User guide

for

Using the ALEX^{123} framework to identify lipid molecules detected by MALDI-based high-resolution FTMS^{1} and ITMS^{2} analysis

Used in the publication:

Ellis et al. (2018, in review)

Version 180213

Table of contents

1. Introduction

ALEX^{123} (Analysis of Lipid Experiments) is a software framework for processing, management and visualization of direct infusion (shotgun) and MALDI/imaging-based lipidomics datasets acquired using high-resolution FTMS^1, MS^2 and MS^3 analysis (Almeida et al. (2015), Ellis et al. (2018, in review)).

This user guide describes how to execute the routine used for automated high confidence identification of lipid molecules detected by MALDI-based high-resolution FTMS^1 and low resolution ITMS^2 analysis executed on an LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific), as described in Ellis et al. (2018, in review).

The ALEX^{123} framework uses a set of distinct modules (Figure 1):

i) that make spectral data in proprietary data files (.RAW) searchable (executed by the ALEX^{123} converter),

ii) that matches detected mass spectral features to *m/z* values of intact lipid precursors and fragment ions (executed by the ALEX^{123} target list generator, ALEX^{123} extractor, ALEX^{123} isotope compiler and ALEX^{123} unifier),

iii) that performs automated high confidence lipid identification (by data filtering) (executed by SAS® Enterprise Guide-based scripts), and

iv) that support visual analysis (quality control) of raw data (executed by visualization in Tableau® Desktop).

In this manual we first describe installation requirements, provide download instructions, and describe each module of the ALEX^{123} framework. In the second part of the manual (section 0) we provide a step-by-step guide on how to use the ALEX^{123} framework for automated lipid identification using MALDI-based FTMS^1 and ITMS^2 data.

2. ALEX^{123} core modules and data processing workflow

The ALEX^{123} framework uses of six core modules (grey colored boxes with red text in Figure 1) and two auxiliary data processing modules, that are executed using SAS® Enterprise Guide and Tableau® Desktop (white boxes with blue text in Figure 1).

The six core ALEX^{123} modules are:

- the **ALEX^{123} converter**: converts proprietary .RAW data files to searchable .txt data files,
- the **ALEX^{123} lipid database**: library with curated lipid ionization and fragmentation information (described in Pauling, Hermansson et al. (2017)),
- the **ALEX^{123} target list generator**: shortlists lipid ionization and fragmentation information that the user wants to search for in the mass spectral data,
- the **ALEX^{123} isotope compiler**: adds ^{13}C isotope information to searches (optional),
- the **ALEX^{123} extractor**: searches spectral data (.txt) files for the user-defined lipid ionization and fragmentation information, and
- the **ALEX^{123} unifier**: concatenate results from multiple searches into a single result file.

The output of executing all the core ALEX^{123} modules is a tabulated result file (named “Results_unified.tab) that contains information about detected lipid species (FTMS^1 data) and fragment ions (MS^2 data and MS^3 data, if acquired), and their intensities and measured *m/z* values across all processed .RAW files (i.e. samples) and MS scan ranges (termed ”scan filter” in the proprietary software Xcalibur (Thermo Fisher Scientific)).

The result file is organized in “database table format”, which facilitates robust down-stream data processing for high confidence lipid identification by SAS®

Enterprise Guide and for dynamic visualization of all raw spectral data using Tableau® Desktop.

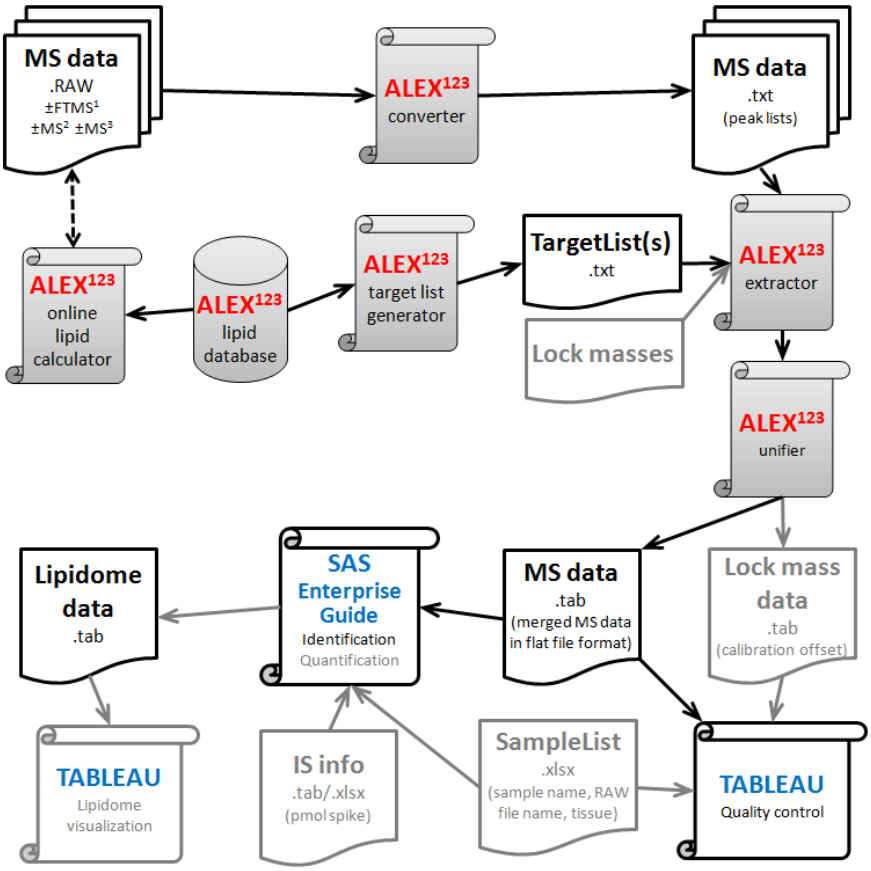


Figure 1. Overview of the ALEX^{123} framework. Core ALEX^{123} modules are highlighted with grey colored boxes and red text. Auxiliary data processing modules are highlighted with white boxes and blue text. Boxes with grey text exemplify additional functionalities that are used for processing high resolution shotgun lipidomics data, and are not described in this user manual (which is focused on processing of MALDI-based FTMS[1] and ITMS[2] data).

3. Requirements

The ALEX^{123} framework is operational on desktop and labtop computers running Microsoft™ Windows™ 7 or 10 (Professional 64-bit), and having at least a 2.7 GHz dual core processor with at least 8 GB RAM. The user should have administrator rights.

Install **Java (64-bit)** to use Java-based ALEX^{123} software modules (target list generator, extractor, isotope compiler and unifier). Download here.

Install **MSFileReader (64-bit)** ("MSFileReader_x64_3.1 SP4.exe") (Thermo Fisher Scientific) to use the Python-based ALEX^{123} converter. Download here.

Install **Python 3.6.1 (64-bit)** and **comtypes** to use the Python-based ALEX^{123} converter. Click here for instructions.

Install **SAS® 9.2 with SAS® Enterprise Guide 5.1** to use scripts (file extension .epg) for automated high confidence lipid identification. This proprietary software is typically free of charge to academic institutions (often used for teaching statistics and business analytics). More information is available here.

Install **Tableau® Desktop** (version 10.2 or later) to use and modify data visualization templates (file extensions .twb). This proprietary software is typically free of charge to academic institutions. More information is available here.

4. Download of ALEX^{123} software modules

Note that all the ALEX^{123} modules can be downloaded together as part of the Example dataset. This dataset can be downloaded for testing local installations of the ALEX^{123} framework and is as described in section 0).

Each ALEX^{123} core module, and the example dataset, can also be downloaded at www.msLipidomics.info | software.

Note that the core ALEX^{123} software modules do not need to be installed; they are executable scripts.

Note that the ALEX^{123} modules should be executed from distinct "search folders". See section 5 and 0). In other words, the file manager is the overarching GUI for executing the ALEX^{123} modules.

5. Using the ALEX^{123} framework

Identifying lipid molecules using FTMS^{1} and ITMS^{2} data requires executing six distinct processing steps (see step-by-step guide in section 0). To perform the processing it is highly recommended to use the specific file management structure exemplified in Figure 2.

Note that the file management structure is also used in the Example dataset (see section 0).

The six processing steps are (Figure 2):

1) Converting proprietary .RAW files to searchable averaged peak lists saved as .txt files (done in folder "02_RAW_files_and_data_conversion" using ALEX^{123} converter),
2) Searching FTMS^{1} data (done in folder "03_ALEX123_ms1_data_search" using ALEX^{123} target list generator, ALEX^{123} isotope compiler, ALEX^{123} extractor and ALEX^{123} unifier),

3) Searching ITMS^2 data (done in folder “04_ALEX123_ms2_data_search” using ALEX^{123} target list generator, ALEX^{123} extractor and ALEX^{123} unifier),
4) Visualization of raw FTMS^1 and ITMS^2 data (optional quality control) (done in folder “05_TABLEAU_quality_control_MSn” using Tableau® Desktop),
5) Performing deisotoping of FTMS1 data (done in folder “06_SAS_ms1_deisotoping” using SAS® Enterprise Guide), and
6) Doing high confidence lipid identification (done in folder “07_SAS_msn_lipid_identification” using SAS® Enterprise Guide)

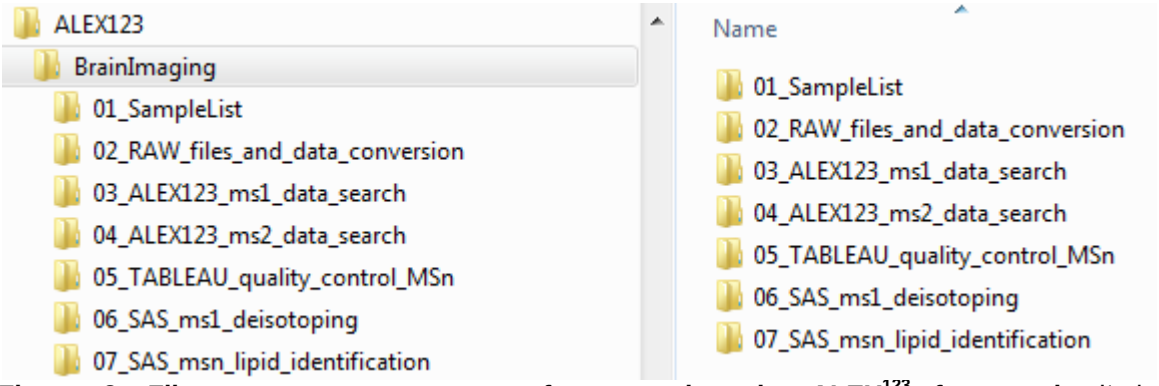


Figure 2. File management structure for executing the ALEX^{123} framework. It is recommended to create and use a file management structure with specific folders for executing each step of ALEX^{123} data processing pipeline. Using numbering of folders (e.g. 00-06) will keep the folders in the right order when sorting them by name.

The individual file folders have the following purposes/functions:

- **01_SampleList:** the folder contains a sample list (in Microsoft® Excel format). Information regarding .RAW file names (termed RAW_ID), sample number, sample name, tissue type, etc., should be specified on this sample list.

- **02_RAW_files_and_data_conversion:** This folder contains the ALEX^{123} converter (section 6.1) and associated files. The subfolder “raw” should contain all proprietary .RAW files that should be processed. It is recommended to store .RAW files with positive and negative ion mode data in separate subfolders termed “pos” and “neg”, respectively. FTMS^1 and ITMS^2 data is converted by

executing the ALEX^{123} converter files “convert_FTMS1_data.cmd” and “convert_ITMS2_data.cmd”, respectively (see section 6.1 for further details). The output of the ALEX^{123} converter (.txt files) will be located in the subfolders “txt_FTMS1” (containing FTMS^{1} data) and “txt_ITMS2” (containing ITMS^{2} data).

- **03_ALEX123_ms1_data_search:** This folder contains all information related to searching FTMS^{1} data using the ALEX^{123} target list generator (section 6.3), ALEX^{123} extractor (section 6.5), ALEX^{123} isotope compiler (section 6.4), and ALEX^{123} unifier (section 6.6). The folder can contain multiple subfolders with specific settings for searching different lipid classes and different FTMS^{1} scans (i.e. scan filters) in the acquired .RAW files.

- **04_ALEX123_ms2_data_search:** This folder contains information related to searching ITMS^{2} data using the ALEX^{123} target list generator (6.3), ALEX^{123} extractor (6.5) and ALEX^{123} unifier (section 6.6). The folder can contain multiple subfolders with specific settings (files) for searching for different lipid classes and different ITMS^{2} scans (i.e. scan filters) in the acquired .RAW files.

- **05_TABLEAU_quality_control:** This folder contains pre-built visualization templates (.twb files) for visualizing FTMS^{1} and ITMS^{2} data using Tableau® Desktop. This quality control analysis is typically performed in two steps: a) visual inspection of FTMS^{1} data across all processed .RAW files and polarities, and b) visual inspection of ITMS^{2} data across all processed .RAW files and polarities.

- **05_SAS_ms1_deisotoping:** This folder contains a script (.epg file executed by SAS® Enterprise Guide) that reads the output file with FTMS^{1} data (stored in “Results_unified.tab” under folder “03_ALEX123_ms1_data_search”). This script checks whether identified monoisotopic lipid ions are subject to isotope interference from a neighboring ion with the mass equal to that of a [13]C

atom. If isotope interference is found, then deisotoping is performed. The script produces an output file with deisotoped (recalculated) intensity values for lipid species identified by $FTMS^1$.

- **06_SAS_msn_lipid_identification:** This folder contains a script (.epg file executed by SAS® Enterprise Guide) that generates an output file with a list of lipids identified by $ITMS^2$-based detection of lipid class-selective fragment ion(s) and $FTMS^1$ analysis (termed "lipid species"), and lipids identified by $ITMS^2$-based detection of molecular lipid species-specific fragment ion(s) and $FTMS^1$ analysis (termed "molecular lipid species"). The input for the script includes:
    - the output file with $ITMS^2$ data ("Results_unified.tab" in folder "04_ALEX123_ms2_data_search"),
    - the output file with deisotoped $FTMS^1$ data ("02_SASoutput_ms1_data_w_deisotoping.tab" in folder "05_SAS_ms1_deisotoping"),
    - a Microsoft® Excel-based file ("02_SASinput_FragmentGuide.xlsx") that supports the lipid identification routine, and
    - a Microsoft® Excel-based file that prioritizes which adducts ions to use for cross-validating lipids identified by $FTMS^1$ and $ITMS^2$ analysis ("03_SASinput_Lipid_class_adducts_for_high_confidence_ID.xlsx").

6. Descriptions of individual $ALEX^{123}$ modules

6.1. $ALEX^{123}$ converter

6.1.1. General information

The $ALEX^{123}$ converter uses Python 3.6.1 (64-bit) and comtypes, and MSFileReader (64-bit) (Thermo Fisher Scientific).

The ALEX[123] converter is executed from the command-line prompt, using executable .cmd files. There is no GUI for the ALEX[123] converter.

The ALEX[123] converter uses the proprietary dynamic-link library MSFileReader (Thermo Fisher Scientific) to export averaged spectral peak lists of specific MS scan ranges (i.e. scan filters) in .RAW files, and saves these peak lists in .txt format (that can be searched by the ALEX[123] extractor).

The ALEX[123] converter output consists of ".txt" files named according to the originating .RAW input files that are stored in folders named according the MS scan filter they derive from (Figure 3).

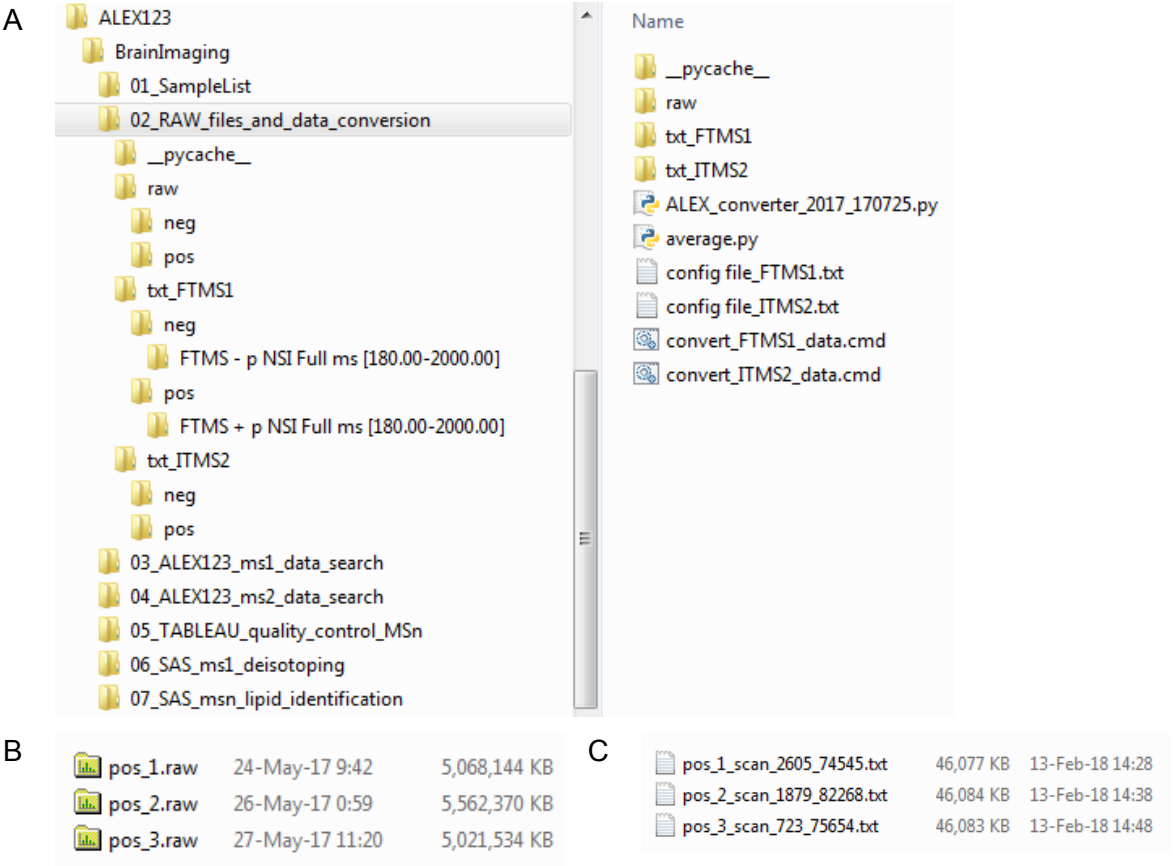


Figure 3. **(A)** File management structure for executing the ALEX[123] converter. The folders "..\raw\pos" and "..\raw\neg" should contain proprietary .RAW files with positive and negative ion mode data, respectively. The folders "..\txt_FTMS1" and "..\txt_ITMS2" contain .txt files (averaged peak lists) with FTMS[1] and ITMS[2] data, respectively. **(B)** Example of .RAW files with positive ion

mode data stored in the folder "..\raw\pos". **(C)**. Example of content in the folder "..\txt_FTMS1": this folder contains .txt files organized and sorted by subfolders named according to acquired scan filter (e.g. "FTMS + p NSI Full ms [180.00-2000.00]") and file name of the originating .RAW file (followed by underscore "_" and the scan number interval used for spectral averaging).

6.1.2. Notes

The ALEX^{123} converter uses a set of Python (.py) files (ALEX_converter.py, average.py, and average.cpython-36.pyc located in the folder "__pycache__"). These files must be located in the file management structure as shown in Figure 3A.

Running the ALEX^{123} converter requires a configuration file (e.g. "config file_FTMS1.txt"), which instructs the ALEX^{123} converter what to export from input .RAW files (Figure 3A).

The configuration file (e.g. "config file_ITMS2.txt") is a txt file which can be opened using Notepad++ and consists of following parameters (Figure 4):

- **input_dir/output_dir:** Set the input ("raw") and output (e.g. "txt_FTMS1") directory. These folders need to be in the same directory as the configuration file.

- **state_file:** (e.g. state_ms1.txt) is a file recording the settings that each .RAW file has been processed with. Files that already have been processed will not be processed again, if the settings have not changed. Delete the state_file "skipped_scans.txt", if you want all files to be reprocessed.

- **include_filters**: this option allows of export data from specific 'scan filters'. For example, using "FTMS*" will only export FTMS data, and not ITMS data.

For example, using “*ms2*” will only export MS2 data, and not MS1 and MS3 data.

- **data_format**: this option allows you to export either “profile” or “centroid” mode data.

- **msfr_method**: this option allows you to export data using either the MSFileReader functions:

  “GetAverageMassList” (should be used for exporting data in profile mode), or

  “GetAveragedLabelData” (should be used for exporting data in centroid mode).

- **named_averages**: this option enables you to export data from specific scan numbers or intervals. For example, [“myavg”, 13,15,17] will average scans 13, 15 and 17, and write an output file with a suffix _myavg. Another example, [“otheravg”, [30,70]] will average all scans between 30 and 70, and write an output file with a suffix _otheravg.

- **skip_scans**: specifies how many scans to skip at the beginning and end of the RAW files. The setting is specified as a nested structure representing the input directory tree. Any level allows a “(default)” item overriding any higher level default values. A single global setting can be specified by: “skip_scans” : { “(default)” : [1,1] } or for individual files as : {“file name”: [2,2]}.

```
{
  "input_dir" : "raw",
  "output_dir" : "txt_FTMS1",
  "state_file"  : "state_ms1.txt",
  "include_filters" : "FTMS*",
  "data_format" : "profile",
  "msfr_method" : "GetAverageMassList",
  "centroid_peakwidth" : "0",
  "named_averages" : {
    "neg"  : {
      "neg_1.raw" : [["scan_4687_66645", [4687,66645] ] ],
      "neg_2.raw" : [["scan_8294_72427", [8294,72427] ] ],
      "neg_3.raw" : [["scan_5851_57638", [5851,57638] ] ]
    },
    "pos"  : {
      "pos_1.raw" : [["scan_2605_74545", [2605,74545] ] ],
      "pos_2.raw" : [["scan_1879_82268", [1879,82268] ] ],
      "pos_3.raw" : [["scan_723_75654", [723,75654] ] ]
    }
  },
  "skip_scans" : {
    "(default)" : [0,0],
    "pos" : {
      "acE8_pos_direct_151_23.RAW" : [18,2],
      "acE8_pos_direct_151_16.RAW" : [14,2],
      "acE8_pos_direct_151_28.RAW" : [13,2],
      "acE8_pos_direct_151_05.RAW" : [2,35]
    },
    "neg" : {
      "acE8_neg_direct_21_01.RAW" : [27,2],
      "acE8_neg_direct_21_23.RAW" : [2,14]
    }
  }
}
```

Figure 4. Example of a configuration file for the ALEX^{123} converter.

The ALEX^{123} converter can be executed by double clicking on the .cmd files located in the folder:

→ Convert FTMS^{1} data by executing the file “convert_FTMS1_data.cmd”.

→ Convert ITMS^{2} data by executing the file “convert_ITMS2_data.cmd”.

Depending on the .RAW file size this process may take several minutes. If needed, the process can be terminated using “ctrl+c”.

6.2. ALEX^{123} lipid database

6.2.1. General information

The ALEX^{123} lipid database features curated ionization and fragmentation information for more than 430,000 lipid molecules from 47 lipid classes covering five lipid categories (PMID: 29161304). The database is used by the ALEX^{123} target list generator (section 6.3) and the online ALEX^{123} lipid calculator (www.alex123.info, section 0).

6.3. ALEX^{123} target list generator

6.3.1. General information

The ALEX^{123} target list generator uses Java. Moreover, to use the ALEX^{123} target list generator requires internet access in order to access the online ALEX^{123} lipid database.

The ALEX^{123} target list generator ("ALEX123_TargetListGenerator.jar") generates target lists by querying the ALEX^{123} lipid database. Target lists consist of selected lipid molecules (that should be searched for) and specific information about adduction, *m/z* values of lipid molecules and their fragment ions, name of fragment ions, chemical formula of intact lipid molecules and fragment ions, lipid category, lipid class and other features.

Target lists are saved as .txt files (e.g. "targetlist.txt). User-defined search parameters (queries in the ALEX^{123} lipid database) are saved as .lxt files.

6.3.2. GUI

The ALEX^{123} target list generator is operated using the GUI shown in Figure 5.

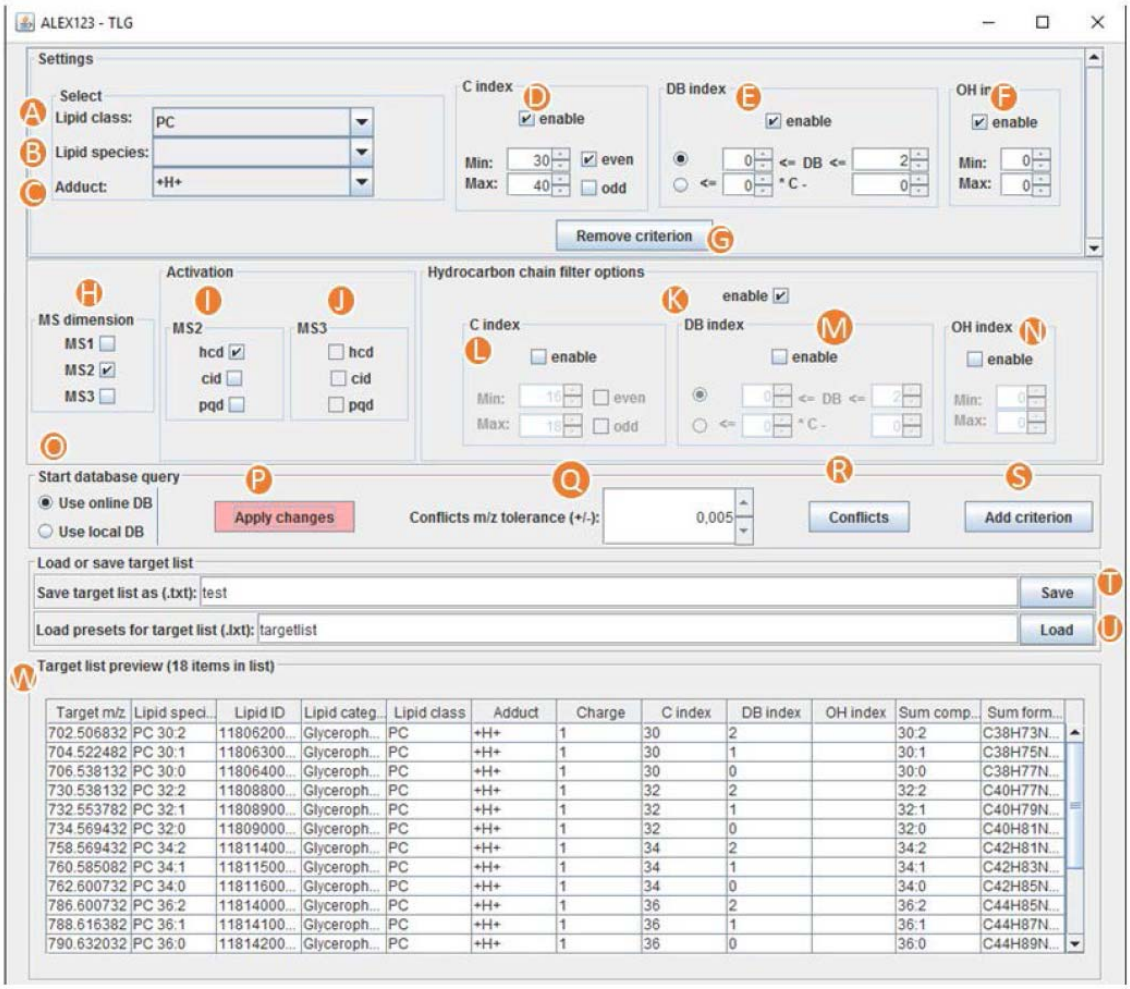


Figure 5. Screenshot of the ALEX[123] target list generator, which allows users to select lipid classes and lipid species to be searched for using criteria such as lipid class, adduction, C index, DB index and OH index. Individual lipid species (including internal standards) can also be selected. The ALEX[123] target list generator outputs a .txt file with a shortlist of selected lipid species and related information including *m/z* values and adduction. See text for description of each of the GUI elements.

Description of GUI elements:

- Field A is a dropdown-list of lipid classes that can be selected,
- Field B is a dropdown-list with lipid species that be selected,
- Field C is a dropdown-list with all adducts that can be selected,

- Field D sets the range of total number of C atoms in the hydrocarbon-based chain of lipid species (sum composition) as well as even/odd numbers,
- Field E sets the total number of double bonds in lipid species (sum composition). The upper option defines a range while the lower option applies a linear regression to select the total number of double bonds per lipid species (sum composition) as function of the total number of C atoms in the hydrocarbon-based chain of the lipid species,
- Field F sets the range of total number of hydroxyl groups in lipid species (sum composition),
- Field G can be clicked to remove the specific search criterion,
- Field H selects the MS dimension of the information on the targetlist,
- Field I is available if MS dimension MS2 or MS3 is chosen. Searching scan filters with the following fragmentations can be selected: hcd, cid or pqd.
- Field J is available if MS3 is chosen, and will specify which fragmentation method used for the second fragmentation,
- Field K can be checked to enable the hydrocarbon chain filter (for molecular lipid species) and makes field L, M and N available.
- Field L selects the number of C atoms in hydrocarbon-based chains of molecular lipid species,
- Field M selects number of double bonds in hydrocarbon-based chains of molecular lipid species,
- Field N selects number of hydroxyl groups in hydrocarbon-based chains of molecular lipid species,
- Field O selects whether to queried information from the online ALEX^{123} lipid database or a local database on the computer.
- Field P, clicking this object will show a preview of the information queried to be included on the target list,
- Field Q (optional) allows specifing a *m/z* tolerance for resolving searches for isomeric and isobaric lipids (see shortlist using Field R).

- Field R, if clicked, will open a window showing searches for isomeric and isobaric lipids. Inside this window it is possible to select which lipid species the user want on the target list. The deselected lipid species will be added to the target list under the column “Conflicts. Note that this option cannot be used if also using the ALEX^{123} isotope compiler.
- Field S adds additional search criteria to the query,
- Field T allows the user to specify the file name of the target list (saved as .txt). This file will be saved in the folder where the ALEX^{123} target list generator was exacted from. Clicking Field T will also saved the search settings (saved as a .lxt file),
- Field U can be filled out to load previously saved search settings (stored in a .lxt file). When loaded, then click the Field P to show the preview.
- Field W shows the target list preview and makes it possible to see what will be saved to the target list when save is pressed (note that MS2 and MS3 fragmentation information cannot be previewed, but will be saved to the .txt target list file).

6.3.3. Output

The target list (.txt file) contains the information that the ALEX^{123} extractor (see section below) is using for searching MS data. This information is organized in tabulated format (Figure 5), and includes information on

- “Polarity” : specifies whether searches should be made for positive or negative ion mode data,
- MS dimension: specifies whether MS^1, MS^2 or MS^3 data should be searches,
- “Target m/z” : the calculated *m/z* value of lipid species with a given adduct ion or a fragment ion *m/z* value from a given lipid precursor,
- “Fragment name” : name of fragment ions (see PMID: 29161304),
- “Structure information” : specifies what information the fragment ion conveys (can be lipid class-selective or molecular lipid species-specific),
- “Lipid species” : name of lipid species (at the sum composition level),

- “Molecular lipid species” : name of molecular lipid species (at the level of defined hydrocarbon chains),
- ”Lipid class” : defines the lipid class,
- “MS2 precursor m/z” :defines the m/z value of the intact lipid precursor ion,
- “MS2 activation” :defines the fragmentation type (hcd, cid, pqd) used for MS2 analysis,
- “MS3 precursor m/z” :defines the m/z value of the fragmented precursor ion,
- “MS3 activation” :defines the fragmentation type (hcd, cid, pqd) used for MS3 analysis,
- ”Adduct” : specifies the adduct information of the intact lipid precursor ion,
- “Lipid category” : specifies the LipidMAPS category of the lipid molecule,
- “Conflicts” : specifies whether there is any i) isomeric or isobaric conflicts (see above description of GUI elements Field Q and R) or ii) whether it is a search for a ^{13}C isotope (if using the ALEX^{123} isotope compiler, see next section),
- “Charge” : specifies the charged of the lipid precursor or fragment ion.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Detector	Polarity	MS dimension	Target m/z	Fragment name	Structure information	Lipid species	Molecular lipid species	Lipid class	MS2 precursor m/z	MS2 activation	MS3 precursor m/z	MS3 activation	Adduct	Lipid ID	Lipid category	Conflicts	Charge
86	FTMS	+	ms	759.683695			DAG 44:0		DAG					+Na+	30324600000000	Glycerolipid		1
87	FTMS	+	ms	763.527194			DAG 46:12		DAG					+Na+	30326000000000	Glycerolipid		1
88	FTMS	+	ms	765.542844			DAG 46:11		DAG					+Na+	30326100000000	Glycerolipid		1
89	FTMS	+	ms	767.558494			DAG 46:10		DAG					+Na+	30326200000000	Glycerolipid		1
90	FTMS	+	ms	769.574144			DAG 46:9		DAG					+Na+	30326300000000	Glycerolipid		1
91	FTMS	+	ms	771.589794			DAG 46:8		DAG					+Na+	30326400000000	Glycerolipid		1
92	FTMS	+	ms	773.605444			DAG 46:7		DAG					+Na+	30326500000000	Glycerolipid		1
93	FTMS	+	ms	775.621095			DAG 46:6		DAG					+Na+	30326600000000	Glycerolipid		1
94	FTMS	+	ms	777.636745			DAG 46:5		DAG					+Na+	30326700000000	Glycerolipid		1
95	FTMS	+	ms	779.652395			DAG 46:4		DAG					+Na+	30326800000000	Glycerolipid		1
96	FTMS	+	ms	781.668045			DAG 46:3		DAG					+Na+	30326900000000	Glycerolipid		1
97	FTMS	+	ms	783.683695			DAG 46:2		DAG					+Na+	30327000000000	Glycerolipid		1
98	FTMS	+	ms	785.699345			DAG 46:1		DAG					+Na+	30327100000000	Glycerolipid		1
99	FTMS	+	ms	787.714995			DAG 46:0		DAG					+Na+	30327200000000	Glycerolipid		1
100	FTMS	+	ms	668.353403			PS 26:3		PS					+Na+	10700900000000	Glycerophospholipid		1
101	FTMS	+	ms	670.369053			PS 26:2		PS					+Na+	10701000000000	Glycerophospholipid		1
102	FTMS	+	ms	672.384703			PS 26:1		PS					+Na+	10701100000000	Glycerophospholipid		1
103	FTMS	+	ms	674.400353			PS 26:0		PS					+Na+	10701200000000	Glycerophospholipid		1
104	FTMS	+	ms	694.369053			PS 28:4		PS					+Na+	10703400000000	Glycerophospholipid		1
105	FTMS	+	ms	696.384703			PS 28:3		PS					+Na+	10703500000000	Glycerophospholipid		1
106	FTMS	+	ms	698.400353			PS 28:2		PS					+Na+	10703600000000	Glycerophospholipid		1
107	FTMS	+	ms	700.416003			PS 28:1		PS					+Na+	10703700000000	Glycerophospholipid		1

Figure 6. Screenshot of a target list (.txt file) generated by the ALEX^{123} target list generator (visualized in Excel).

6.3.4. Notes

- It is recommended to make separate target lists for searching FTMS^1, and MS^2 and MS^3 data, and to execute these searches from separate subfolders (i.e.

"search folders") (Figure 7). It is also recommended to only have one single target list (.txt file) per "search folder".

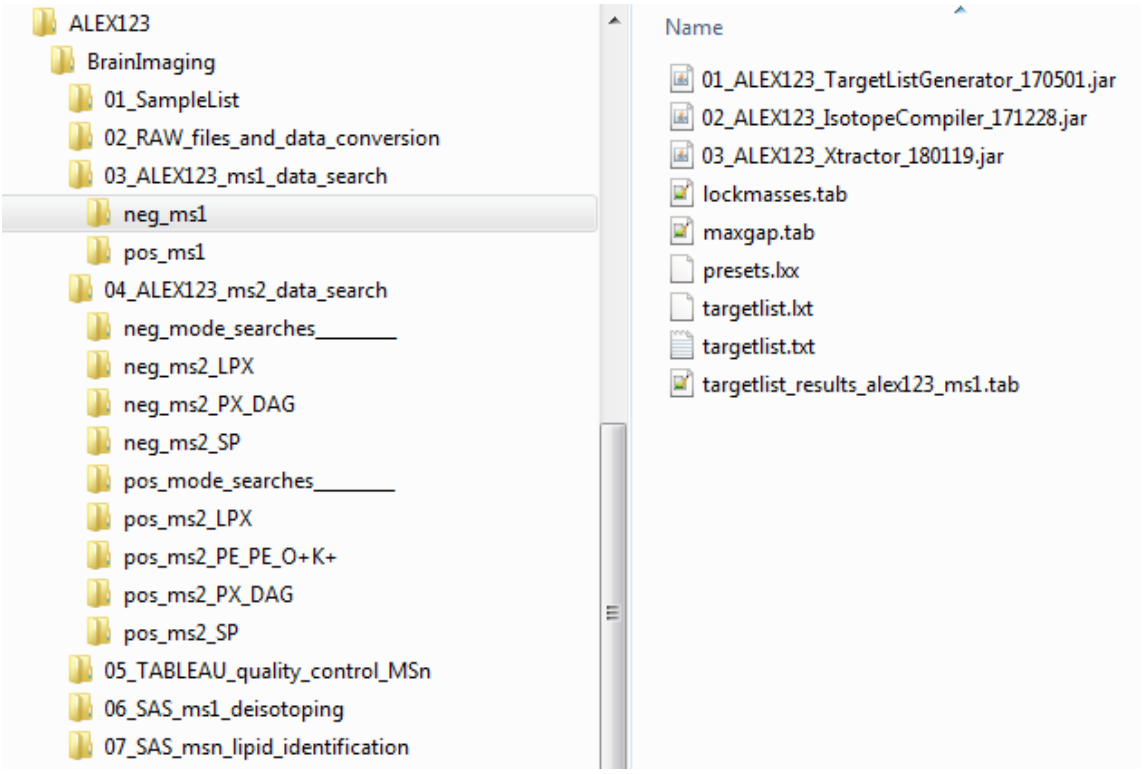


Figure 7. File management structure for executing the ALEX[123] target list generator, and the ALEX[123] extractor, ALEX[123] isotope compiler, and the ALEX[123] unifier. Searching of FTMS[1] and ITMS[2] data are done in separate "search folders". For example, the folder "03_ALEX123_ms1_data_search" contains all searches of FTMS[1] data, which are stored in individual subfolders. For example, searches for lipids in negative FTMS[1] data are executed from the subfolder "neg_ms1").

- Experienced users are able to modify the information in target list .txt files. As such, experienced users can use, for example, Microsoft Excel to compute *m/z* values of lipid and fragment ions that might not be available in the ALEX[123] lipid database.

- Users can limit the "search space" by restricting the total number of C atoms, double bonds and OH group in lipid molecules and fragment ions (see field D, E F and K). For example, searching for lipids with polyunsaturated fatty acyl chains

in certain bacteria and yeast might not be meaningful. Moreover, a linear equation-based filter is also available for increasing the number of double bonds as function of increasing number of C atoms.

6.4. ALEX^{123} isotope compiler

6.4.1. General information

The ALEX^{123} isotope compiler uses Java.

The ALEX^{123} isotope compiler ("ALEX123_IsotopeCompiler") (Figure 8) serves to automatically add ^{13}C isotope information to target lists (.txt files) (generated using the ALEX^{123} target list generator, see previous section).

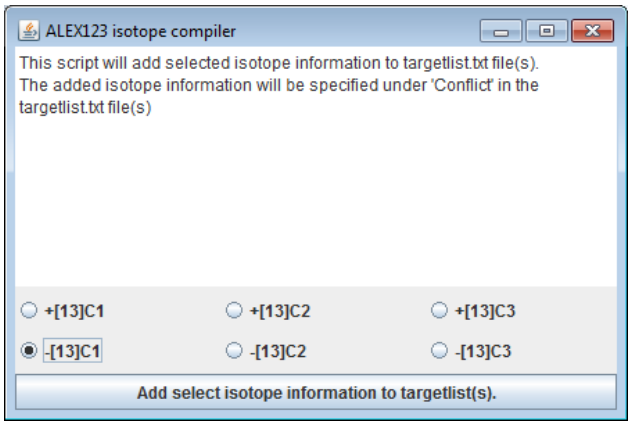


Figure 8. Screenshot of the ALEX^{123} isotope compiler. This script allows adding ^{13}C isotopes to searches. For example, selecting "-[13]C1" will, for each entry on the target list, subtract the mass of a ^{13}C atom from the monoisotopic *m/z* value, and add to the field "Conflicts" the information ""-[13]C1". Effectively, this will allow searches for both monoisotopic *m/z* values and potential ^{13}C isotope interferences. Potential ^{13}C isotope interferences are corrected for a SAS-based script (see section 9.6).

6.4.2. Notes

- This script will automatically overwrite existing target lists (.txt files) located in the same folder from which the ALEX^{123} isotope compiler is executed. In addition, the script makes a backup of the original target list file, saved without a file

extension (e.g. Input: targetlist.txt → Output: targetlist.txt (with ^{13}C isotope information) and targetlist (no extension)). Note the increase in file size:

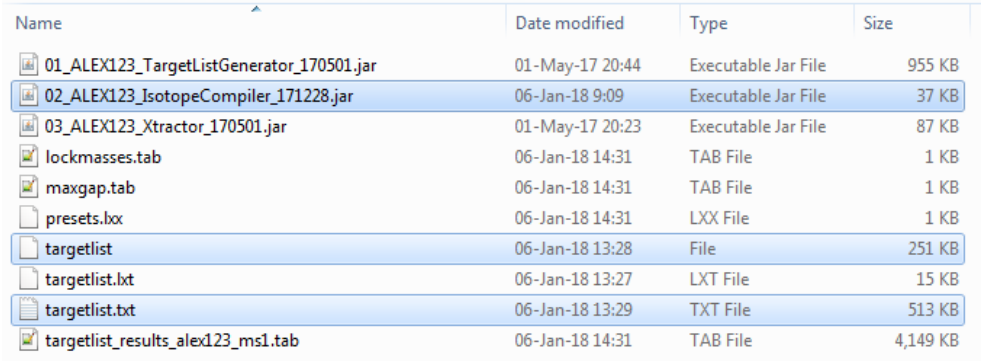


Figure 9. Executing the ALEX^{123} isotope compiler will a) add selected ^{13}C isotope information to txt-based target lists located in the same folder, b) overwrite the same of the txt-based target lists, and c) generate a backup of the original, unmodified txt-based target lists, which will be saved without file extension.

6.5. ALEX^{123} extractor

6.5.1. General information

The ALEX^{123} extractor uses Java.

The ALEX^{123} extractor (Figure 10) uses information in txt-based target lists (generated by the ALEX^{123} target list generator) to search for lipid species and fragment *m/z* values in averaged spectral peak lists (.txt files) (generated by the ALEX^{123} converter).

6.5.2. GUI

The ALEX^{123} extractor is operated using a GUI shown in **Figure 10**:

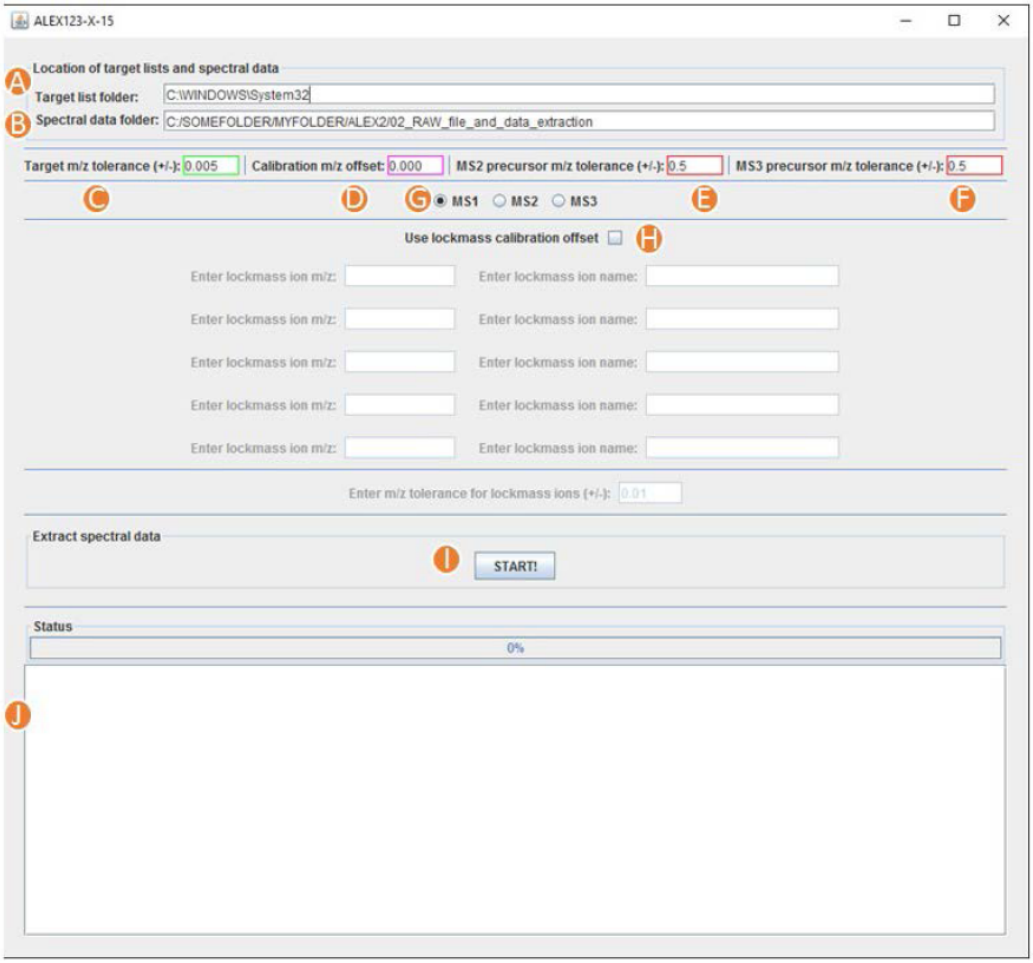


Figure 10. Screenshot of the ALEX[123] extractor.

Description of GUI elements:

- Field A specifies where the "search folder" and target list .txt file is located. By default this will always be the folder from where the ALEX[123] extractor is executed,
- Field B specifies the absolute path/folder where averaged peak lists generated by ALEX[123] converter spectral are located. This data is typically located under:

  ..\ 02_RAW_files_and_data_conversion\txt_FTMS1, and

  ..\ 02_RAW_files_and_data_conversion\txt_ITMS2
- Field C specifies the *m/z* tolerance for identifying *m/z* values of lipid species and fragment ions.

- Field D possibility to specify a constant *m/z* offset to correct searches for a calibration offset,
- Field E specific the m/z tolerance for the MS^2 precursor m/z,
- Field F specifies the m/z tolerance for the MS^3 precursor m/z,
- Field G specifies the MS dimension to search (MS^1, MS^2 or MS^3),
- Field H option to use automatic lock mass adjustment to correct $FTMS^1$ searches for calibration drifts,
- Field I clicking this field starts the searches.

6.5.3. Output

The $ALEX^{123}$ extractor outputs up to three tabulated result files:

- A main result file with identified lipid and fragment ion *m/z* values, and intensities and other metadata. This file is named as:

  <name of target list>_results_alex123_<MS dimension>.tab

  (example: ” targetlist_results_alex123_ms1.tab” (**Figure 11**)).
- (*only for $FTMS^1$ data*) A ”quality control” file that reports the minimum, average, median and maximum *m/z* width between all data point the averaged $FTMS^1$ peak list. This file is named ”maxgap.tab”.
- (*only for $FTMS^1$ data*) Another ”quality control” file that reports the calibration of the searched $FTMS^1$ scan filter. This file is named ”lockmasses.tab”.

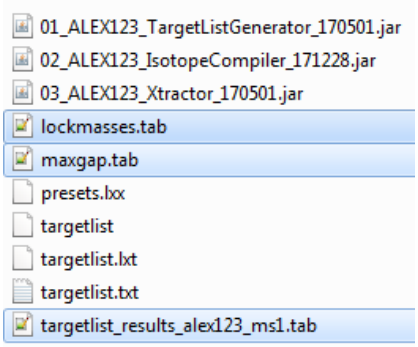


Figure 11. Executing the $ALEX^{123}$ extractor will produce three result files i) targetlist_results_alex123_ms1.tab, ii) maxgap.tab and iii) lockmasses.tab. See main text for explanation of their contents.

6.5.4. Notes

- There should only be single .txt file (targetlist) in each “search folder”, otherwise the ALEX^{123} extractor might not work.
- There is no option to specify an output folder. By default results of searches will be saved in the same “search folder” as the ALEX^{123} extractor is located in. Executing a new search will overwrite the results of a previous search.
- To specify m/z tolerances (Field C) requires a profound understanding of mass resolution and its ability to resolve peaks!
- m/z tolerances for MS2 and MS3 precursor m/z values is typically done with ‘unit’ resolution (i.e. using a tolerance of ±0.25 amu).
- Using automatic lock mass adjustment (Field H) requires specification of well-characterized and ubiquitous lock mass ions in order to estimate the FTMS^{1} calibration offset.
- Searching FTMS1 requires specifying the absolute path/folder *including* the “scan filter” (Field B). For example:

  “..\02_RAW_files_and_data_conversion\txt_FTMS1\neg\FTMS - p NSI Full ms [180.00-2000.00]” ← scan filter is underlined!
- Searching MS^{2} and MS^{3} data requires specifying the absolute path/folder *excluding* the “scan filter”. For example:

  “..\02_RAW_files_and_data_conversion\txt_ITMS2\neg\” ← NO scan filter is specified!

6.6. ALEX^{123} unifier

6.6.1. General information

The ALEX^{123} unifier uses Java.

The ALEX[123] unifier concatenates result (.tab) files (generated by ALEX[123] extractor), that are located in all subfolders, and outputs a single tabulated output file named ”Results_unified.tab”.

The ALEX[123] unifier also concatenates all target list (.txt), lockmass (.tab) and maxgap (.tab) files located in all subfolders, and saves these as ”Targetlist_unified.txt”, ”Lockmass_unified.tab” and ”Maxgap_unified.tab”, respectively.

6.6.2. Notes

- The ALEX[123] unifier should be located and executed from a folder that is above all ”search folders” (e.g. at ”..\ 03_ALEX123_ms2_data_search”) (Figure 12).

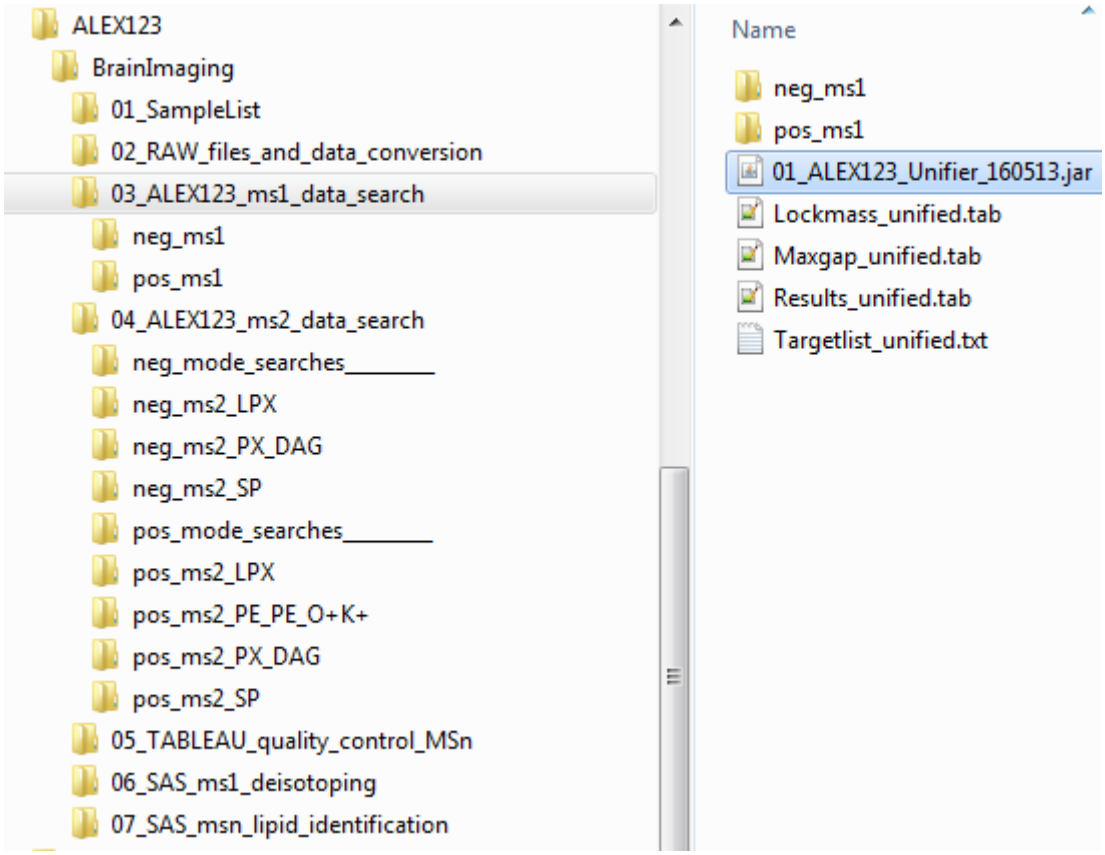


Figure 12. The ALEX[123] unifier should be execute from a folder level higher than all "search folders". This will generate up to four concatenated (unified) results files: i) Results_unified.tab, ii) Targetlist_unified.txt, iii) Lockmass_unified.tab (only for FTMS[1] data) and iv) Maxgap_unified.tab (only for FTMS[1] data). See main text for explanation of their contents.

7. SAS® Enterprise Guide

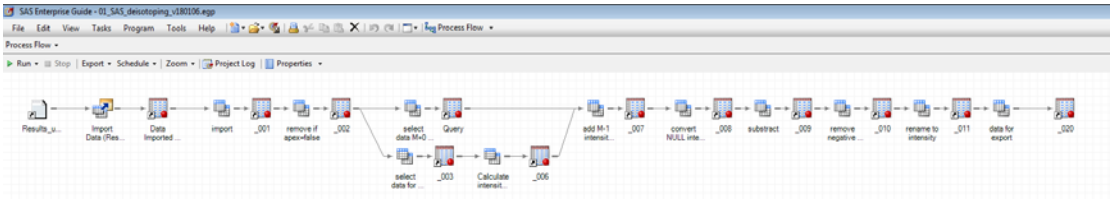
7.1.1. General information

SAS® Enterprise Guide is proprietary software that supports visual programming (https://www.sas.com/en_us/software/enterprise-guide.html). In this user guide we specify how to execute scripts in SAS® Enterprise Guide to

- check for potential ^{13}C isotopic interferences and do deisotoping of FTMS[1] data, and
- to do high confidence lipid identification using FTMS[1] and ITMS[2] data.

7.1.2. Deisotoping of FTMS[1] data

The data processing pipeline, termed "01_SAS_deisotoping.egp" (Figure 13A) located in folder "..\05_SAS_deisotoping" (Figure 13B), automatically checks for ^{13}C isotopic interferences, and if present, performs deisotoping.

A 

B

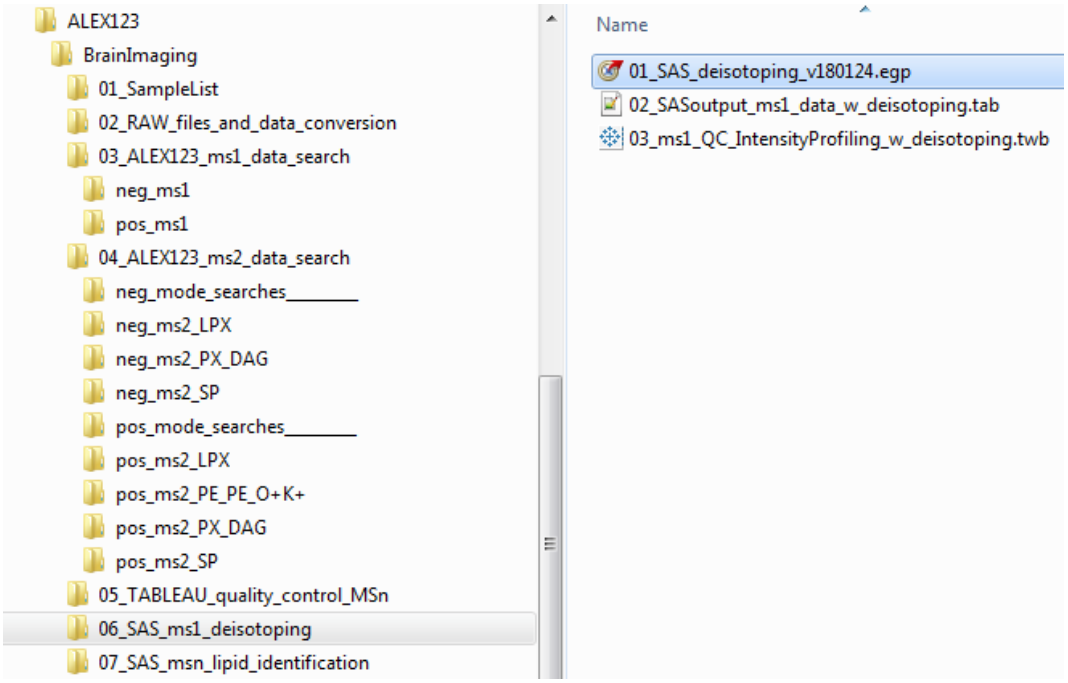


Figure 13. (A) Screenshot of script for automated deisotoping of FTMS[1] data. **(B)** Screenshot of file management structure with highlight of location/folder from where the SAS-based deisotoping is performed.

- **INPUT:** result file with FTMS[1] data (generated by ALEX[123] unifier), named "Results_unified.tab" and located in folder "03_ALEX123_ms1_data_search".

- **OUTPUT:** tabulated result file, named "02_SASoutput_ms1_data_w_deisotoping.tab" and located in folder "..\05_SAS_deisotoping".

- (optional) OUTPUT data can be accessed for visual inspection (quality control) by Tableau® Desktop.

7.1.3. Lipid identification

The script, termed "01_SAS_lipid_identification.egp" located in folder "..\06_SAS_msn_lipid_identification" (Figure 13B), automatically performs identification of lipid molecules detected by a) lipid class-selective fragment ions (annotated by lipid species sum composition) and b) molecular lipid species-specific fragment ions (annotated as molecular lipid species).

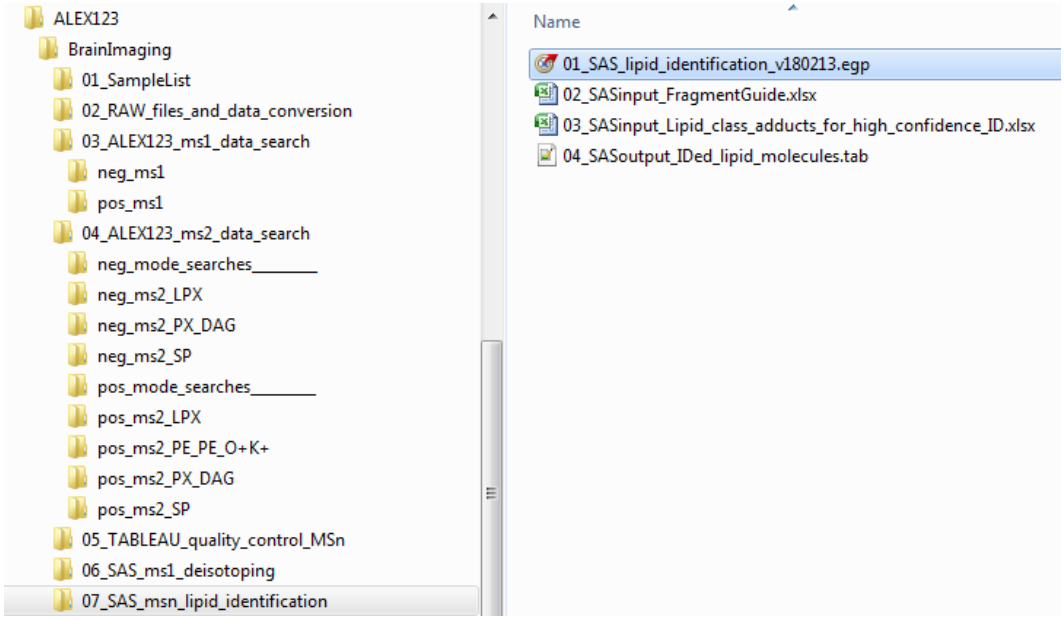


Figure 14. Screenshot of file management structure with of location/folder from where the SAS-based lipid identification routine is executed.

- **INPUT:** result file with ITMS^2 data (generated by ALEX^{123} unifier), named "Results_unified.tab" and located in folder "04_ALEX123_ms2_data_search".

- **INPUT:** result file with deisotoped FTMS^1 data, named "02_SASoutput_ms1_data_w_deisotoping.tab" and located in folder "..\06_SAS_deisotoping" (generated by using SAS).

- **INPUT:** Microsoft® Excel file, named "02_SASinput_FragmentGuide.xlsx" and located in folder "..\07_SAS_msn_lipid_identification".

- **INPUT:** Microsoft® Excel file, named "03_SASinput_Lipid_class_adducts_for_high_confidence_ID.xlsx" and located in folder "..\07_SAS_msn_lipid_identification".

- **INPUT:** concatenated target list file, named "Targetlist_unified.txt" and located in folder "..\04_ALEX123_ms2_data_search".

  INPUT: Microsoft® Excel file, named "SampleList.xlsx" and located in folder "..\01_SampleList".

- **OUTPUT:** Tabulated (.tab) result file with list of identified lipid molecules named "04_SASoutput_IDed_lipid_molecules.tab" and located in folder "..\07_SAS_msn_lipid_identification".

8. Tableau Desktop

Tableau® Desktop (www.tableausoftware.com) is an interactive data visualization software that can be dynamically linked to ALEX^{123}- and SAS-based output files. Data can be sorted and visualized in different display formats depending on the question (Figure 15).

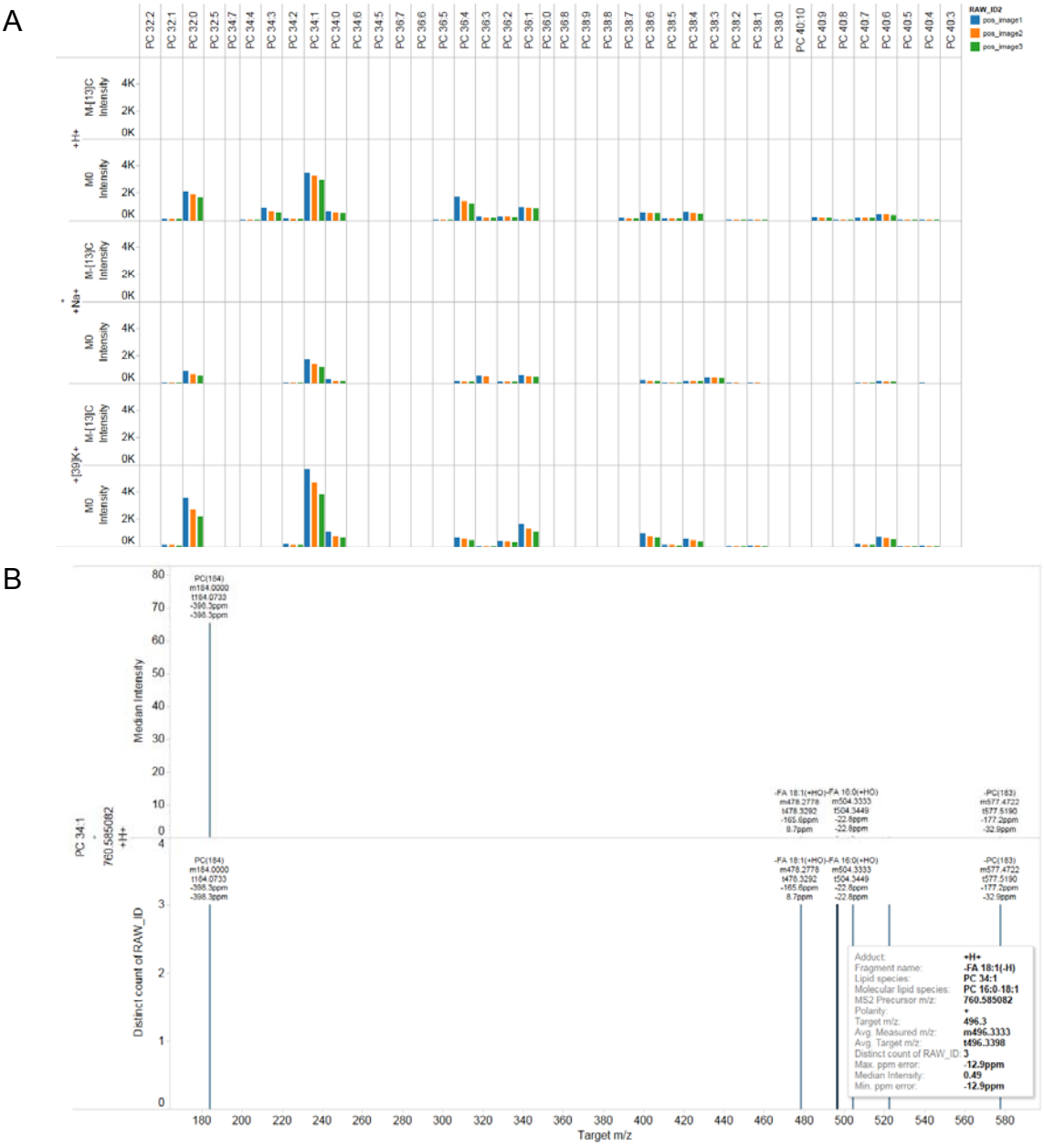


Figure 15. Data visualization by Tableau Desktop. (A) Combined visualization of ionization information and potential ^{13}C isotope interferences for PC molecules detected in three distinct rat brains. Note that raw FTMS[1] intensity data is shown for three different adduct ions ($+H^+$, $+Na^+$ and $^{39}K^+$), and that every second panel shows intensity data for potential ^{13}C isotope interferences (M-[13]C intensity) and monoisotopic lipid species (M0 intensity). **(B)** Visualization of concatenated fragment ion data for protonated PC 16:0-18:1, detected in three distinct rat brains (by ITMS[2]). The upper panel shows the median fragment ion intensity across the three samples. The low panel shows how many times each fragment ion was were detected.

9. Example dataset

9.1. General information

The example dataset is from a MALDI-based lipid imaging study described in Ellis et al. (2018, in review). In this study we acquired high resolution FTMS[1] data and low resolution ITMS[2] data of rat brain sections in both positive and negative ion mode using an LTQ Orbitrap Elite mass spectrometer.

9.1.1. Requirements and downloads

- Software listed in section 3 must be installed.
- The folder "C:\ALEX123\" should be generated.
- The ALEX[123] framework, available as a .zip file, should be downloaded: www.mslipidomics.info | software, and saved in the folder "C:\ALEX123\".
- Unzip the .zip file (with the ALEX[123] framework). This will setup the ALEX[123] file management system (Figure 16).
- The following six .RAW files should also be downloaded from MetaboLights: https://www.ebi.ac.uk/metabolights/reviewerb78b2bf1-aeb6-4244-b967-368e18ed806d
    - Go to the folder "Study Files" and download:

neg_1.raw	pos_1.raw
neg_2.raw	pos_2.raw
neg_3.raw	pos_3.raw

- Put the files neg_1.raw, neg_2.raw and neg_3.raw into the file folder: "C:\ALEX123\BrainImaging\02_RAW_files_and_data_conversion\raw\neg\"
- Put the files pos_1.raw, pos_2.raw and pos_3.raw into the file folder: "C:\ALEX123\BrainImaging\02_RAW_files_and_data_conversion\raw\pos\"

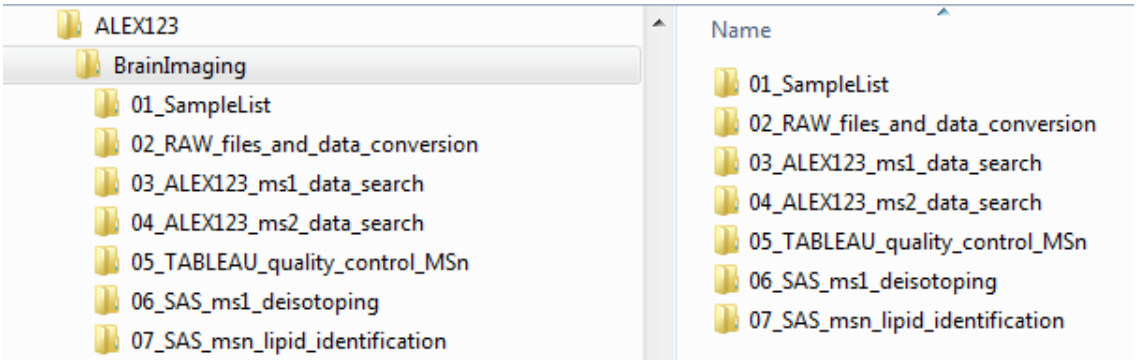


Figure 16. File management system used for executing the ALEX[123] framework.

9.2. Step 1: Raw files and data conversation

- Place .RAW files with negative mode data in the folder (see Figure 3, Figure 17):

"..\02_RAW_files_and_data_conversion\raw\neg\"

- Place .RAW files with positive ion mode data in the folder (see Figure 3, Figure 17):

"..\02_RAW_files_and_data_conversion\raw\pos\"

- Go to folder:

"..\02_RAW_files_and_data_conversion\"

- Execute (double click on) the file:

"convert_FTMS1_data.cmd"

This will launch the ALEX[123] converter and generate averaged peak lists with FTMS[1] data that are saved in the folder "..\txt_FTMS1".

- Once the conversion of FTMS[1] data is done,

then execute (double click on) the file:

"convert_ITMS2_data.cmd"

This will launch the ALEX[123] converter and generate averaged peak lists with ITMS[2] data that are saved in the folder "..\txt_ITMS2".

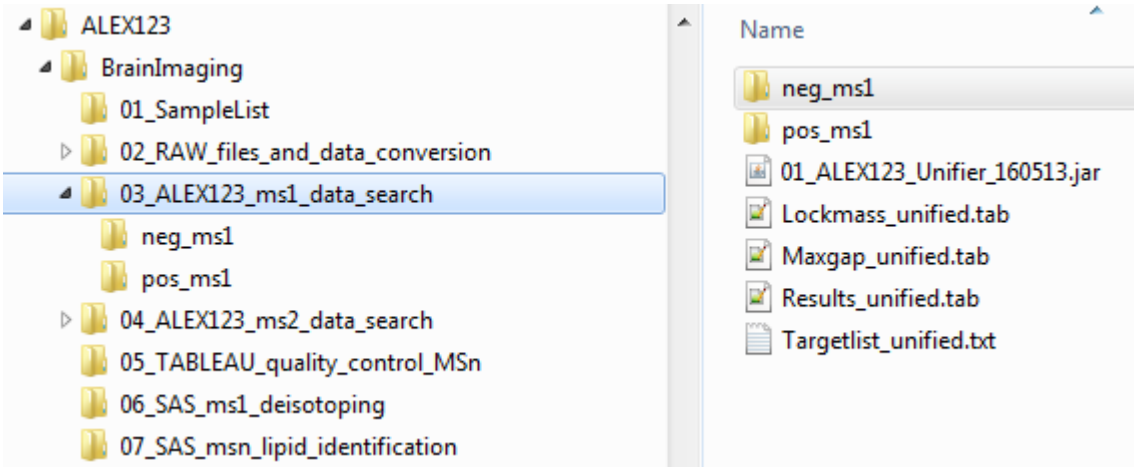


Figure 17. File management structure for executing FTMS[1] searches. Note that searches are organized by polarity, and that i) searches are setup using the ALEX[123] target list generator and ALEX[123] isotope compiler, and executed using the ALEX[123] extractor, present in the subfolders 'neg_ms1' and 'pos_ms1'.

9.2.1. Notes

- The output of the ALEX[123] converter is controlled by settings specified in the configuration file (e.g. "config file_FTMS1.txt"). See section 6.1 for further details on how to change the output of the ALEX[123] converter (e.g. changing folders for input and output data, export of averaged peak lists in profile or centriod mode).

9.3. Step 2: Search FTMS[1] data

The downloaded ALEX[123] framework comes with pre-configured settings for searching FTMS[1] data. Searches are executed from distinct "search folders" (i.e. subfolders) located in "..\03_ALEX123_ms1_data_search\" (Figure 7).

Note that the pre-configured search settings are specified in the following files:

- "targetlist.txt": contains *m/z* values of lipid species and related information. This file is generated by the ALEX[123] target list generator (section 6.3) and ALEX[123] isotope compiler (section 6.4).

- "presets.lxx": includes information on *m/z* search tolerance, location of .txt-based peak lists that should be searched. This file is used and generated by the ALEX^{123} extractor (section 6.5).

- To search negative ion mode FTMS^{1} data, using pre-configured search settings, do the following:

  - Go to search folder: "..\03_ALEX123_ms1_data_search\neg_ms1".
  - Execute (double click on) the file: "03_ALEX123_Xtractor.jar".
  - This will launch the ALEX^{123} extractor GUI (see Figure 10, Figure 18).
  - Specify in the input field "Spectral data folder" the absolute path for the folder with .txt-based peak lists for negative ion mode FTMS^{1} data:

    "C:\ALEX123\BrainImaging\02_RAW_files_and_data_conversion\txt_FTMS1\neg\FTMS - p NSI Full ms [180.00-2000.00]".
  - Use the pre-defined "Target m/z tolerance".
  - The GUI of the ALEX^{123} extractor will automatically specify the folder where the file "targetlist.txt" is located.
  - Click on "START!" to search the FTMS^{1} data.
  - This will generate the output file: "targetlist_results_alex123_ms1.tab" with results of searching the negative FTMS^{1} data.

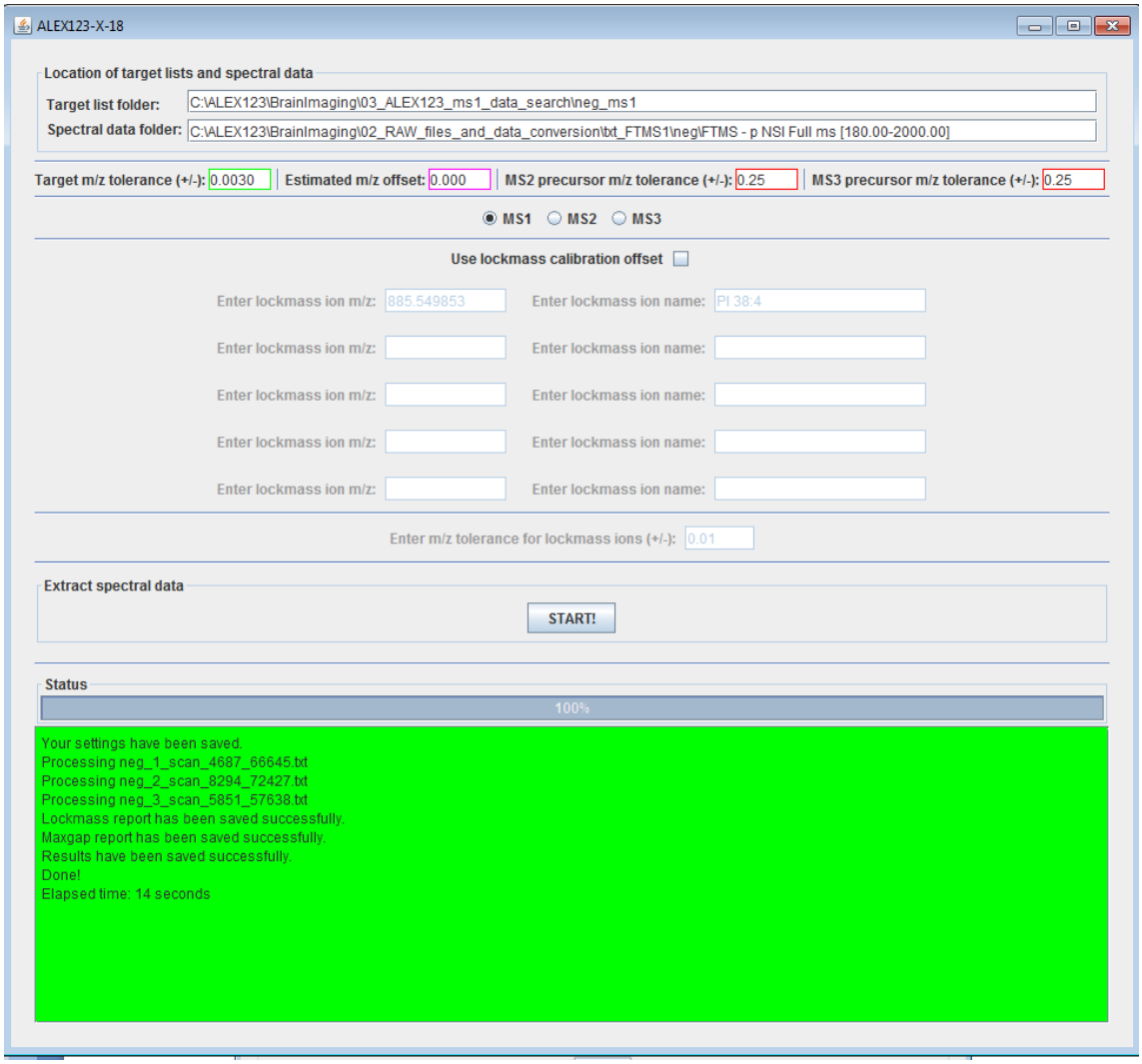


Figure 18. Screenshot of the ALEX[123] extractor. For searching FTMS[1] data the user needs to specify an appropriate value for the "Target m/z tolerance" (typically the average full width ($\Delta m/z$) at half maximum peak intensity for a particular scan filter). Moreover, the user should also manually specify the path for averaged peak lists (.txt). The ALEX[123] extractor will automatically find the path of the targetlist.txt (i.e. the folder from which the ALEX[123] extractor is launched).

- To search positive ion mode FTMS[1] data, using pre-configured search settings, do the following:

  - Go to search folder: "..\03_ALEX123_ms1_data_search\pos_ms1".
  - Execute (double click on) the file: "03_ALEX123_Xtractor.jar".

- This will launch the ALEX^{123} extractor GUI (see **Figure 10**, **Figure 18**).
- Specify in the input field “Spectral data folder” the absolute path for the folder with .txt-based peak lists for negative ion mode FTMS^{1} data:

  “C:\ALEX123\BrainImaging\02_RAW_files_and_data_conversion\txt_FTMS1\pos\FTMS + p NSI Full ms [180.00-2000.00]”.
- Use the pre-defined “Target m/z tolerance”.
- The GUI of the ALEX^{123} extractor will automatically specify the folder where the file “targetlist.txt” is located.
- Click on “START!” to search the FTMS^{1} data.
- This will generate the output file: “targetlist_results_alex123_ms1.tab” with results of searching the positive FTMS^{1} data.

- Next, combine the results from searching the negative and positive FTMS^{1} data:
  - Go to folder: “..\03_ALEX123_ms1_data_search\.
  - Execute (double click on) the file: “01_ALEX123_Unifier.jar” (see Figure 12).
  - This will execute the ALEX^{123} unifier (no GUI) and
  - Generate an output file “Results_unified.tab” with the results from the searches of negative and positive FTMS^{1} data located in underlying search folders.

9.3.1. Notes

- The ALEX^{123} target list generator (named “01_ALEX123_TargetListGenerator.jar” and located inside each search folder) is used for setting up and modifying the searches for lipid molecules. See section 6.3 for instructions on how to use the ALEX^{123} target list generator.

- It is highly recommended to make separate searches, executed in separate "search folders", for negative and positive FTMS[1] data, and to concatenate the search results using the ALEX[123] unifier.

- The ALEX[123] isotope compiler (named "02_ALEX123_IsotopeCompiler.jar" and located inside each search folder) is used for adding ^{13}C information to searches for lipid molecules. Executing the ALEX[123] isotope compiler will add ^{13}C information to the "targetlist.txt" and overwrite the original .txt file. A backup of the original .txt file will be made and named "targetlist" (with file extension). See section 6.4 for instructions on how to use the ALEX[123] isotope compiler.

- The ALEX[123] extractor (named "03_ALEX123_Xtractor.jar" and located inside each search folder) is used for searching for and retrieving lipid *m/z* information in averaged peak lists. See section 6.5 for instructions on how to use the ALEX[123] extractor.

9.4. Step 3: Search ITMS^2 data

The downloaded ALEX[123] framework also comes with pre-configured settings for searching ITMS^2 data. Searches are executed from distinct "search folders" (i.e. subfolders) located in "..\04_ALEX123_ms2_data_search\" (see Figure 12, Figure 19). Note that these search folders refer to specific groups of lipid classes and polarity. For example, negative ITMS^2 data pertaining to lyso-glycerophospholipids are searched for in the folder "..\neg_ms2_LPX". Similarly, positive ITMS^2 data pertaining to sphingolipids are searched for in folder "..\pos_ms2_SP".

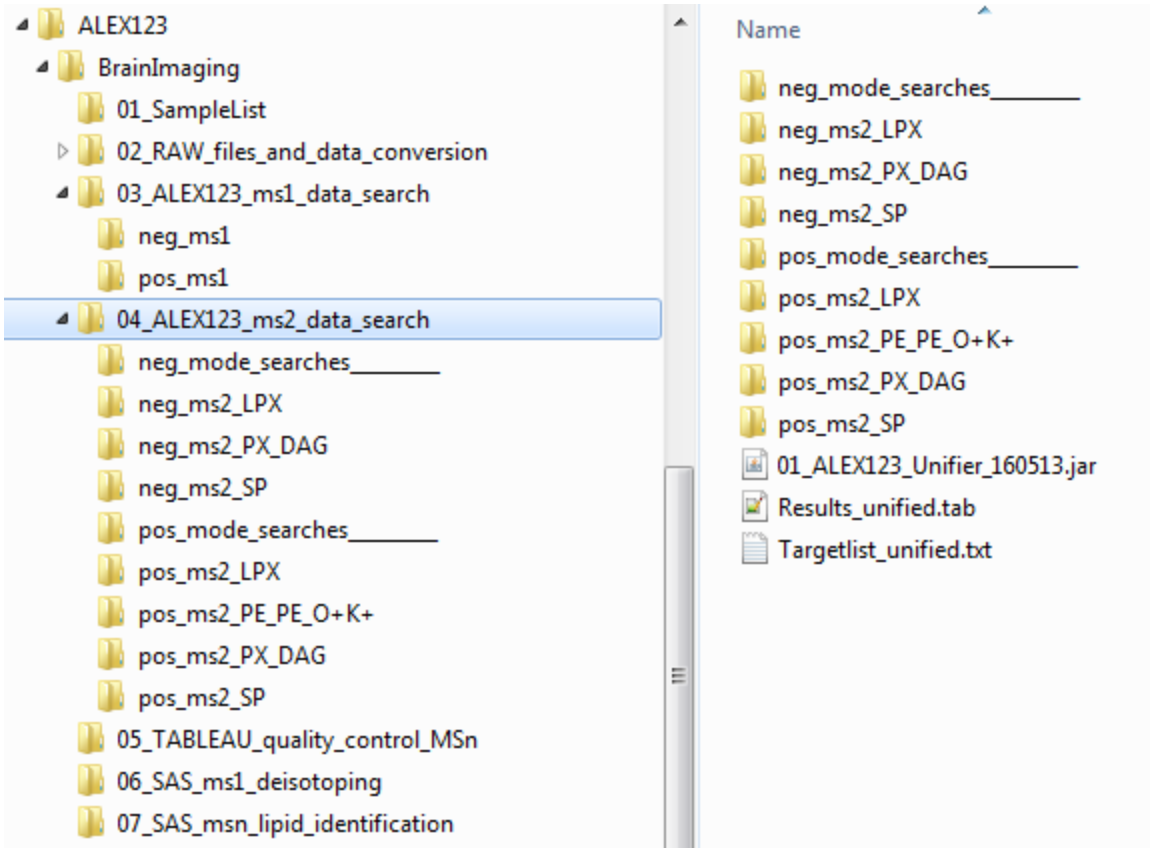


Figure 19. File management structure for executing ITMS^2 searches. Note that searches are organized by polarity and lipid classes/categories. Searches are setup using the ALEX^{123} target list generator and ALEX^{123} isotope compiler, and executed using the ALEX^{123} extractor, present in the subfolders.

Note that the downloaded ALEX^{123} framework features pre-configured search settings for the ITMS^2 data, and are specified in the following files:

- "targetlist.txt": contains *m/z* values of lipid fragments and related information. This file is generated by the ALEX^{123} target list generator (section 6.3) and ALEX^{123} isotope compiler (section 6.4).
- "presets.lxx": includes information on search tolerance for *m/z* values of fragment ions and precursors, and location of .txt-based peak lists that should be searched. This file is used and generated by the ALEX^{123} (section 6.5).

- To search ITMS^2 data, using pre-configured search settings, do the following:
  - Go to search folder: "..\04_ALEX123_ms2_data_search\neg_ms2_LPX".

- Execute (double click on) the file: “03_ALEX123_Xtractor.jar”.
- This will launch the ALEX^{123} extractor GUI (Figure 18).
- Specify in the input field “Spectral data folder” the absolute path for the folder with .txt-based peak lists of negative ITMS^2 data: e.g. “C:\ALEX123\BrainImaging\02_RAW_files_and_data_conversion\txt_ITMS2\neg”.
- Use the pre-defined “Target m/z tolerance”.
- The GUI of the ALEX^{123} extractor will automatically specify the folder where the file “targetlist.txt” is located.
- Click on “START!” to search the ITMS^2 data.
- This will generate the output file: “targetlist_results_alex123_ms2.tab” with results of the ITMS^2 search.

- The user should repeat the above-listed steps for each search folder located inside the folder with all ITMS2 searches: “..\04_ALEX123_ms2_data_search”

- Next, combine the results of all ITMS^2 searches using ALEX^{123} unifier:
  - Go to folder: “..\04_ALEX123_ms2_data_search\.
  - Execute (double click on) the file: “01_ALEX123_Unifier.jar” (see Figure 19).
  - This will execute the ALEX^{123} unifier (no GUI) and
  - Generate an output file “Results_unified.tab” with the results from the all searches of negative and positive ITMS^2 data, located in the underlying search folders.

9.5. Step 4: Visual quality control

The results of searching FTMS^1 and ITMS^2 data can be visualized to provide important information about, for example, intensities of intact lipid ions and fragment ions, the preferred adduction of specific lipid classes, the spectral profile of all lipid species for a particular lipid class, etc.

The downloaded ALEX[123] framework comes with two templates for visualizing FTMS[1] and ITMS[2] data (using Tableau® Desktop). These templates are located inside the folder:

"..\05_TABLEAU_quality_control_msn" (Figure 20),

and are named:

02_ms1_QC_IntensityProfiling.twb

03_ms2_QC_IntensityProfiling.twb

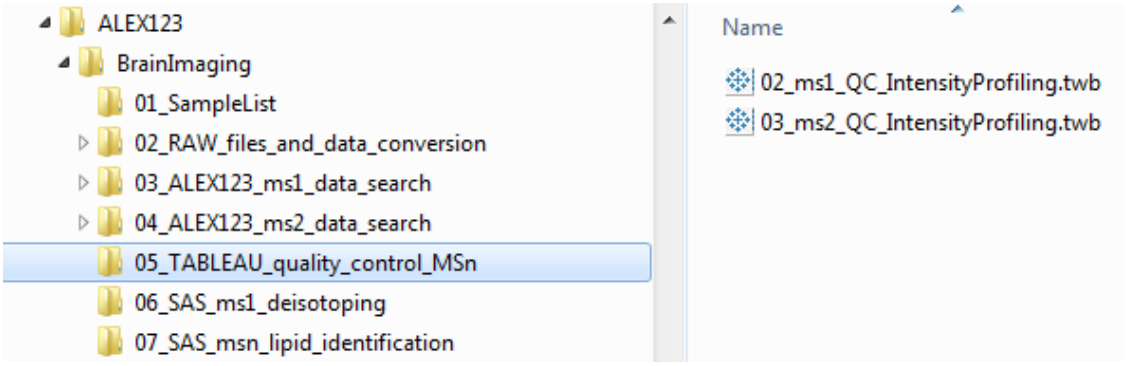
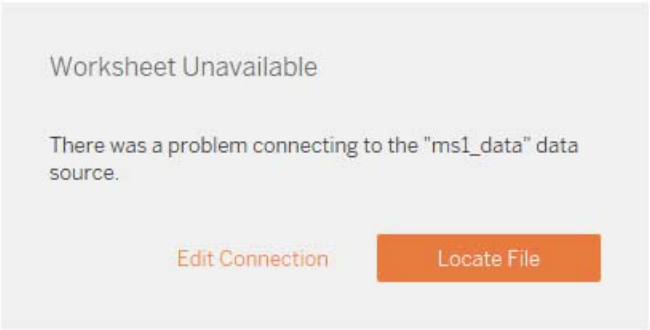


Figure 20. File management structure for Tableau-based visual quality control of FTMS1 and ITMS[2] searches.

9.5.1. Visualize FTMS[1] data

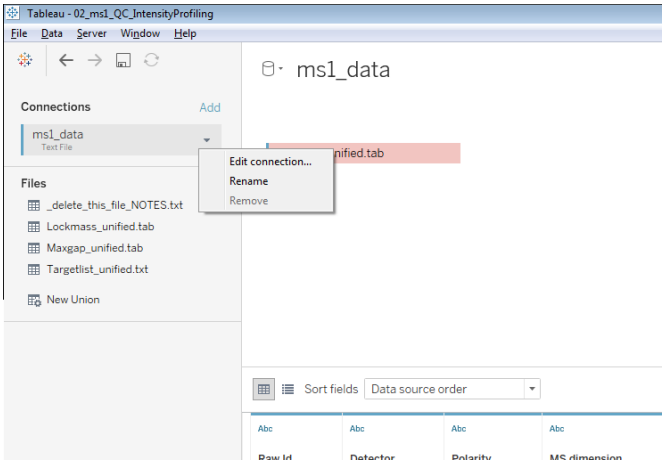
- To visualize FTMS[1] data, using the appropriate template file, do the following:
  - Go to folder: "..\05_TABLEAU_quality_control_msn".
  - Execute (double click on) the file: "02_ms1_QC_IntensityProfiling.twb"

    → This will open the Tableau file and probably prompt the following message:



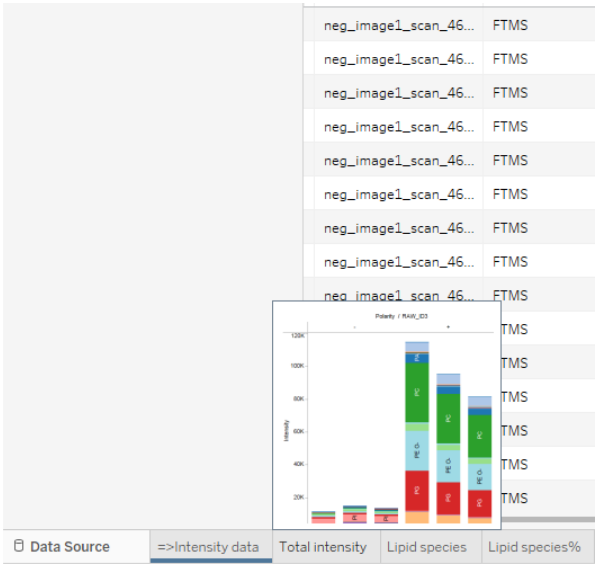
  - Click on "Edit Connection"

→ This return the following window:



- Right-hand click on the field with “ms1_data”
- Choose “Edit connection”
- Go to folder: “..\03_ALEX123_ms1_data_search”
- Choose the file: “Results_unified.tab”
- Click “Open”

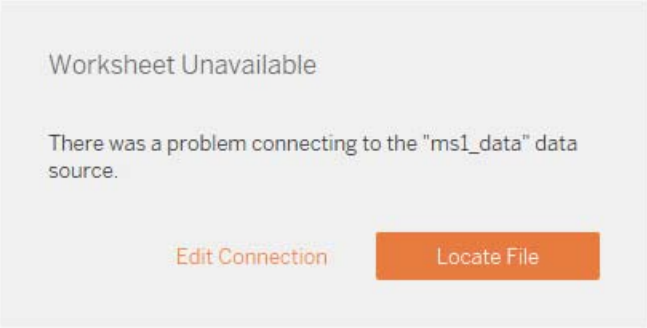
  → This should load the result file with negative and positive FTMS[1] data
- Click on the below-listed “sheets” (e.g. “Total intensity) to visualize different subsets of the FTMS[1] data:



9.5.2. Visualize ITMS^2 data

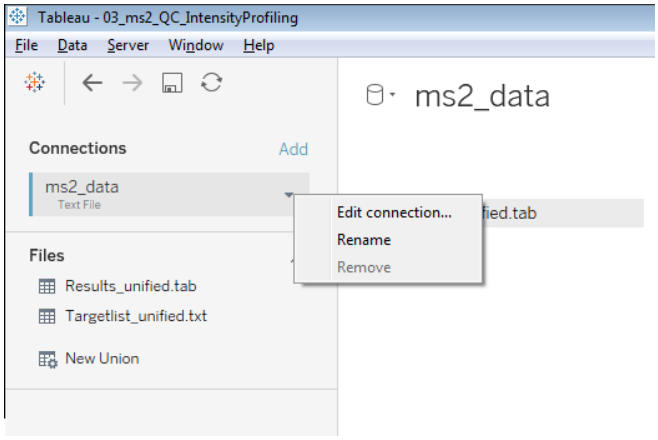
- To visualize ITMS^2 data, using the appropriate template file, do the following:
  - Go to folder: "..\05_TABLEAU_quality_control_msn".
  - Execute (double click on) the file: "03_ms2_QC_IntensityProfiling.twb"

    → This will open the Tableau file and probably prompt the following message:



  - Click on "Edit Connection"

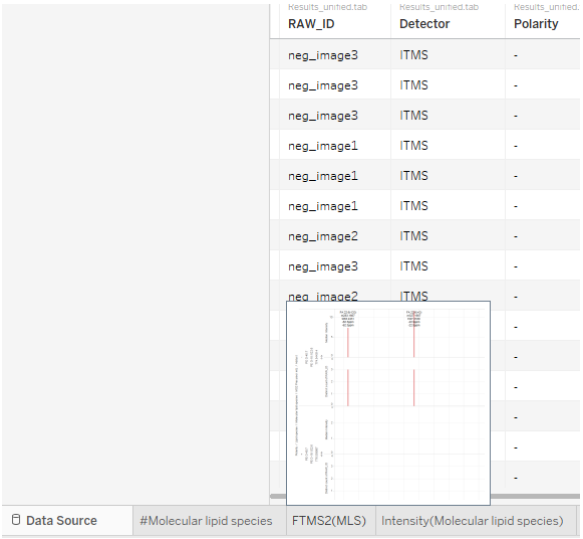
    → This return the following window:



  - Right-hand click on the field with "ms2_data"
  - Choose "Edit connection"
  - Go to folder: "..\04_ALEX123_ms2_data_search"
  - Choose the file: "Results_unified.tab"
  - Click "Open"

    → This should load the result file with negative and positive ITMS^2 data

- Click on the below-listed “sheets” (e.g. “Total intensity) to visualize different subsets of the ITMS^2 data:



9.5.3. Notes

- For more information on how to use Tableau visit www.tableausoftware.com and the online help at http://onlinehelp.tableau.com/current/pro/desktop/en-us/help.htm

9.6. Step 5: Deisotoping of FTMS^1 data

The unified result file from searching positive and negative FTMS^1 data is used as an input for checking for potential isotopic interferences from neighboring ^{13}C isotopes, and if present, doing deisotoping.

The downloaded ALEX^{123} framework comes with pre-configured script for doing deisotoping of FTMS^1 data (using SAS® Enterprise Guide). This script is located inside the folder:

“..\06_SAS_ms1_deisotoping” (Figure 21),

and is named:

“01_SAS_ms1_deisotoping.egp”

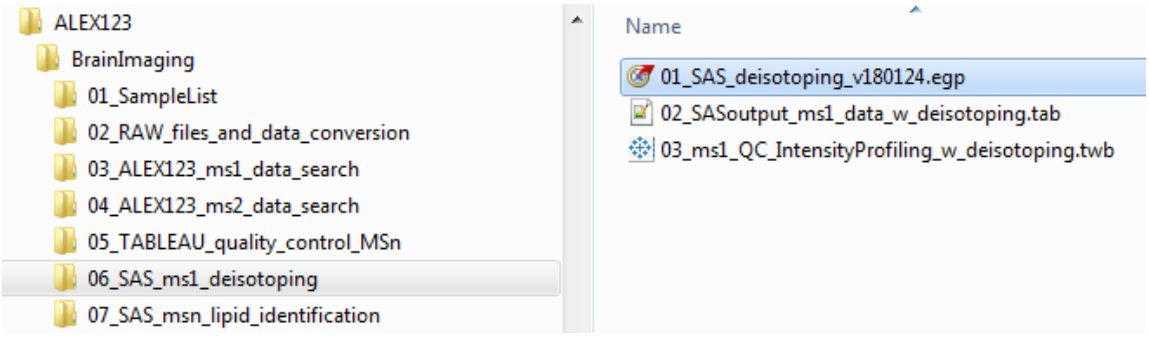
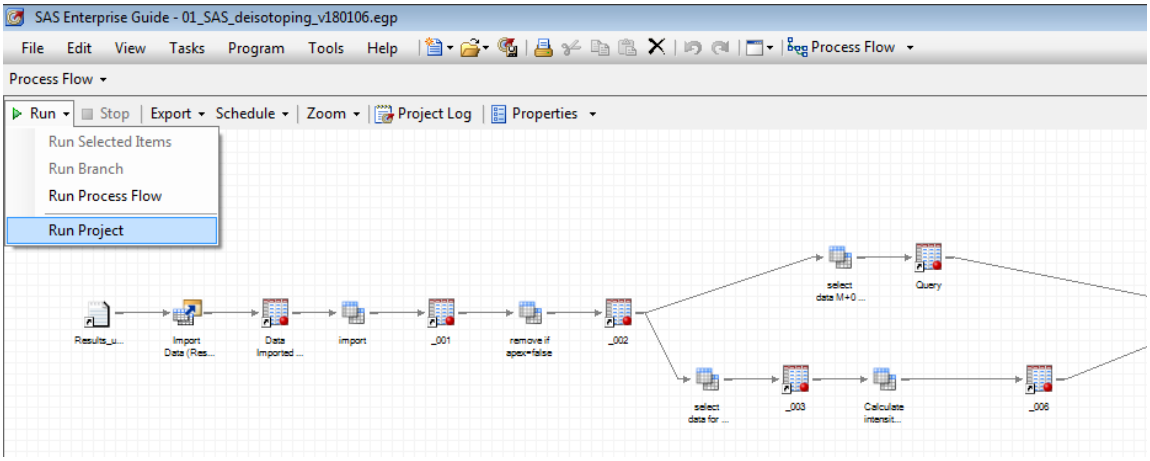


Figure 21. File management structure for deisotoping of FTMS[1] data using the SAS Enterprise Guide-based script: "01_SAS_ms1_deisotoping.egp".

9.6.1. Run script

- To run the script for deisotoping FTMS[1] data the user need to do the following:
  - Go to folder: "..\06_SAS_ms1_deisotoping".
  - Execute (double click on) the file: "01_SAS_ms1_deisotoping.egp"

  → This will open SAS® Enterprise Guide and show the data processing pipeline:



  - Click on "Run" and subsequently "Run Project"

  → This will run the script and includes

    - reading the result file with FTMS[1] data: "Results_unified.tab" located in folder "..\ 03_ALEX123_ms1_data_search".
    - writing a result file deisotoped FTMS[1] data: "02_SASoutput_ms1_data_w_deisotoping.tab" located in folder "..\06_SAS_ms1_deisotoping".

- The output file with deisotoped FTMS[1] data serves as an input file in the subsequent data processing step (Step 6: high confidence lipid identification).

9.7. Step 6: High confidence lipid identification

For high confidence lipid identification the ALEX[123] framework uses information from searches of positive and negative ITMS[2] data, and the deisotoped FTMS[1] data (generated in Step 5). The output is a list with identified 'molecular lipid species' (annotated with identified hydrocarbon-based chains, e.g. PC 16:0-18:1) and 'lipid species' (annotated with sum of C atoms, double bonds and OH groups in hydrocarbon-based chains, e.g. PC 34:1). The identification routine is executed using a SAS® Enterprise Guide-based script.

The downloaded ALEX[123] framework comes with a pre-configured script for doing the lipid identification (using SAS® Enterprise Guide). This script is located inside the folder:

"..\07_SAS_msn_lipid_identification" (Figure 22),

and is named:

"01_SAS_lipid_identification.egp"

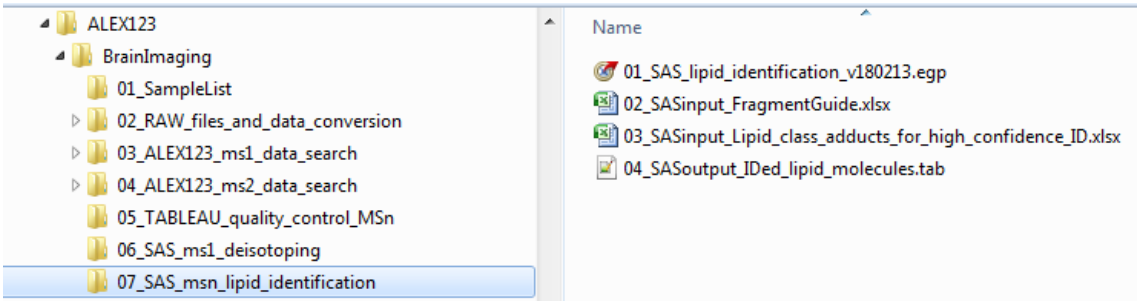


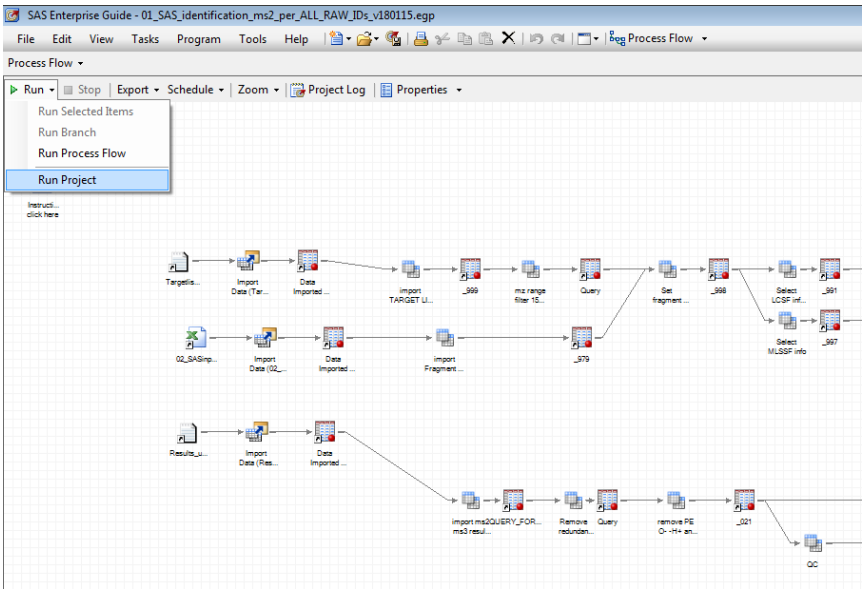
Figure 22. File management structure for high confidence lipid identification using the SAS Enterprise Guide-based script: "01_SAS_lipid_identification.egp".

9.7.1. Run script

- To run the script for high confidence lipid identification the user need to do the following:

  - Go to folder: "..\07_SAS_msn_lipid_identification". (Figure 22)
  - Execute (double click on) the file: "01_SAS_lipid_identification.egp".

  → This will open SAS® Enterprise Guide and show the data processing pipeline:



  - Click on "Run" and subsequently "Run Project"

  → This will run the script and includes

    - reading the result file with ITMS2 data: "Results_unified.tab" located in folder "..\04_ALEX123_ms2_data_search".
    - reading the file: "02_SASinput_FragmentGuide.xlsx" located in folder "..\07_SAS_msn_lipid_identification". This file assists the lipid identification routine.
    - reading the file: "03_SASinput_Lipid_class_adducts_for_high_confidence_ID.xlsx" located in folder "..\07_SAS_msn_lipid_identification". This file specifies adduct ions that must be detected by $FTMS^1$ analysis for verifying MS^2-based lipid identifications.

- writes a result file with identified lipid species and relevant metadata that supports their identification: “04_SASoutput_IDed_lipid_molecules.tab” located in folder “..\07_SAS_msn_lipid_identification”.

- The information on the output file (“04_SASoutput_identified_lipids.tab”) can be easily viewed using Microsoft® Excel.