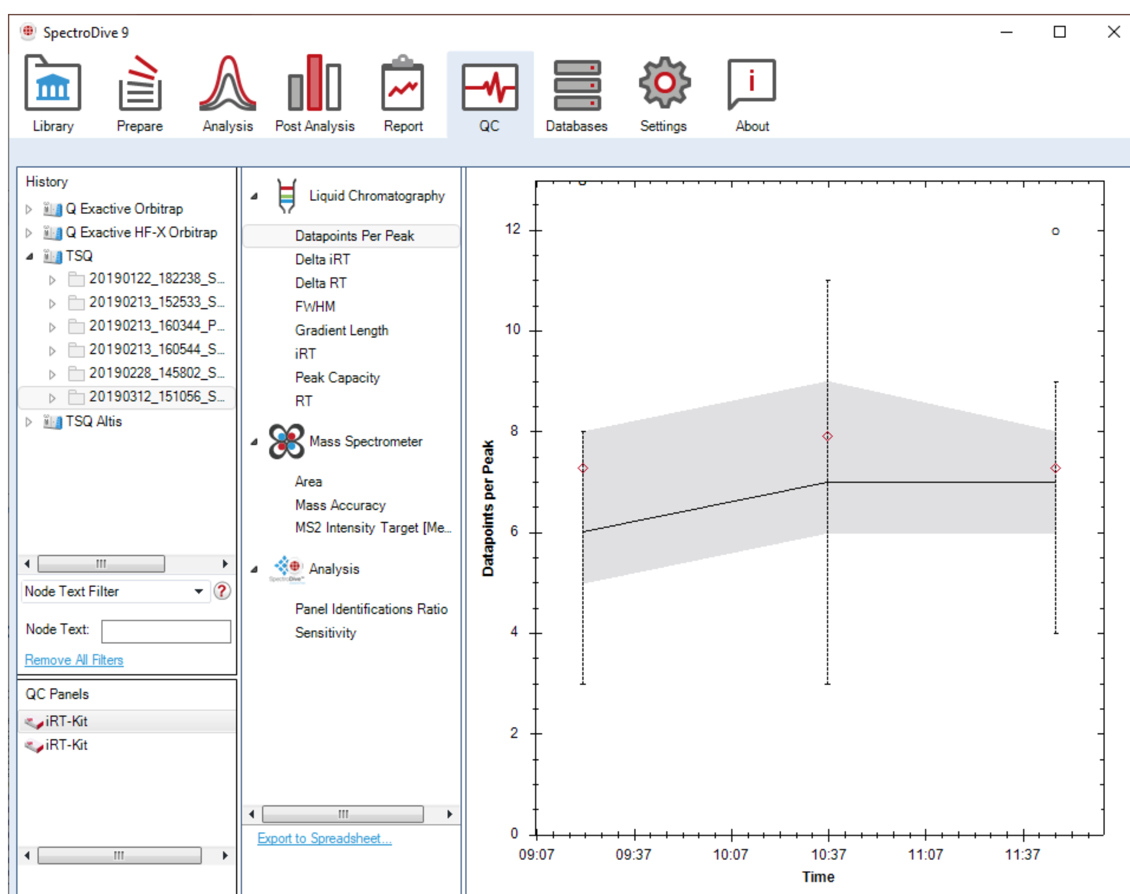

Figure 33 QC Perspective. Runs in which QC panel is detected are saved in the history tree. You can monitor instrument performance with help if many plots related to several aspects of the experement, form LC to SpectroDive analysis.

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5.10 Databases Perspective

The Databases Perspective allows you to store and manage information that you will need to use when setting up analyses. This includes protein databases (FASTA files), peptide modifications and protein digest rules.

5.10.1 Protein Databases

This section of the Databases Perspective lets you import and manage your protein sequence databases. SpectroDive uses protein databases (FASTA files) to make Pulsar searches for library generation and to perform protein inference. The protein databases contain all the sequences, as well as metainformation extracted from the FASTA protein headers using the specified parsing rule. SpectroDive already contains the UniProt parsing rule, and you can add a new rule by clicking "New Rule" in the Protein Databases page or during the import of a new protein sequence database (Figure 34).

In order to import a new protein database from a FASTA file, click on "Import…" in the bottom left corner (Figure 34). While importing, SpectroDive will try to find the appropriate parsing rule for this header line format from the already specified rules. Should there be no matching parsing rule found, you will be asked to specify a new one. Once your new protein database is imported, it will be available in the Databases tree.

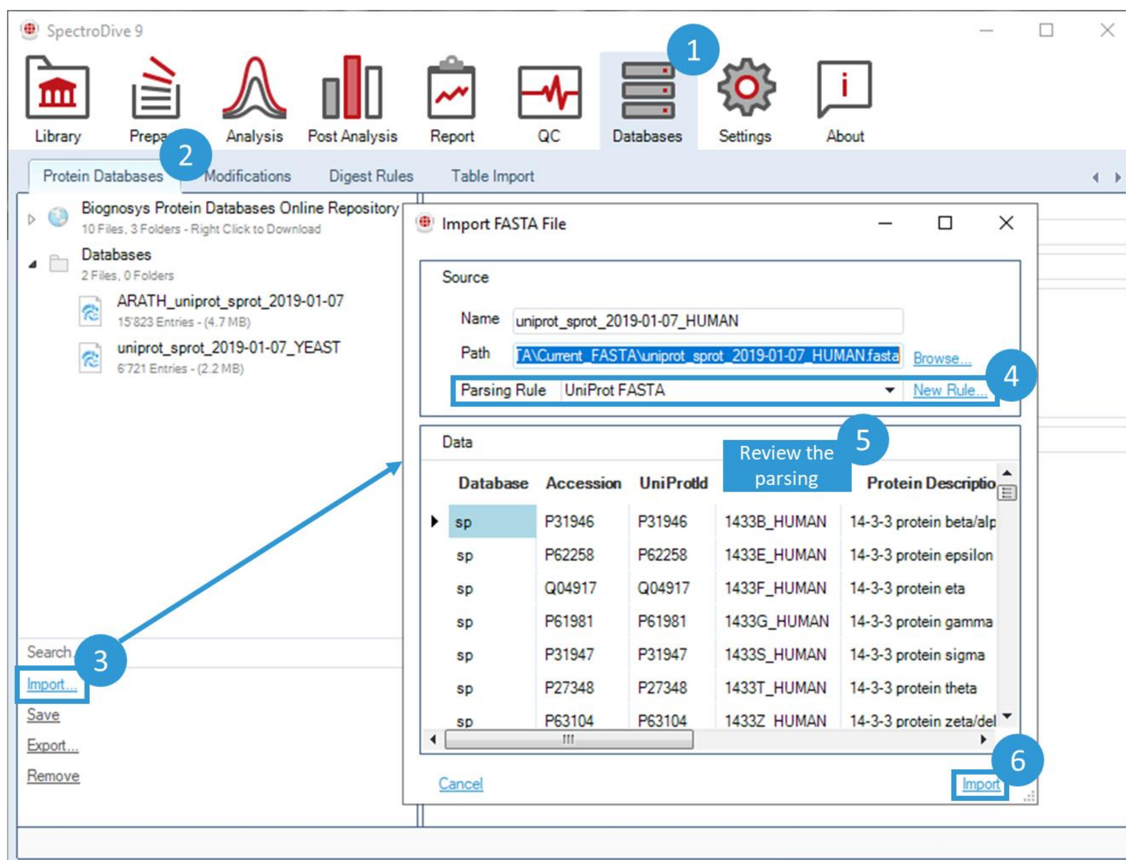

Figure 34. Importing a new FASTA file into SpectroDive. Your new database will appear in the Databases tree and will be available for setting-up analyses.

5.10.2 Modifications

The SpectroDive modifications panel provides a central location to manage modifications. Modifications contained here are used to automatically detect modified sequences in a search engine output when creating a spectral library from an external search engine. They are also used to detect modified sequences encoded in an external spectral library by means of synonyms. We recommend that modifications in a spectral library are mapped to SpectroDive modifications for optimal downstream analysis. If a library is generated using SpectroDive, this is taken care of automatically. SpectroDive comes with a database of default modifications for all search engines. If you use special modifications, please import the corresponding modifications file into SpectroDive.

5.10.2.1 *Importing Modifications from Search Engine*

To import non-default modifications into SpectroDive, you can batch import:

- For MaxQuant, using the modifications.xml file in its bin folder

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- For ProteinPilot, using Unified Modification Catalog.xlsx, located in the ProteinPilot/Help folder in the Program Files
- For Proteome Discoverer, no action is required
- For Mascot, non-default modifications have to be created as custom modifications, see below.

When possible, SpectroDive will merge identical modification from multiple sources and save only the necessary search engine specific mapping information. However, if it is not able to unambiguously merge two or more modifications, you will be asked to resolve any conflicts at the time of import. You can tell if a modification has been mapped to multiple search engines by looking at the "Mapped to" data grid in the panel.

5.10.2.2 *Creating custom modifications*

It is also possible to specify a new modification. This action has two main applications:

1. Incorporate modifications for Mascot searches which are not in the *Unimod* database (non-default ones).
2. Add a new label to generate a labeled library

To create a new modification, click "new" in the bottom left corner, give a name to your modification, and click "OK" (Figure 35). Edit your new modification as desired and click "Save" in the bottom left corner. You can also modify an existing modification by clicking "Save As…".

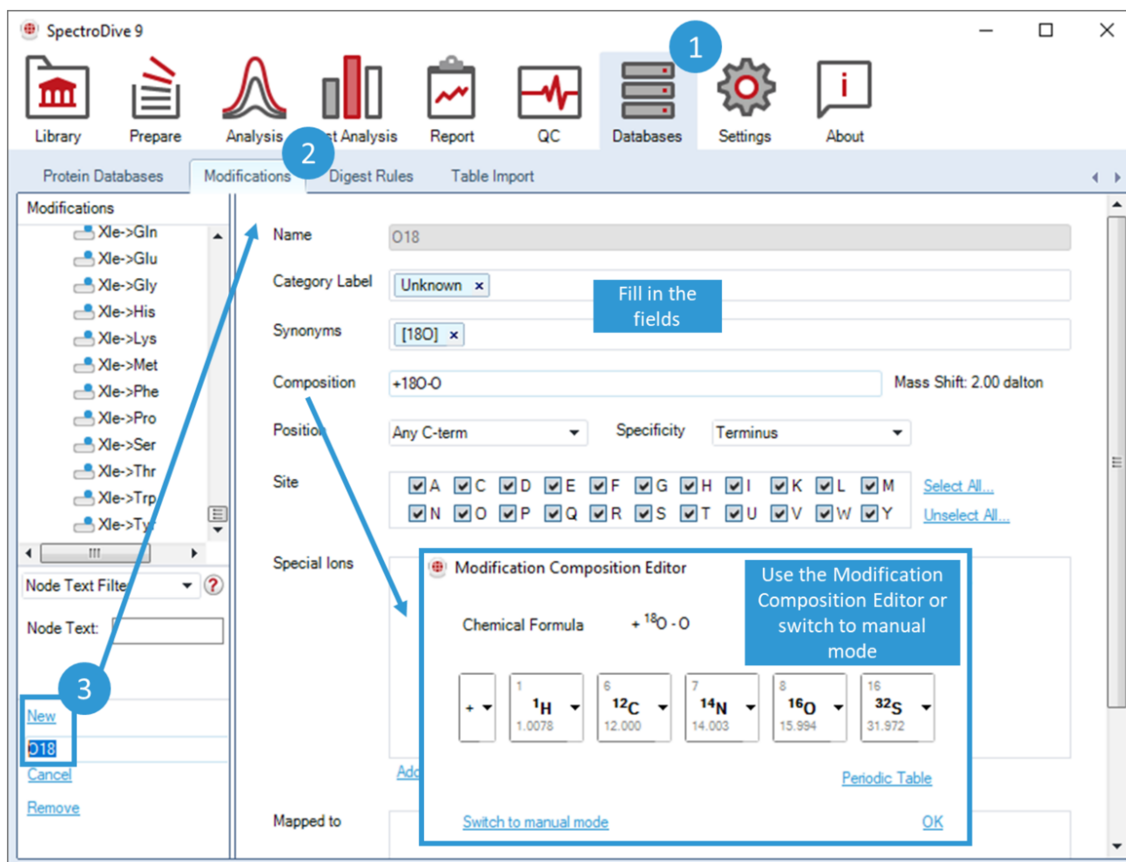

Figure 35. Adding a new modification to the database.

5.10.3 Cleavage Rules

This tool lets you define the rule to *in silico* digest your proteins from the protein database(s). Digest rules are applied whenever you do a Pulsar search (in library generation). The most frequent rules are already included in SpectroDive, such as Trypsin, Trypsin/P, and LysC.

To design your own rule, you have to click on an existing one, modify it and click on "Save as…" in the bottom left corner (Figure 36). The rules are defined by which sites are cleaved by the enzyme. In the Digest Rule page, you will see a 20 x 20 matrix containing all possible combinations of amino acids. Select the combination where your enzyme cleaves (Figure 36). At the bottom, you will see a preview of how a sequence will look after being cleaved following your digest rule. You can also include a description.

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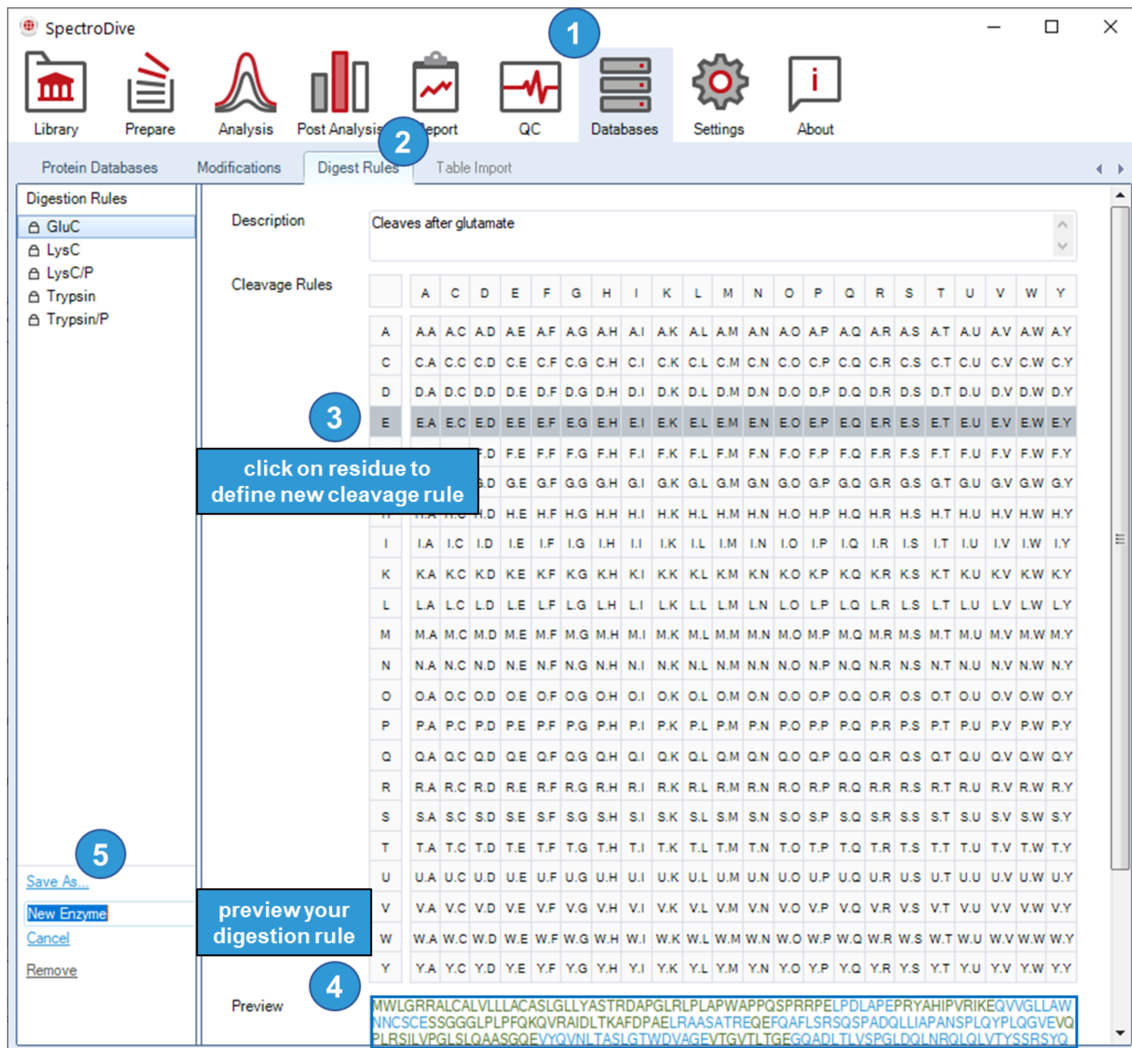

Figure 36. Define a new Cleavage Rule. The Cleavage Rule editor will allow the generation of new cleavages rules in a very friendly manner.

5.10.4 Table Import

SpectroDive™ can remember column names in user spectral libraries. Once you import a new library format into SpectroDive, it will ask you whether it should store novel synonyms for column header. You can manage those synonyms in the column recognition settings tab.

5.11 Settings Perspective

The Settings Perspective is used to define custom settings schemas for any of the processes performed by the software. In this perspective, you will see a tab corresponding to each of these processes: Analysis, Library Generation and Pulsar Search (Figure 37). In addition, you can alter global settings of SpectroDive in the Global page (see below). Detailed information regarding each setting option can be obtained by hovering the pointer over the label of a specific settings option (Figure 37).

You can make your own Setting schema by modifying one of the predefined ones. Go throughout the nodes and edit the corresponding settings. Once you are done with the customization, click "Save as…" in the bottom left corner to give a name to your schema, and click "OK" (Figure 37). Your new schema will appear in the tree and it will be available to be selected during the set-up of your next analysis. Please see the Appendixes for detailed information about the numerous settings within each process.

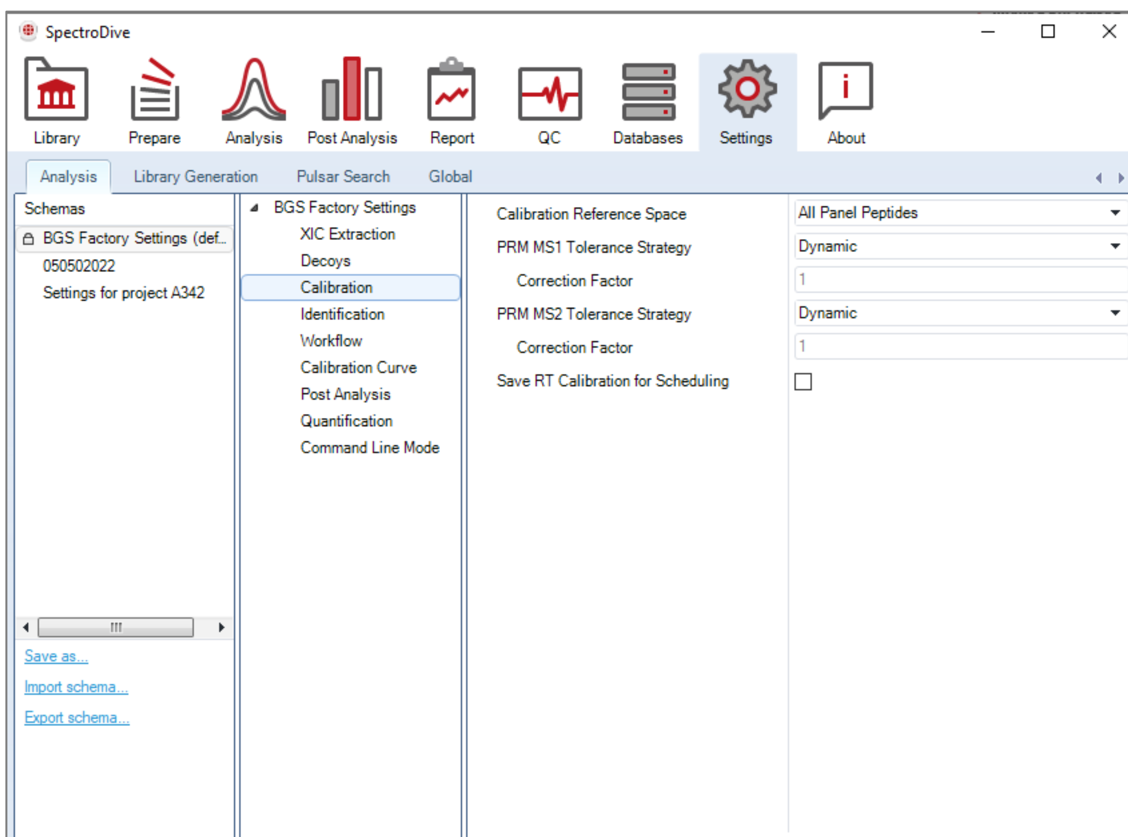

Figure 37 The settings Perspective. Make a custom schema for your analysis. The new schemas will be available during the subsequent analysis set ups.

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5.11.1 Analysis Settings

The MRM/PRM Analysis Settings define the details of how SpectroDive will analyze the data, from raw data extraction to post-analysis calculations. These settings will specify important metrics, such as FDR cutoffs, decoy set generation to estimate statistics scores, quantification settings and workflow to be used (label-free, labeled, spike-in), among many others. Find details of each setting in Appendix 1. MRM/PRM Analysis Settings (Section 7.3).

5.11.2 Pulsar Search Settings

These settings define how Pulsar will create the search-space when performing a search. You can specify the expected peptide characteristics (digest enzyme used, length, modifications, etc.). Find details of each setting in Appendix 4. Pulsar Search Settings (Section 7.4).

5.11.3 Library Generation Settings

This set of settings defines the Library Generation process, both from search results generated by Pulsar or by an external search engine. You can specify metrics such as MS1 and MS2 mass tolerances, FDR cutoffs for identification confidence, peptide-based filters for your library, among others. Find details of each setting in Appendix 4. Library Generation Settings (Section 7.5).

5.11.4 Global Settings

The Global settings tab in the Settings Perspective will allow you to change parameters that can be considered analysis unspecific. Here you will find options regarding general settings, working directories, plotting and other settings.

5.11.4.1 General

This section contains settings options that allow you to modify the default behavior of SpectroDive. For more information about these options use the pointer to hover over each individual entry. One important aspect of this settings is the File Name Parsing Schema to let SpectroDive read information directly from the run file name. One of the most relevant uses of this function is the Conditions annotation. Parsing of the file names requires the file annotation to be separated by a special

character, by default this is the "_" character. By defining the meaning of the different segments in the file name, SpectroDive can obtain this information automatically (Figure 38).

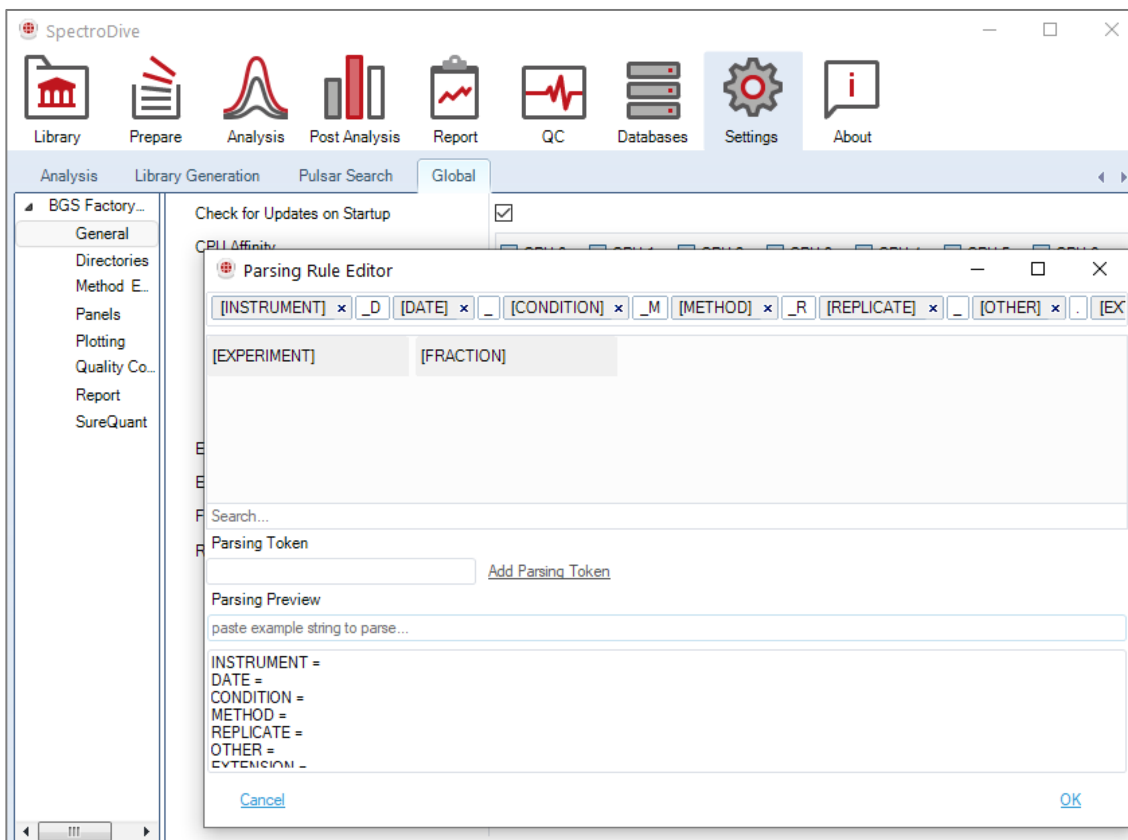

Figure 38. Parsing Rule Editor in the General Settings of the Global Settings page. Setting this rule properly will let SpectroDive read annotation information directly from the run file name.

5.11.4.2 Directories

Here you can setup the different storage paths for data managed by SpectroDive. Should you have a central storage location for all your raw data, you can specify this location here. This will allow SpectroDive to automatically map the correct acquisition runs during the setup of the library generation pipeline (see Box 3). See some recommendation for optimal performance in section 3.6.

NB! *All changes within the "Directories" section will require a restart of SpectroDive in order to take effect.*

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5.11.4.3 Method Export

The Method Export section allows you to customize the *m/z* range when generating and exporting an acquisition method for different MS instruments.

5.11.4.4 Panels

Here you can set up the calibration history (how many RT calibration runs are stored), data extraction (percent of matching transitions between the panel and the acquired data) as well as the directory where panel kit files are located.

5.11.4.5 Plotting

The plotting section allows you to customize the look and feel of most plotting options used in SpectroDive. You can specify whether XIC plots should show the integration boundaries, as well as the expected elution time. Additionally, you can also apply smoothing to your plots. For more information about these options use the tool tip hover for each individual entry.

5.11.4.6 Quality Control

This section allows you to specify two things: the maximum number of runs to be used in the QC plot and the QC results storage path.

5.11.4.7 Report

Here you can specify the number of rows that will be displayed in the Report Perspective.

5.11.4.8 SureQuant

You can specify here global defaults for the SureQuant acquisition method generation. These settings are mainly related to the intensity threshold of the reference peptide which will trigger acquisition for the target one.

5.11.5 SpectroDive Command Line Mode

In addition to the visual pipeline mode, SpectroDive is also capable of running the pipeline from command line. To run SpectroDive in command line mode you can simply call the SpectroDive.exe file using the following parameters.

`-r` Adds a raw file to the experiment. Any file format that is also supported during the analysis setup from the user interface is possible. This command can be used multiple times to add additional files.

`-d` Adds all raw files of a specified directory to the experiment. This command can be used multiple times to add additional directories.

`-a` Assigns an assay panel to every run in the experiment.

`-s` [OPTIONAL] Selects a settings schema (the schema must already exist). If not specified, whatever is selected as the default schema will be used. This command should be provided with a settings schema name, or a path to the schema file (*.prop).

`-o` [OPTIONAL] Specifies an output directory for the reports. If this parameter is not present, reports will be generated in *%AppData%/SpectroDive/results.* This directory should already exist.

`-n` [OPTIONAL] Specifies the name of this experiment. If not provided, SpectroDive will automatically generate an experiment name from the selected run file names.

`-rs` [OPTIONAL] Selects a report schema (the schema must already exist). If this schema is not present, then the default schema will be used.

`-con` [OPTIONAL] The path to condition setup file as exported via the SpectroDive Conditions Setup.

`-pq` [OPTIONAL] Selects a peptide information file for peptide quantification with calibration curve (the peptide information file must be generated prior in GUI).

`-ccs` [OPTIONAL] Selects a calibration curve file (the calibration curve file has to be generated in the GUI or by command-line (ccs file))

An example:

```
SpectroDive.exe

-d "C:\PRM\PQ500_Plasma_Depleted"

-a "C:\04_Resources\panels\PQ500.kit"

-n "PQ500_MQLive_Depleted" -o "C:\Results"
```

If you encounter problems with the automatic parsing of your spectral library, please first try to load the spectral library using SpectroDive's graphical user interface and make sure that all necessary columns are recognized automatically.

6 References

Benjamini Y, and Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc* 57:289–300.

Bruderer R, Bernhardt OM, Gandhi T, and Reiter L (2016) High-precision iRT prediction in the targeted analysis of data-independent acquisition and its impact on identification and quantitation. *Proteomics* 16.

Callister SJ, Barry RC, Adkins JN, Johnson ET, Qian W, Webb-Robertson B-JM, Smith RD, and Lipton MS (2006) Normalization Approaches for Removing Systematic Biases Associated with Mass Spectrometry and Label-Free Proteomics. *J Proteome Res* 5:277–286.

Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen J V, and Mann M (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 10:1794–1805.

Dunn OJ (1961) Multiple Comparisons Among Means. *J Am Stat Assoc* 56:52–64.

Escher C, Reiter L, Maclean B, Ossola R, Herzog F, Chilton J, Maccoss MJ, and Rinner O (2012) Using iRT, a normalized retention time for more targeted measurement of peptides. *Proteomics* 12:1111–1121.

Zhang B, Chambers MC, and Tabb DL (2007) Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. *J Proteome Res* 6:3549–3557.

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7 Appendixes

7.3 Appendix 1. Analysis Settings

XIC Extraction

The XIC RT Extraction Window defines if iRT should be used to predict the elution of a peptide:

- Dynamic (default setting): Spectronaut will determine the ideal extraction window dynamically depending on iRT calibration and gradient stability. Sections of the gradient that show higher variability during the calibration step will automatically be extracted using wider windows.
- Static: Spectronaut will use a fixed width (in min).
- Full: Spectronaut will use the full gradient width to find the target.

Select how SpectroDive should determine MS1 and MS2 mass tolerances for the XIC extraction and scoring:

- Dynamic: Let SpectroDive determine the ideal tolerance based on the extensive mass calibration
- Relative: Select a relative mass tolerance in ppm for the targeted ion mass
- Static: Select a fixed mass tolerance in Thompsons

Select how SpectroDive should perform XIC extraction for data acquired with PASEF: Use fixed PASEF ion mobility range or use PASEF ion mobility range obtained directly from the acquisition runs.

Decoys

Defines which decoy generation strategy should be used for the generation of PRM decoys. As a default the option Mutated is selected, which could be changed to Inverse or Scrambled.

Calibration

Calibration Reference Space	Specify which peptides are used for iRT and mass calibration. Either all panel peptides or only the iRT kit peptides. Using all panel peptides is recommended.
PRM MS1 Tolerance Strategy and PRM MS2 Tolerance Strategy	Specify how the MS1 and MS2 mass tolerance is determined and scored. • Dynamic: Let SpectroDive determine the ideal tolerance based on the extensive mass calibration • Relative: Select a relative mass tolerance in ppm for the targeted ion mass • Static: Select a fixed mass tolerance in Thompsons
Save RT Calibration for Scheduling	Select this if you want to save the RT to iRT calibration for each run to be saved in the prepare perspective for using it to schedule future runs. When you select the "Set up Analysis for Method Scheduling", this will be automatically turned on.

Identification

Basic Settings	
Identification Method	Qvalue-based The protein inference, report generation and differential abundance will be performed for all the identifications that pass the ***q*-value cut-off**. **Use Machine learning** - when checked, applies mProphet-like algorithms to determine the data dependent score weights.
Reference must be identified	If selected, the SpectroDive will apply the q-value threshold to both the target and reference channel in a spike in workflow.
Advanced Settings	
Run based	When checked, calculated the *q*-values for each run individually, when not checked, performs global *q*-value calculation including all runs of the experiment.

Workflow

Here you can specify if you are running a label-free analysis or a different kind of quantification

Basic Settings	
Multi-Channel Workflow Definition	• **From Library Annotation**: will take the definition annotated in the library. Any precursors containing only one channel specification will automatically be treated as label-free. You can set what is the fallback option if the annotation detection fails. • **Labeled**: peak detection and scoring will be applied to all channels. Quantification in post-analysis will be performed on the light to heavy ratio. • **Spike-in**: peak detection will be performed on only the reference (default is set to heavy) channel. Scoring and identification will be performed on the target (light) and reference (heavy) channels. The heavy channel is expected to be easily detectable and considered a peak-picking aid in this experiment. Quantification in post-analysis will be performed on the target to reference ratio. **Inverted**: If selected, the light channel is considered the reference channel, if not selected, the heavy channel is considered the reference channel. **Reference based Identification**: If selected, will perform peak detection and identification on the reference channel, and if not selected, will perform peak detection on the reference channel, and identification on the target channel.
iRT Refinement Experiment	Use this option when you are analyzing a file acquired using the “iRT Refinement” based method export in the Prepare Perspective.

Advanced Settings	
Profiling Strategy	The profiling workflow allows the user to carry over the measured iRT of peptides that could be identified (Qvalue ≤ 0.01) in certain runs to fix integration boundaries in runs where an identification could not be achieved: • **Template Correlation Profiling**: takes the best peptide signal in all runs as a template to find low abundant signals in the rest of the runs. • **iRT profiling**: takes the best peptide signal in all runs as a template and translates the empirical iRT to the integration boundaries of the low abundant signals in the rest of the runs. Profiling Row Selection: choose a row-wise *q*-value threshold for to choose which rows to profile (Minimum, Average or none). Profiling Target Selection: specify which precursors should be readjusted (non-identified or automatic).
SureQuant/HybridDIA Settings	**Method Detection** – you can specify if you would like to force SpectroDive to use SureQuant preprocessing or HybridDIA preprocessing, or use regular PRM preprocessing (use SureQuant Pipeline, HybridDIA Pipeline or use regular PRM Pipeline, respectively). Otherwise you could leave default option – automatic, which will allow automatic detection of the file type and automatic choice of applicable pipeline. **Scan Matching Threshold** – specifies the strategy which is used for the matching of SureQuant scans to the panel peptides, which is either by matching to the Precursor Window Center or to the Entire Precursor Window.

Quantification

Basic Settings	
Precursor Filtering	Decide how to apply the q-value filter on the precursors in an experiment-wide manner to quantify protein groups: • **Identified (Qvalue)** (default): only those precursors passing the q-value cut-offs will be reported (considered as quantified) and, accordingly, used for statistical testing of differential abundance. Those cases not passing the cut-off will be tagged as "Filtered". By the default there will be no imputation of missing values. • **Identified in All Runs** (complete): the peptide precursor needs to pass the q-value threshold in all the samples to be reported. This is the most stringent filter and produces the smallest data matrix. • **Identified in % of Runs:** the peptide precursor needs to pass the Qvalue threshold in the user defined percentage of all experimental samples. For instance, if you set a 50th percentile cutoff (0.5), the peptide precursor needs to pass the Qvalue in 50% or more of your samples to be reported. In the samples where it was not identified below the significance threshold (default ≤ 0.01), the specified Imputing Strategy will be applied.
Use Reference Normalization	When selected, SpectroDive will perform a cross run normalization using labeled reference peptides. The signal of each target peptide will be divided by signal of label reference and multiplied by average signal of that reference across the runs.
Major Group Quantity	Specify how you want the minor groups to be used to calculate the major group quantities.
Major (Protein) Grouping	Specify what should be considered as a protein (Protein Group Id or Gene Id).
Minor (Peptide) Grouping	Specify what should be considered as a peptide (Stripped Sequence, Modified Sequence or Precursor).
Minor Group Quantity	Specify how you want the precursors to be used to calculate the minor group quantities.

Quantity Type	Decide which feature of the peaks should be used for quantification: area under the curve within integration boundaries or peak height.
Quantity MS-level	Specify MS level that should be used for the quantification. Precursor quantities are calculated as a sum of all selected ions that were not removed or excluded.
Advanced Settings	
Major Group Top N	Use a specific range of the best minor group elements to calculate the major group quantities.
Minor Group Top N	Use a specific range of the best precursors to calculate the minor group quantities.

Command Line Mode

When selected, SpectroDive should save SpectroDive Experimental file (SPE file) automatically. This option applies only if SpectroDive is run via command line.

7.4 Appendix 2. Pulsar search settings

Configure the conditions for Pulsar search:

- **Peptides**

Specify settings related to the peptide sequences.

Enzyme/Cleavage Rules	Proteases used for *in silico* digest of the protein sequences from the protein database(s). Defined in Databases → Cleavage Rules
Digest Type	Specific: both N- and C-terminus follow the specified digest rules Semi-specific: only one of the termini follows the specified digest rules Unspecific: no digest rules
Max Peptide Length	Maximum number of amino acids allowed for a peptide
Min Peptide Length	Minimum number of amino acids allowed for a peptide
Missed Cleavages	How many consecutive cleavage sites the protease could miss

Toggle N-terminal M	Pre-processing of the protein database by removing the N-terminal M (when there is one)

❖ **Labeling**

Labeling applied	If checked, there are up to 3 channels where you can specify which modifications are in which channel.

❖ **Modifications**

Maximum Variable Modifications	Maximum number of variable modifications allowed to happen in one peptide at the same time. The higher this number, the more possible combinations, and the bigger the search space and the longer the calculation time
Select Modifications	Fixed: the amino acid always contains the modification Variable: the amino acid might or might not be modified. The more variable modifications, the bigger the search space and the longer the calculation time

❖ **MS2**

Ion Types	You can choose which ions should be generated in silico
MS2 Demultiplexing	Allows the processing of alternating shifted MS2 windows as presented in D.Amodei et al. 2019

7.5 Appendix 3. Library Generation Settings

Configure the library generation settings. Most settings are described below. For further information, there are also helpful text hovers directly in the software.

Tolerances

SpectroDive™ will, by default, calculate the ideal mass tolerances. SpectroDive performs two calibration searches: based on the first-pass calibration (rough calibration), the ideal tolerance for the second-pass calibration is defined; based on the second-pass calibration (finer calibration), the ideal tolerance for the main search is defined. SpectroDive will do this under default settings (Dynamic).

SpectroDive also allows you to set you preferred tolerances for the different MS instruments. Hence, for both the calibration search (second-pass, finer calibration), and the main search, you can define your tolerances:

- **Dynamic**: determined by SpectroDive based on the precedent search (default). You can set a correction factor for MS1 and MS2 levels (default is no correction = 1)
- **Relative**: set a relative mass tolerance in ppm for both MS1 and MS2 levels
- **Static**: set a fix mass tolerance in Thomson for both MS1 and MS2 levels

Identification

You can specify the search engine scoring type and thresholds

Pulsar	• Filter on PTM Localization: select this option when interested in including modified peptides only if they fulfill a PTM site localization probability (default 0.75). • Peptide FDR Cut off: Specify the FDR threshold on peptide level • Protein FDR Cut off: Specify the FDR threshold on protein level • PSM FDR Cut off: Specify the FDR threshold on PSM level
MaxQuant	• FDR Threshold: default set to 0.01
Proteome Discoverer	• Peptide Confidence Level: default set to High • Protein Confidence Level: default set to High • PSM Confidence Level: default set to High
ProteinPilot	• Peptide Confidence Level: default set to 99% • Protein Unused Score: default set to 0
Mascot	• Ion Score: default set to 0

	• OneinXprobRnd: Ion score threshold value for e PSM being a random match based on 1 in p probability of random match

Protein Inference

You can have this option activated or deactivated. If you let SpectroDive do your proteins inference, you can refine your sequence settings further in these settings:

Digest Rule	Proteases used to *in silico* digest the proteins from the protein database(s). Defined in Databases → Cleavage Rules
Digest Type	Specific: both N- and C-terminus follow the specified digest rules Semi-specific: only of the termini follows the specified digest rules Unspecific: no digest rules
Toggle N-terminal M	Pre-processing of the protein database by removing the N-terminal M (when there is one)

Spectral Library Filters

You have a number of options to filter the search engine results for library generation. There are filters at the level of fragment ion and at the level of precursor. The filters are quite self-explanatory. Please, use the hover text-tools if you need more information. Find below some of the most relevant.

Fragment Ions	Filter peptides not fulfilling the conditions specified regarding fragment ions. Find more details by hovering over the option in the software
Amino Acids	Filter peptides containing specified amino acids
Modifications	Filter peptides according to modifications. Find more details by hovering over the option in the software

iRT Calibration

Set your preferences for iRT calibration:

Basic Settings	
Calibrate from empirical RT	If selected, SpectroDive will generate iRTs based on the empirical RTs during the library import.
iRT Reference Strategy	Define how the reference iRT is derived for iRT calibration: • **Empirical iRT Database** (Default setting). Use Spectronaut's internal empirical iRT reference database of more than 100.000 iRT reference peptides from multiple sources. • **Deep Learning Assisted iRT Regression.** Use the new Deep Learning algorithm to generate the iRT reference set. This is useful when working with non-model organisms hardly covered in Spectronaut's internal empirical iRT reference dataset. • **Use RT as iRT**. No iRT calibration will be performed. It should only be used if the peptide separation method is very stable, homogeneous and non-standard, such as capillary zone electrophoresis (CZE). Select Deep Learning Assisted iRT Regression as a backup option when no non-linear iRT regression is possible with a primary option. **Use stripped sequence to identify Reference Peptides** – if selected, SpectroDive will ignore all modifications when matching peptide to the iRT reference peptides database.
Advanced Settings	
Minimum Rsquare	Choose how strict you want to be to accept the fit of the iRT calibration of your data

Workflow

Fragment Ion Selection Strategy: defines the strategy to be used for selecting the top N fragment ions per peptide precursor • **Intensity Based**: Prioritizes fragment ions by their intensity in the consensus spectra • **Evidence Based**: Prioritizes fragment ions by how often they have been observed in the experiment for the same precursor • **Maximize sequence coverage**: Groups fragment ions by type and position and ranks them based on the best fragment ion per group (either by intensity of by evidence) in an iterative manner.
In-Silico Generate Missing Channels: if generating a library for an isotopically labeled sample, you can activate this option to *in-silico* generate an assay for a missing label pair. For example, if you have a sample of only spiked-in heavy aqua peptides, it will make a light/heavy library where the label-free channel is generated *in-silico* based on the heavy channel and with a consistent fragment ion selection. This option works with n-channel search results as long as you are working with isotopic labels. The workflow options are: Spike in workflow: will create a light channel for all heavy (SIS) peptides that are identified without a light counterpart. Labeled workflow: will detect the labeling setup of an experiment and add the channels that are missing for a given peptide Inverted spike-in: will create a heavy channel for all light peptides that are identified.
Use DNN Ion Mobility decides if ion mobility should be predicted based on deep neural network (DNN) for library generation. • **Auto** will always predict Ion Mobility during library generation. Only if empirical ion mobility value is not available for the peptide, a predicted value will be used. • **Always use predicted Ion Mobility** – a library will contain predicted ion mobility values, regardless if empirical information is available or not. • **Never predict Ion Mobility** – ion mobility prediction will not be performed. Library will contain only empirical ion mobility values (if available).

7.6 Appendix 4. Global Settings

General

This section contains settings that are modifying the default behavior of SpectroDive. You can allow here automatic check for updates with every software start up. Next you determine how many CPUs will be used by the software. Finally, you can define rules for an automatic experiment naming.

Directories

Here you can setup the different storage paths for data managed by SpectroDive. Please note that all changes within the "Directories" section will require a restart of software in order to take effect. You can specify directories for the Local Search Archives, Protein Database Storage, Shotgun Raw repository, Spectral Library Storage and Temporary Directory.

Method Export

Allow to specify maximum and minimum m/z value for the MRM or PRM acquisition for the instruments supported by the software. Transitions that are below or above that range will not be present in exported method.

Panels

Here you can determine settings relevant for the construction of panels and their analysis.

Auto-detect iRT Kit peptides	When selected, SpectroDive will automatically look for iRT Kit peptides
Calibration History	Determines how many (n) last calibration files will be stored at your computer, available for performing panel calibration.
Matching Fraction	Defines how many transitions from your panel have to be found in the analysis run to allow a positive match.
Panel Store	Specifies additional directory for the panel kits.

Plotting

The plotting section allows you to customize the look and feel of most of the plotting options used in Spectronaut. You can specify whether XIC plots should show the integration boundaries, as well as the expected elution time. Additionally, you can also apply smoothing to your plots. Finally, you can select Line Strength for the plots in pixels.

Quality Control

Those settings allow you to determine how many runs should be displayed in QC plots and where you would like to store QC results.

Report

Preview Row Count determines number of rows which will be previewed in the Report Perspective. In order to see full data matrix, generated report must be exported.

SureQuant Settings

These settings allow you to define global defaults for the SureQuant acquisition method generation.

Default Intensity Threshold	Allows you to define the intensity threshold in the situation when peptide was not detected in a survey scan.
Global Minimum	Defines the minimum intensity threshold used for peptides
Minimum Fragments	Determines the recommended number of fragments that peptide should have for triggering. If that number is not met, the peptide will be listed with a warning that could be seen in ReadMe file in the SureQuant method export.
Peak Fraction	Defines the percentage of monoisotopic peak heigh that will be used for determination of intensity threshold.

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7.7 Appendix 5. Analysis Perspective Plots

7.7.1 Run Node Plots

On the run node level, one can access different plots which give you detailed information about the RT Calibration, Run overview information and cross run RT Overlay.

RT Calibration Chart

This chart shows the status of run calibration. Initially, only showing the peptides of the used calibration kit. SpectroDive™ now also supports non-linear gradients using a refined calibration based on the initial calibration and detailed information in the user library.

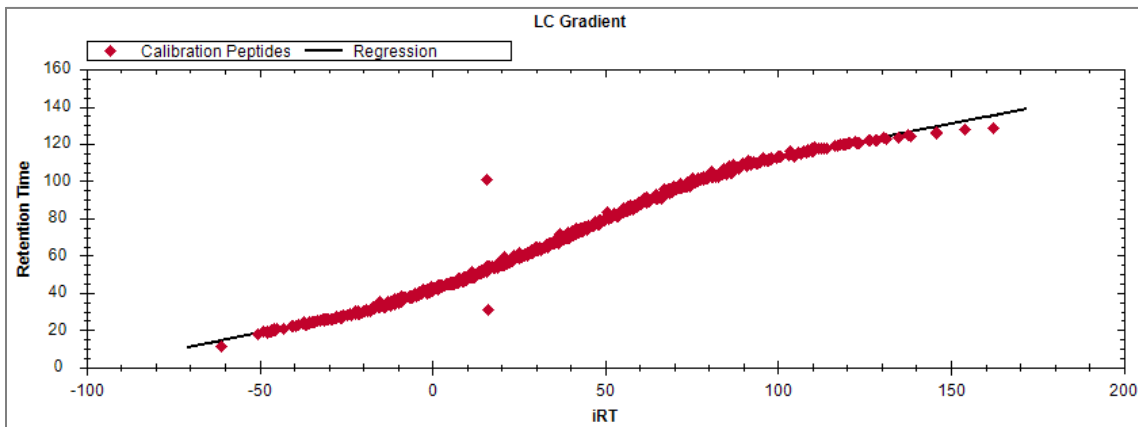

Figure 39. iRT Calibration Chart showing the non-linear regression of the predicted retention times. The chart is shown after extended non-linear calibration has been performed. The extended calibration allows you to correct even small gradient fine-structure fluctuations in order to get the most accurate retention time prediction for your library.

Run Overview & RT Run Overlay Plot

The Run Overview chart (Figure 40, top panel) shows the extracted chromatograms of all targeted peptides of this run. The RT Run Overlay (Figure 40, bottom panel) combines the information of all runs of your experiment in one plot for better insight into instrument stability. You can right-click on these plots and select “Show Point Labels” to annotate the peaks with the matching peptide sequence. It is possible to toggle the x-axis between retention time and iRT scale (select “Use iRT scale”).

If more than one panel were used for the analysis, above information could be also visualized for each of the panels separately. In order to do you can go to the panel of the

interest in the analysis tree and choose Panel Overview Chart and RT Panel Overlay Chart respectively.

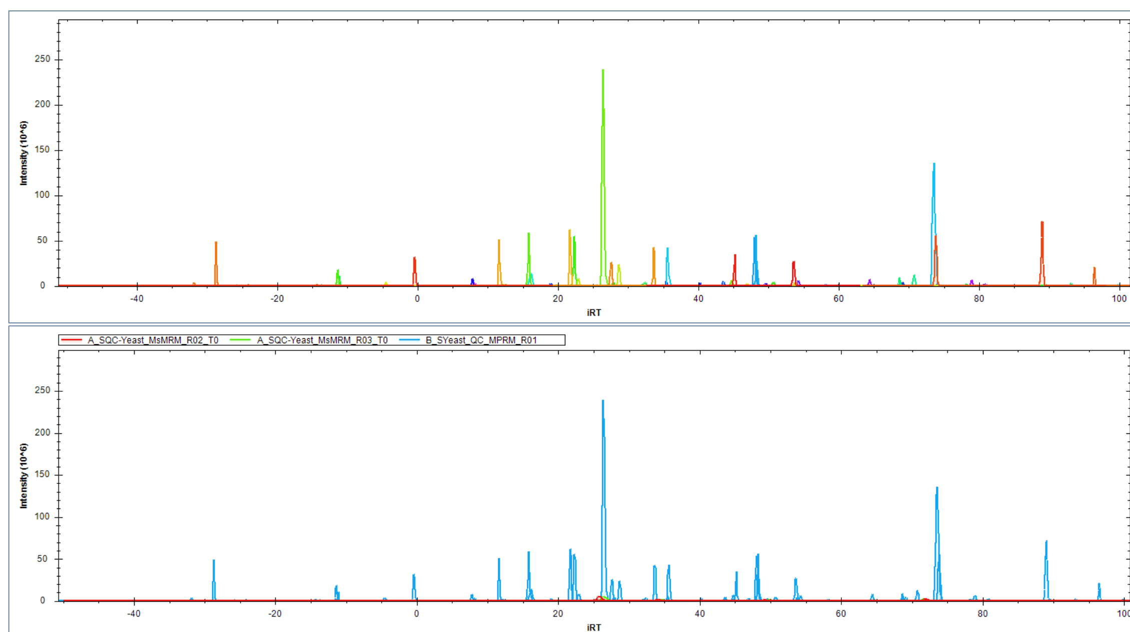

Figure 40. The Run Overview chart and RT Run Overlay Chart showing the ion current of all runs in your experiment in a single plot, giving insight into instrument stability and amount of sample injected.

7.7.2 Peptide and Fragment Plots

MS2 XIC

MS2 XIC is a default plot on Elution Group (EG or peptide), Transition Group (TG or peptide precursor) and Transition (T or fragment ion) level. This plot shows the extracted ion current chromatogram of the selected peptide. The plot contains the XICs for all fragments present in the library. Additionally, the expected retention time is marked (black dotted line) and the currently selected peak for this peptide (green area). On the Elution Group and Fragment Group level the coloring of the fragments is based on its expected relative intensity ranging from red (expected most intense fragment) to blue (expected least intense fragment). You can directly change integration boundaries in the plot. On fragment level, this plot only shows the selected fragment in color and all others in gray. There are several options available upon right-clicking in the plot such as switching the y-axis to log scale, toggling accept/reject the peak, changing x-axis scale to iRT, showing normalized intensities and showing the XIC chromatogram for the whole gradient.

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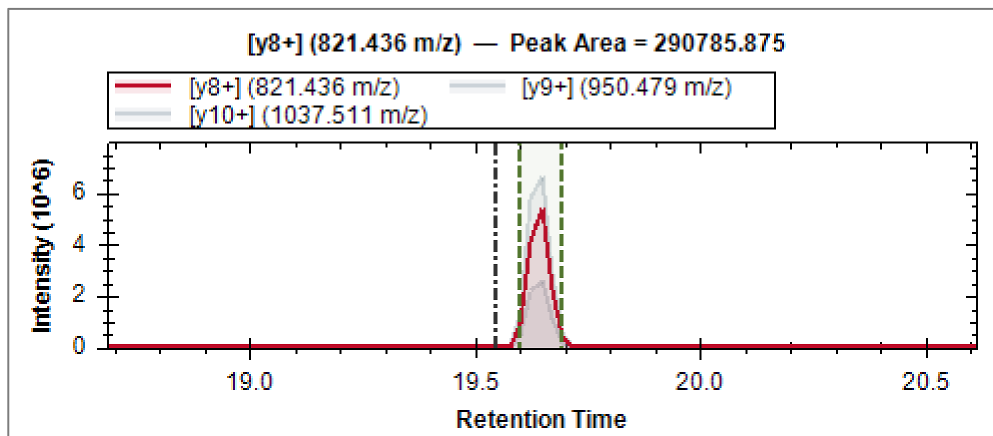

Figure 41 MS2 XIC chromatogram for the peptide ETVESESSQTALSK++. A chosen transition is highlighted in red color. For each of the transitions you can read Interference Score value in the Peak Info summary (in the range -1;1). Interference score of higher value indicates non-interfering fragment ion. You can right-click on the interfering transition after manual agreement and select Exclude In all runs and apply those changes to the experiment by right-clicking on the experiment tab and selecting "Refresh Post-Analysis." You can also select to remove the interfering transition from the panel completely by refining it.

MS2 XIC Sum

The XIC Sum chromatogram chart shows the selected peptides quantitative information. The XIC shown is the sum of all fragment XICs that qualified for quantitation. All fragments that were excluded due to interfering signals are not used to calculate the sum XIC chromatogram.

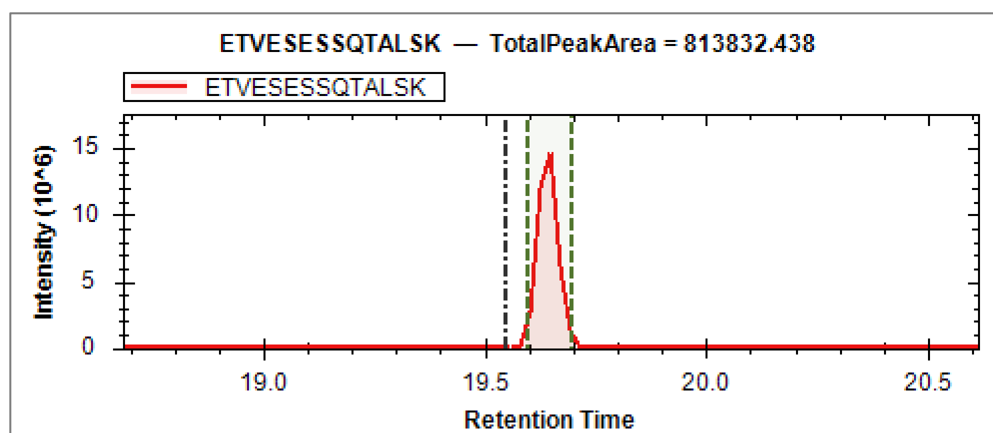

Figure 42. The MS2 XIC Sum plot of the peptide ETVESESSQTALSK++.

MS2 Intensity Correlation

This plot shows detailed information about the correlation of predicted and measured relative fragment ion intensity. The predicted values in red correspond to the relative intensities provided by the spectral library. The black lines correspond to the relative measured intensity of each fragment ion. Fragment ions with potential interferences are displayed as dotted lines.

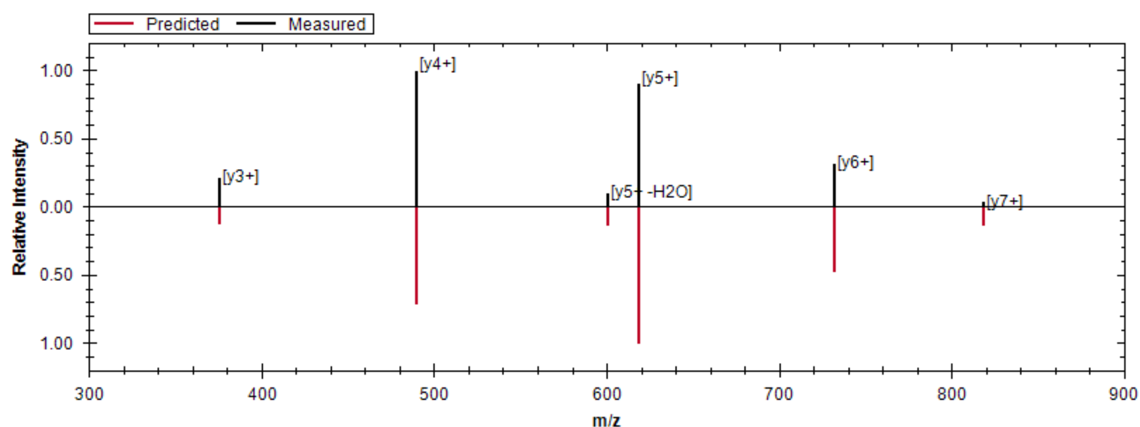

Figure 43. The MS2 Intensity Correlation plot for a given peptide precursor. The plot indicates a very good correlation between the expected relative intensities (red) and the measured intensities of the library fragments.

MS2 XIC Alignment

The MS2 XIC Alignment plot allows you to visualize the extracted ion chromatograms of a single peptide across all your runs. You can directly change integration boundaries in the plot.

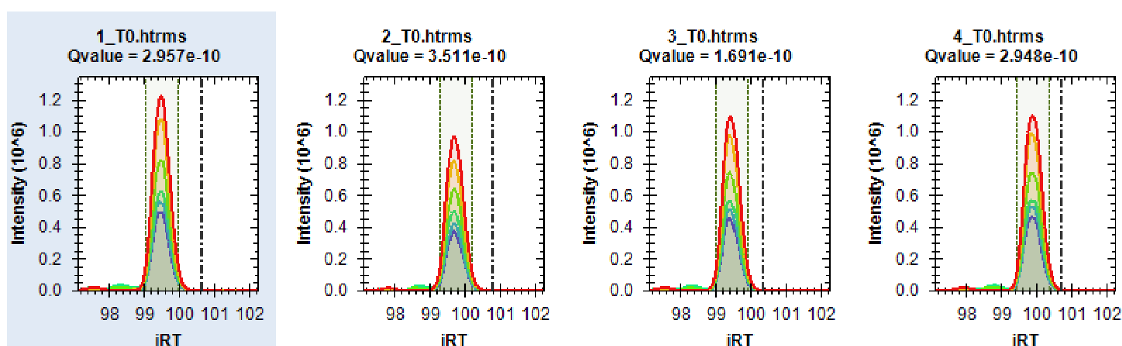

Figure 44. The MS2 XIC Alignment of the peptide IILDLISESPIK++ across 4 runs. The x-axis is automatically changed to iRT to reduce chromatographic variance. The axis can be changed to retention time by right-clicking on the plot and un-selecting the "Use iRT Scale" option.

iRT XIC Sum Overlay

This plot allows you to show all sum XIC chromatograms of your selected peptide from all runs as an overlay plot. The x-axis scale is by default in iRT but on right-click can be changed to actual retention time.

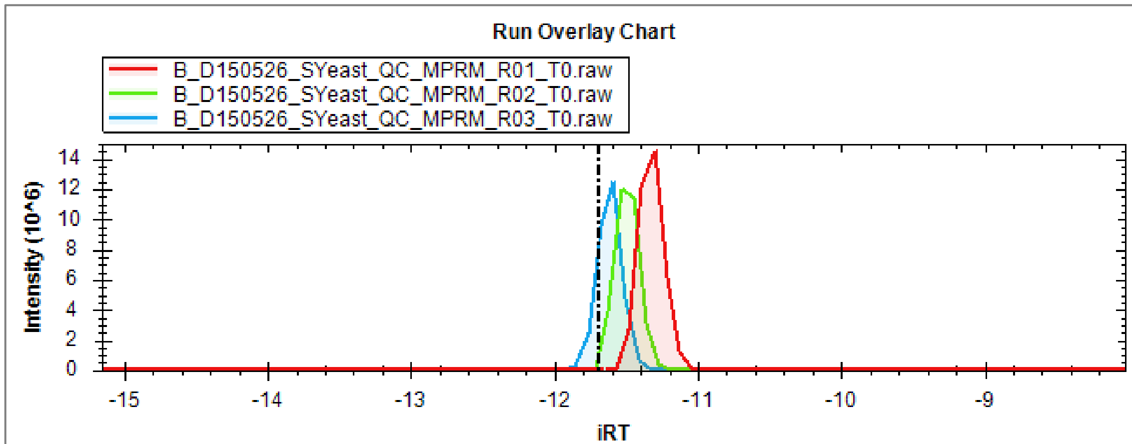

Figure 45. The iRT XIC Sum Overlay chart for the peptide ETVESESSQTALSK++. The 3 XICs correspond to the sum XIC of one peptide in the 3 different runs loaded for this experiment.

MS2 Intensity Alignment

Similar to the MS2 XIC Alignment, this plot gives detailed information about the signal stability for one peptide across several runs. The different bars show the relative abundance of each fragment ion across multiple runs. Using this plot, one can quickly identify an inconsistent signal by the change in the color pattern. Right-click on the plot and deselecting "Normalize" to show intensities on absolute scale.

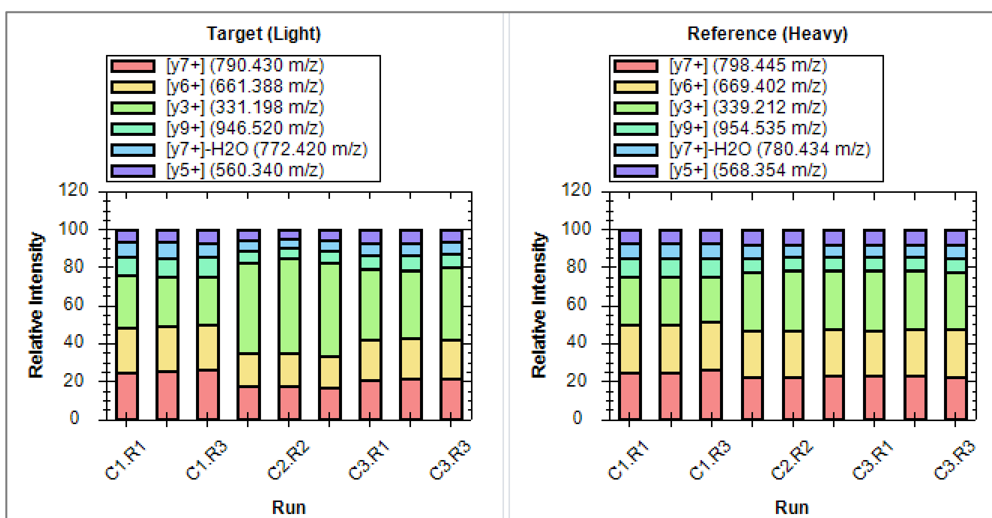

Figure 46. The MS2 Intensity alignment for a peptide containing 6 fragment ions. Each fragment ions relative intensity compared to the total peak height is indicated using a differently colored bar. The

peptide was targeted in 9 runs. An inconsistent signal can be easily identified due to the sudden change in the color pattern.

Cross-Run RT Accuracy

Similar to the MS2 Intensity Alignment chart, the Cross-Run RT Accuracy plot allows one to quickly validate the peak picking across several LC-MS runs. The x-axis shows colored bars that correspond to the peptide in different runs. On the y-axis one can see the retention time in iRT.

The height of each box corresponds to the peak width at the start and the end iRT according to the y-axis. The line through the middle of the box shows the apex retention time in iRT, while the blue colored boxes in the back show the total XIC extraction width. The colors of the bars correspond to the relative intensities of the measured fragment ions. The bar with the green background is the currently selected node. The black, dotted, horizontal line corresponds to the expected retention time in iRT. You can hop to any other node by clicking on the colored bar.

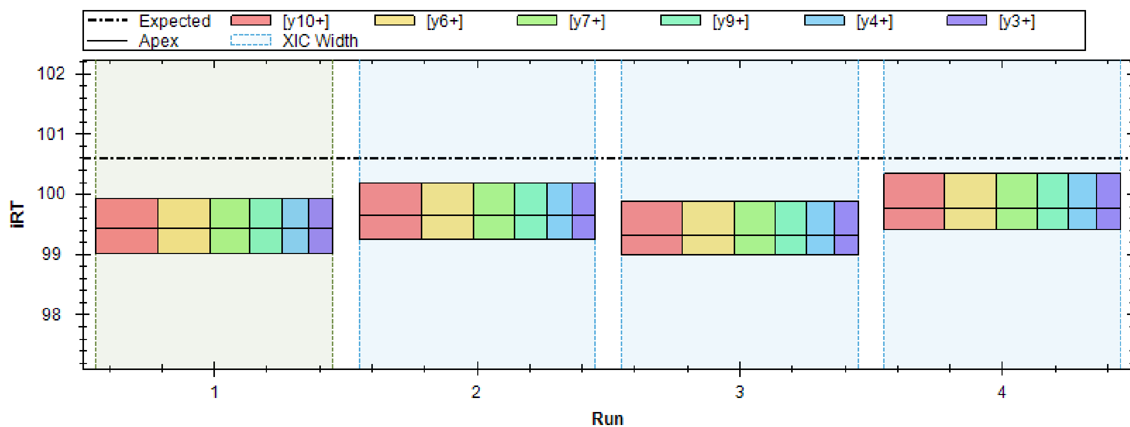

Figure 47. The Cross-Run RT Accuracy plot for a peptide measured in 4 different runs. The multi-colored bars correspond to the detected peak with the colors encoding the relative fragment intensities and the upper and lower boundary of the bar corresponding to the peaks start and end retention time in iRT.

7.8 Appendix 6. Analysis Experiment Tab Options

In the Analysis Perspective, right-click on the experiment tab. A context menu will open with several functionalities to apply to the analysis. The most common actions are available in intuitive icons displayed below the experiment tab.

Add Run	Add runs from the analysis. For the changes to take effect, you need to refresh the Post-Analysis.
Match Panel	Matches a new panel to the MRM/PRM data
Map Missing Runs	If SpectroDive lost the link with the run files, you can map them back.
Recalculate Qvalues	If you manually change the peak picking, by selecting a different peak of changing the integration boundaries, the *q*-values need to be recalculated.
Refresh Post Analysis	If you do any modification on the analysis, such as manually modify a peak or add new runs, the Post-Analysis has to be refreshed.
Export Analysis Settings	Export a report with the settings you used to run your analysis
Order Runs By	Choose a criterion to order you runs
Group by	You can group your data tree under different criteria. The default criterium and recommended for better performance is by precursor window. If you have several workflows in your analysis (e.g., label-free and spike-in), Group by Workflow is very useful.
Refine iRT	Revert the manually modified peak picking back to the automatic one. *q*-values need to be recalculated.
Refine Assay Transitions	If you refined your panel in the Analysis Perspective, the changes will not make effect until your commit the changes.
Export SureQuant Method	You can export your SureQuant method after performing the analysis of survey scan.

Save as and Save Calibration Curves	Save the calibration curves in ccs format for the future use of absolute peptide quantification in the experimental samples.
Save as and Save (Ctrl+S)	SpectroDive will not save your analysis automatically. You need to actively save your experiment. Choose to save with or without XICs.
Settings	This tool allows to explore and **change** many settings of your analysis: • Identification, Quantification and Post Analysis. • You can change the Condition Set-up (annotate replicates and conditions) in the next tab • Finally, in the Summary tab, you can change the name of your analysis. Changing these settings will let you recalculate your analysis, which is significantly less computationally expensive than running it again from scratch.

7.9 Appendix 7. Post Analysis Perspective Plots

Data Completeness

In the data completeness plot the "Cumulative Sparse Profiles" describes the cumulative number of identifications when looking at 1 - *n* runs, where n corresponds to the complete experiment. The "Cumulative Full Profiles" corresponds to identifications that were consistent across 1 - *n* runs, i.e. identified in all the runs currently looked at. The "CVs below X" plot shows the distribution of coefficients of variation CVs in the experiment. On right-click you can change the basis of quantitation between precursor, peptide, protein and protein group.

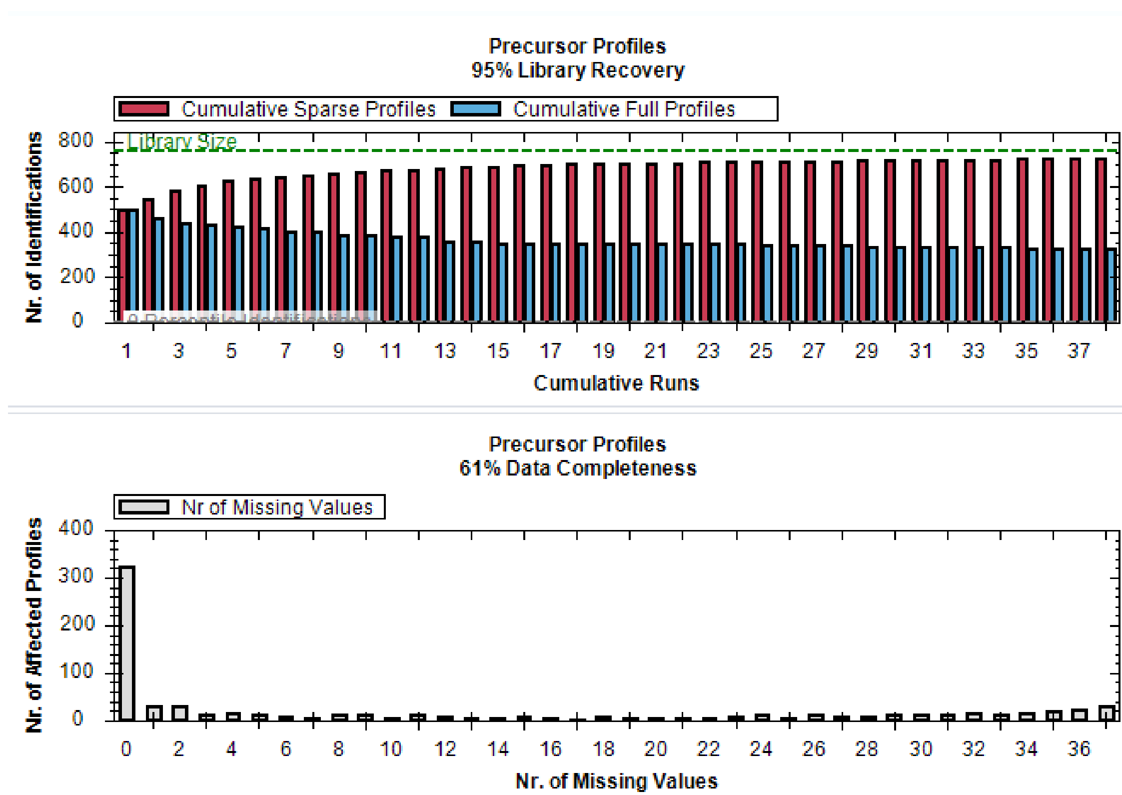

Figure 48. The "Data Completeness" plot. The red bars represent the cumulative precursor identification. For example, if you only consider the first 3 runs (x = 3) 579 precursors were identified at least once in these 3 runs. The blue bars represent the full profiles. For example, after the first 3 runs, only 440 precursors were identified in all 3 previous runs. Adding more runs, the red bars can only ever go up or remain constant while the blue bars can only ever go down or remain constant. The lower plot under the "Data Completeness" node shows the histogram of missing values. In this plot, 325 precursors did not have any missing values and where identified in every run while 31 precursors had one missing value. The last bar represents the number of single-hit precursors. These are the precursors that where only identified in one single run (hence they were only identified in 1 out of 38 runs in this case). As with many other plots you can change the context to show you the missing values on precursor, peptide, protein-group or protein level.

CVs Below X

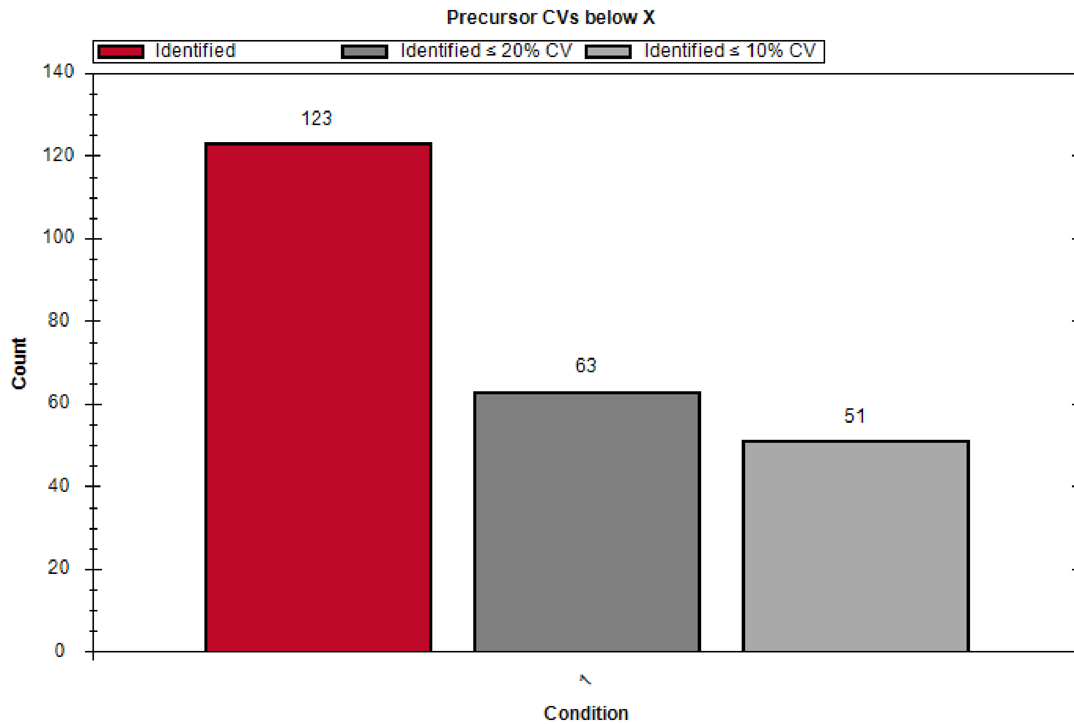

Figure 49. The CVs below X plot shows the number of precursors that were below either 20% or 10% CVs. The red bar shows the number of all identifications for each condition regardless of quantitative precision. The bar for ≤ 20% CVs also includes all counts from ≤ 10% CVs. As with the previous figures, you can change the context of the plot by right-clicking on it to select either precursor, peptide, protein-group, or protein scope.

SureQuant Scans

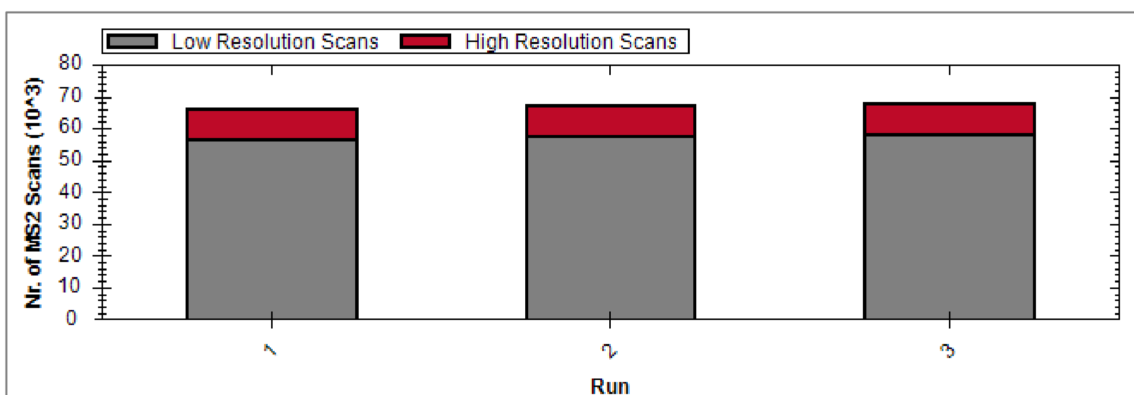

Figure 50 Whenever you process SureQuant data, you will be able to visualize SureQuant Scans graph which is available under the Specialized Workflows noder. The graph shows for each run individually,a number of high resolution scans (in red) and a number of low resolution scans (in grey).

Heatmap

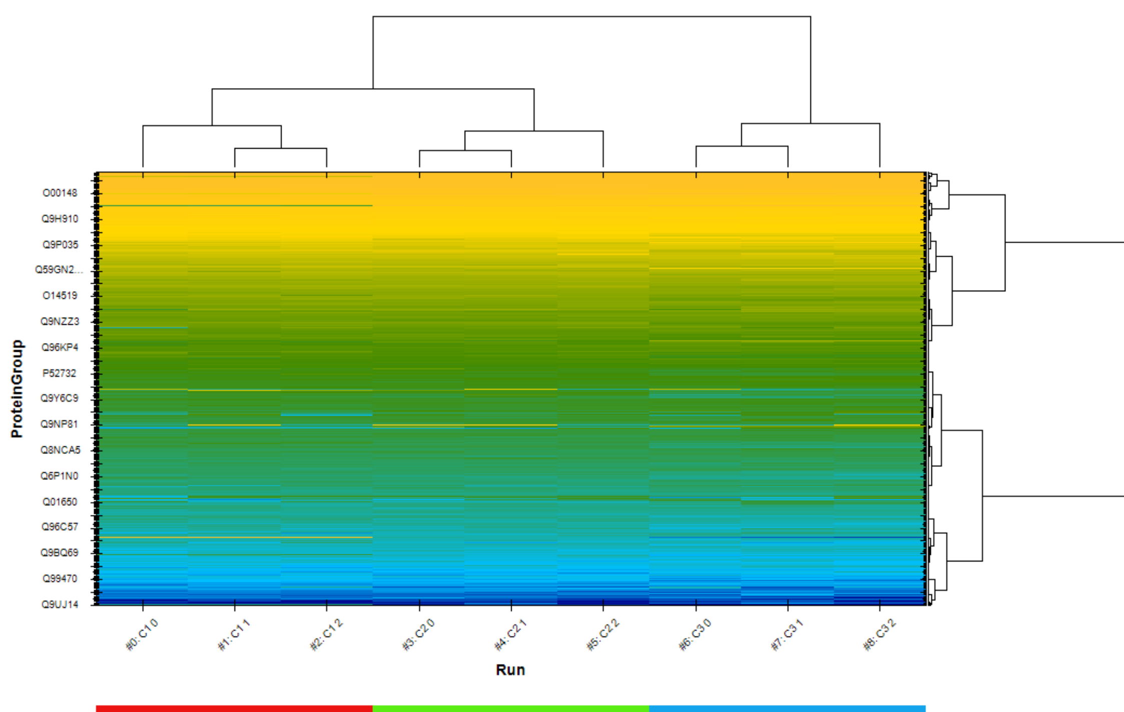

Figure 51. Heatmap showing the clustering of 9 runs from 3 conditions. Runs within the same condition cluster together as illustrated by the condition-based color code in the bottom of the heatmap and the x-axis dendrogram.

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Volcano Plot

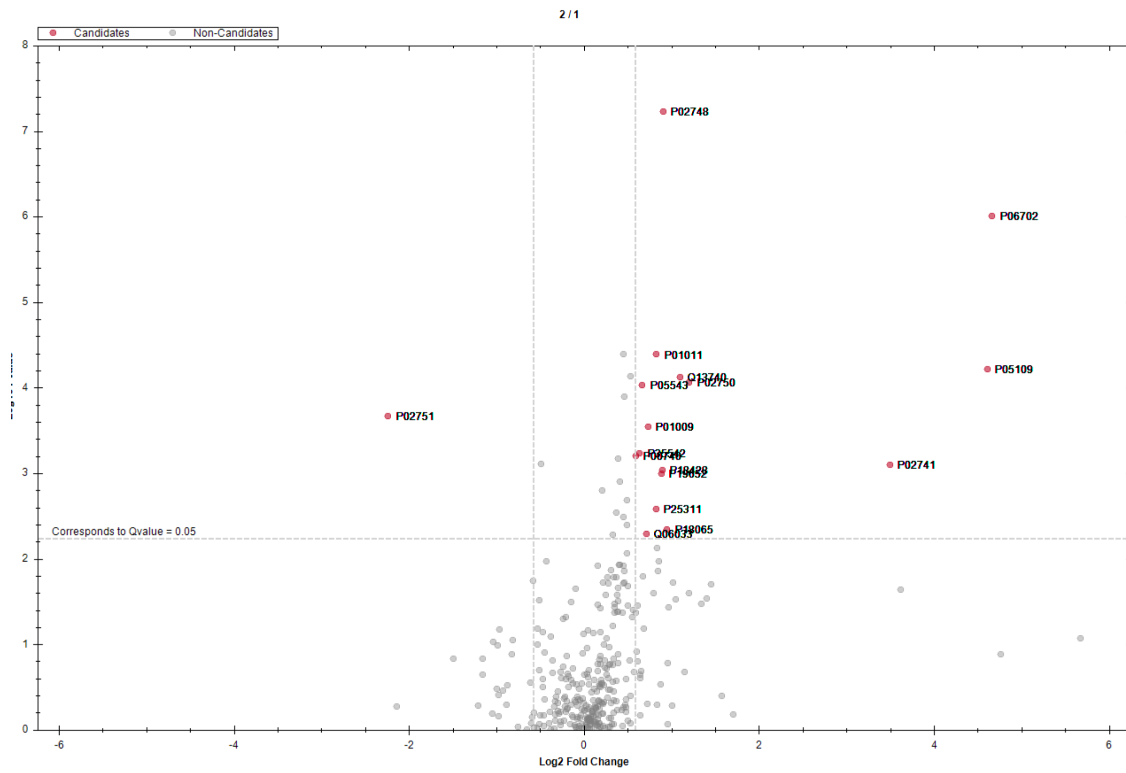

Figure 52. Volcano plot showing the potential candidates of an experiment containing 3 conditions to each 3 replicates. By default, the filters are set to ≥ 1.5 absolute fold change and ≤ 0.05 *q*-value.

7.10 Appendix 8. Most Relevant Report Headers

Many headers have a text hover tool directly in the software. If you don't find the information you are looking for, do not hesitate to contact us at support@biognosys.com.

Protein Group (PG) headers

Headers related to Protein Group (PG) as defined in the settings. Most headers related to PGs are self-explanatory. Here are the most relevant and some which are not too obvious

PG.ProteinId	The protein ID can either originate from the **IDPicker** or from the search engine used to generate the spectral library.
PG.ProteinDescriptions	The protein descriptions in the same order as the protein IDs in "PG.ProteinGroups". The value corresponds to what was defined by the parsing rule. If all the values are identical a unique value is reported.
PG.Quantity	The quantitative value as defined in the settings.

Peptide headers

Headers related to Peptides (PEP) as defined in the settings. Many headers related to Peptides are self-explanatory. Here are the most relevant and some which are not too obvious.

PEP.PrecursorCount	Number of hits (MS1 precursors) found for that peptide
PEP.GroupingKey and PEP.GroupingKeyType	Informs which element is considered as peptide (defined in the settings). Default is stripped sequence.
PEP.IsProteotypic	Informs whether the peptide is proteotypic (belongs to only one Protein ID) – true or is not proteotypic - false.
PEP.Quantity	The quantitative value for that peptide as defined in the settings.

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Elution Group (EG) headers

Headers related to Elution Groups (EG). Many headers related to EG are self-explanatory. Here are the most relevant and some which are not too obvious.

EG.PrecursorId	Unique ID for the precursor: _[modified sequence]_. [precursor charge]
EG.Panel	The name of the panel this assay is based on.
EG.Workflow	The workflow this EG was used in. It can be LABEL_FREE, SPIKE_IN or LABEL
EG.IsUserPeak	Specifies whether the EG was manually integrated in the Analysis Perspective – true or not – false.
EG.Qvalue	The *q*-value (FDR) of the EG
EG.AbsoluteAmount	Absolute amount of endogenoue peptide on a column
EG.AbsoluteAmountRangeLimitted	Quantity reported only when it falls in the linear range of the calibration curve
EG.AbsoluteAmountUnit	Unit of reported absolute amount quantities
EG.CoefficientOfVariationAtLLOQ	Calibration curve coefficient of variation (%CV) at a lower limit of quantification
EG.CoefficientOfVariationLinearRange	Calibration curve coefficient of variation (%CV) in Linear Range
EG.CV	In a multi-run experiment, this value reports the CV of the peptide quantity
EG.LinearRangeLocation	Message that indicates if the absolute quantity falls within, above or below the linear range determined by calibration curve
EG.LLOQ	Calibration curve lower limit of quantification
EG.LOD	Calibration curve lower limit of detection

EG.ProteinAbsoluteAmount	Absolute concentration of protein in the original sample
EG.TotalQuantity	The quantitative value for that EG as defined in the settings
EG.ULOQ	Calibration curve upper limit of quantification
EG.iRTEmpirical	The iRT (Escher et al. 2012) as determined in this specific analytical run
EG.MeanApexRT	The average retention time of the peak apexes across all fragment ions of this peptide
EG.MeanTailingFactor	The average tailing factor of the elution group across all the fragment ions determined at the FWHM
EG. Verified	Reports manual accepting or rejecting of peaks assigned in the Analysis Perspective
EG.Cscore	The SpectroDive identification score, which is based on an advanced mProphet (Reiter et al. 2011) scoring. A high score indicates high quality identifications

Transition Group (TG) headers

Headers related to Transition Group (TG). TG is only relevant in labeled and spike-in workflows. Two TGs belong to one EG. The TG.Id corresponds to the EG ID plus the isotopic labelling.

Many headers related to TG are self-explanatory. Here are the most relevant and some which are not too obvious.

TG.IntMID	A unique ID of the peptide precursor. Corresponds to the EG.Id if the experiment is label free
TG.CompensationVoltage	The compentation voltage that was used for acquisition of a given transition group in the FAIMS PRM experiment.
TG.UILabel	A label of the fragment ion group. The label is not necessarily unique and therefore not intended for structuring data
TG.Charge	The charge state of the peptide precursor
TG.MS1MonoIsotopicHeight	The apex heigh of the MS1 mono-isotopic XIC peak
TG.Quantity	The quantitative value for that TG as defined in the settings (peak area or height)

Transition (T) headers

Headers related to transition (for MRM) or fragment ions (for PRM). If you choose Transition level information in your report, you will have one row per transition. This can make the report considerably large.

Most headers related to transitions are self-explanatory. Here are the most relevant and some which are not too obvious.

T.FrgZ	The charge state of the transition of fragment ion
T.Quantity	The quantitative value calculated as defined in the settings (peak area or height).

8 Glossary

SRM (**Selected Reaction Monitoring**) = **MRM (Multiple Reaction Monitoring)** - Performed on triple-quadrupole and hybrid quadrupole-linear ion trap instruments, where the instrument cycles through a predetermined set of precursor *m/z* (Q1), fragment *m/z* (Q3) pairs called "transitions". It is the most robust and sensitive method for measuring specific peptide quantities.

PRM (Parallel Reaction Monitoring) = tMSMS (Targeted MS/MS) = MRM-HR (MRM-High Resolution) = pSRM (pseudo SRM) – similar to MRM, only performed on a full-scan instrument (ion-trap or Q-TOF). The instrument cycles through a pre-specified set of precursor *m/z* values and collects a full MS/MS fragment ion spectrum for each of them. If the spectra are high-resolution, then extraction can be done using 50-100pm range, making it more selective than MRM.

Scheduled MRM - In order to allow measuring a greater number of transitions per run, transitions are specified with start and end times (or RT and time windows) to allow the instrument to measure each transition for only a fraction of the run, and not thought the entire acquisition run.

tMRM (triggered-MRM) - In order to gain more confidence in the correct identification of a peak in MRM without overly sacrificing quantitative throughput, the instrument measures a set of primary transitions, as in normal MRM until the intensity on those transitions exceeds a defined threshold. When the threshold is exceeded, the instrument takes one or more measurements of a secondary set of transitions usually used only to confirm a peak identity.

Assay Panel – a list of peptide ion properties (transitions) representing the proteins you want to quantify. Generate an inclusion list for the transition of interest including:

- Mono-isotopic precursor *m/z*
- Charge state (*z*)
- Fragment *m/z*
- For scheduling you will need the start and end RT

First, 2 or 3 targeted peptides should be selected from a spectral library or data repository or predicted *in-silico*. The selected peptides should be unique to the protein of interest and easily detected by LC-MS. They also require no missed cleavage sites and no frequently modified amino acids.

Spectral library - a large collection or database of peptide fragment ions assigned to a precursor. The most common way of obtaining the information to build a library is through

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a shotgun approach where data dependent acquisition (DDA) is combined with database searches. The library can be used for generation of an assay panel in MRM/PRM workflows.

DDA - Data-Dependent Acquisition – the acquisition of data after certain conditions have been met in a mass spectrum experiment. Most often it refers to recording of MS2 spectra based on the intensity of the precursor ion signal.

DIA - Data-Independent Acquisition - the unconditional acquisition of data in a mass spectrum experiment. Most often it refers to recording of all MS2 spectra, regardless of the intensity of the precursor ion signal.

PSM - Peptide-Spectrum-Match

FDR - False Discovery Rate, a measure of type I errors in multiple hypothesis testing, used in the control of expected proportion of "false discoveries" and “false rejections” in those tests that result in a significant result (“true discovery”). An FDR adjusted *p*-value is called a *q*-value.

FWHM = Full width at half maximum – the width of a mass spectral peak at a position corresponding to half of the peak height (50% intensity). It is usually used to determine the resolving power of a mass spectrometer.

Dwell Time - the time spent acquiring a specific MRM transition during each cycle. A very short dwell times can be used (5 ms or less). However, longer dwell times are always desirable for better signal/noise and sensitivity.

Duty Cycle - duty cycle is the total amount of time spent monitoring the chosen analytes. If chromatographic resolution requires a particular duty cycle time, then the dwell time for each analyte measured in the duty cycle is inversely related to the number of analytes. For example, if the duty cycle is 500 ms and there are 20 precursors to measure, the dwell time will be 25 msec.

Cycle Time - the duty cycle time for an MRM assay must take into consideration chromatographic peak shape. Ideally, the peak must be sampled 7-9 times as it elutes to get an accurate measurement of its area. So, if a peak is 7 s wide, then the sample time would be every 1 s. For 25 msec dwell times, then 40 MRM transitions can be monitored in one unscheduled run.

BGS – Biognosys

RT – Retention Time

iRT – Indexed Retention Time

TICC – Total Ion Current Chromatogram

XIC – Extracted Ion Chromatogram – a chromatogram for one or more *m/z* values or ranges that have been extracted from the TICC

QC – Quality Control – monitoring of metrics from which the quality of results can be derived (such as consistency, accuracy, precision, specificity, detection limit, quantitation limit, linearity, range, and robustness).

PTM – Post-Translational Modification

EG – Elution Group

TG – Transition Group

LLOQ – Lower Limit of Quantification

LOD – Limit of Detection

ULOQ – Upper Limit of Quantification