MASS SPECTROMETRY METABOLITE LIBRARY OF STANDARDS FREQUENTLY ASKED QUESTIONS (FAQ)

Uses for MSMLS

Q - What is the purpose of MSMLS?

A - MSMLS provides over 600 small metabolite compounds in an easy to use format with enough material for multiple injections for the purpose of building a physical mass spectral library using the conditions that are normally employed in the user laboratory. While many mass spectral libraries are available, and can help identify compounds within a dataset, there is no substitute for generating your own library using your own parameters.

The MSMLS compounds largely represent primary metabolism and ideally suited to build libraries used to identify compounds for metabolic profiling.

The MSMLSDiscovery software provided with the kit facilitates "library building". As each injection sample contains a known set of compounds, the software automatically processes the data for each sample and provides the extracted ion-chromatogram for the expected mass, and for each peak found collects any fragmentation data, centroids the peak and collects all adducts. The software then presents what it finds for a curation step. The curation of the complete library or "dictionary" can be completed in less than a day.

MSMLSDiscovery also provides a "sample analysis" feature that will run a targeted analysis on a centroided data file to find any compounds in the dictionary/library created during "library building".

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Compounds

Q - Are the standards in the MSMLS isotopically labeled according to the IROA technology?

A - No, all the compounds are unlabeled (natural abundance).

Q - Is the amount of material in each well exactly 5µg?

A - 5 µL of an accurately weighed 1 mg/ml solution was placed into the well and dried down. The standard deviation of the scale used to weigh the compounds is +/- 0.1 mg

Q - Why are there duplicated compounds in the library?

A - Compounds are intentionally duplicated for QC purposes which is why the kit is defined as having over 600 "unique compounds". Many compounds placed in the well have different D/L forms, chirality or salts and have different CAS numbers.

Descriptors

Q - What is the Parent CID/ CSID?

A - This usually refers to the KEGG ID (Kyoto Encyclopedia and Genomes) number, where available.

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Q - What does "Charge" of the compound refer to?

A - All MSMLS compounds are in the neutral form except very few that do not exist as neutral (like choline). This way it is much easier to handle the adduct mass calculation. The program assumes the protonated form exists in positive mode. It does not require that it be the dominant ion. It knows if the compound is a quaternary or naturally charged species.

Solubilization

Q - What is the recommended procedure to solubilize the compounds?

A - We recommend adding methanol to begin solubilization and then diluting as required with water.   We suggest adding 5 uL of MeOH, vortex a couple minutes, then added 95 uL of [95/5 water/MeOH] to get to 50 ug/mL of each metabolite in each well. Then multiplexed the samples in each row and inject 5 uL to the LC/MS for both Pos and Neg acquisitions. Good results should be achieved in each mode.

Water is necessary to ensure solubilization of the more polar compounds. For plate 7 we recommend using 1: 1: 0.3 chloroform: methanol: water as these compounds are more lipid-like. If you are not comfortable injecting the multiplexed sample of CHCl3/MeOH on a reverse phase system without adding any aqueous start with about 15-20 uL of ACN/IPA or ACN/EtOH, vortex 5 min, then add 15-20 uL of aqueous starting mobile phase, and final vortex 5 min before injections. If the mobile phase has acid in it then just use the mobile phase minus the acid.

Q - How much volume can I add to each 96 plate well?

A - Each well can hold a volume of 1.2 mL but be careful not to dilute the samples too much otherwise you will get a poor signal.

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Chromatography

Q - What is the best chromatography to use with MSMLS?

A - There is no "best chromatography or ionization method" and this is dependent upon the instrumentation available, i.e. there is no single recommended Mass Spec model and/or protocol. The library is designed to help you tailor your own system and support your ability to build a library using authentic compounds under your source and chromatographic conditions.

Many MSMLS users prefer a basic C-18 column run in both positive and negative mode. A HILIC or Diamond-hydride is often employed to further separate the very water-soluble compounds. This increases the number of injections and the time per analysis.

The University of Michigan and University of Florida NIH Metabolomics facilities have both deposited copies of all of their SOPs, including chromatographic and instrumental with the Metabolomics Workbench (https://www.metabolomicsworkbench.org/). We recommend that you look there and see what works for you. Many other labs also donate their SOPs to the workbench.

Stability and Storage Conditions

Q - What is the stability and best storage conditions for the MSMLS compounds?

A - The shelf life of the kit is 2 years. Once diluted the plates should be resealed and kept at -20°C or -80°C for long-term storage, protected from moisture and light. Avoid repeated freeze/thaw cycles.

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Q - What are the 96-well plates that were used to generate MSMLS.

A - Greiner MASTERBLOCK® #780215, polypropylene deep-well (total volume per well = 1. 2mL) in combination with seals, Greiner VIEWseal #676070.

Mass Spec Analysis

Q - Is there any limit to the number of LC-MS methods you can use with the Discovery software?

A – No, the only limitation is the memory of the computer that is employed.

Q- In our lab we perform MS1 level and/or MS2 level injections. Are there certain methods that must be used, or does the software identify the compounds regardless of the method?

A-Normal DIA works just fine because these are generally the biggest peaks in the chromatogram, and it knows the appropriate masses to look for in ms1 and associated ms2 scans. Data-independent acquisition is whereby all ions from a first stage of mass spectrometry (MS1) are formed in an ion source and separated by *m/z (mass-to-charge.* Then within a selected *m/z* range, ions are fragmented and analyzed in a second stage of mass spectrometry (MS2). (Data-dependent acquisition is whereby a fixed number of precursor ions are selected).

The files should be converted to mzXML files (these are smaller and more efficient than mzML and contain all the same information). When you examine the file structure in Discovery after you load the file you will see that the scans alternate between a single ms1 scan followed by several ms2 level scans. Discovery knows the exact mass of each compound and will predict at least one ion that should be

seen for each compound. It then runs an extracted ion search for this ion and examines any chromatographic peaks found. For each peak, it will examine the average of the three apical scans for any known fragments, adducts, or polymeric peaks. It will determine when the peak starts and when it finishes. Within this time period it sill find all fragmentation scans for the relevant peaks. It will put all of what it finds and presents it to you for approval. It almost always gets it right, so you can scan through them quickly. In general, you need at most the top two peaks found in the extracted chromatographic peaks. (This is to be safe, the top one is almost always the correct correct.)

Q - If I switch polarity (alternating polarity) when analyzing my samples, can the MSMLSDiscovery software still be used?

A – The current MSMLSDiscovery version does not support mixed polarity data files.

Q - Can I multiplex every row in every plate?

A - Occasionally the map plate will change due to the availability of compounds. Although we try to make sure that the compounds of each row have distinct molecular weights and can be multiplexed, users should refer to the plate map before proceeding.

Publications

Q - Are there any publications that reference MSMLS?

A - *MetaboNews* featured and article that described the MSMLSDiscovery. A User Guide and video are provided with the kit. We expect to release an Application Note soon.

MSMLSDiscovery

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Q – We are interested in your mass spec library, but have a few questions. We have an Agilent 6550 and would like to expand our library. We are currently developing both negative and positive mode, HILIC and reverse phase methods. I understand that it comes in 96 well plate format and can be multiplexed. I was wondering how the software interprets the data?

A - The software knows which masses to look for and finds them. The analysis is performed on what it finds.

Q - What operating system should I use and how much memory does the MSMLSDiscovery program require?

A - Microsoft Windows 7 (64-Bit) or higher with a minimum of 8 GB memory, for improved data processing 16 GB memory is preferred. The latest version of Java should be callable.

Q - What should I do if I get a message "unable to load" when I try to download the MSMLS software?

A - This generally means that a corporate firewall is not permitting any downloads to be run. The install may need to be done by the IT department who will download and test the software.

Q - I am having issues with Cluster Finder... it is not accepting my email address at the first use of the newly installed version. Do you know what should I do?

A - The program may have been installed it to a place where you do not have write permission (If installed to Program files Microsoft will not allow write to the database). Please uninstall and install latest version to a place on your computer where Microsoft will not restrict using it.

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Q - I was able to download the installation file and install the program. However, when I try to run the program, the green MSMLS Discover screen loads for a moment then disappears, and the program does not actually start. I tried restarting the computer and also uninstalling and reinstalling the program, neither helped. Do you know what the problem may be?

A – If you are using another language other than English, you may need to change the system language setting to English (see image below).
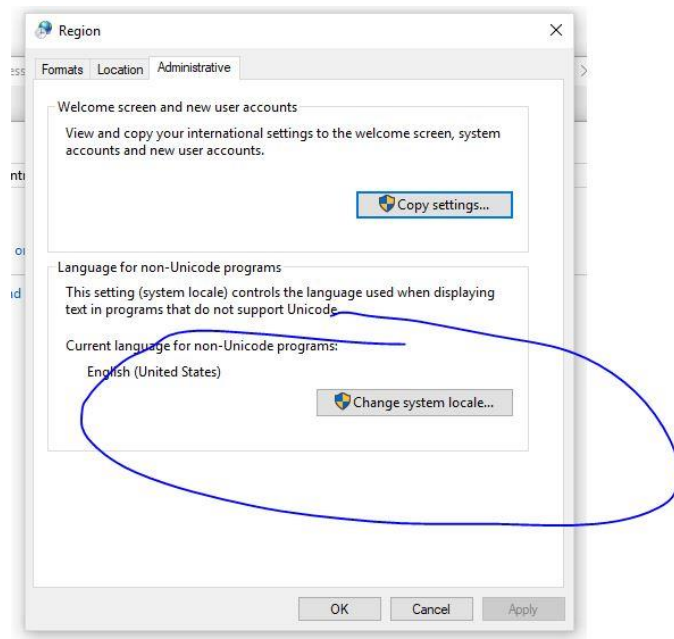
Q - What does it mean if I get the message "GC overhead memory limit exceeded"?

A -   Java has a Garbage Collection (GC) process that tries to free up memory. This message is telling you that you are out of virtual memory. On a 64-BIT operating system you can manually change "HEAP_SIZE=8" TO "HEAP_SIZE=32".

MASS SPECTROMETRY METABOLITE LIBRARY OF STANDARDS FREQUENTLY ASKED QUESTIONS (FAQ)

Q – What if I cannot access the task manager or if it does not allow me to start a new project?

A- This issue may be a result of incompatibility between the windows operating systems and Java. Java apparently transmits a "space" in a name such as "John Smith" as a "%20" character that the underlying operating system no longer interprets.

Please uninstall any Discovery copies on your C drive and install to a place where there will be no spaces in the path to the libraries. Note: the new copy will open old projects so there is no need to make new ones.

If the above solution does not work, take a screen shot of the message screen and send it to info@iroatech.com for further instructions.

Q - What data format is required to upload into MSMLSDiscovery?

A - Convert datafiles to centroided mzXML. Most vendor files can be processed using ProteoWizard msconvert, an open-sourced, cross-platform general data format conversion tool. The software is available from http://proteowizard.sourceforge.net/ and needs to be run under Microsoft Windows. (In msconvert, use the peakpicking option which is the centroid mode and select prefer vendor algorithm). A helpful article is Curr Protoc Bioinformatics. 2014; 46: 13.24.1–13.24.9.

Q - What if I get an error message as shown in the picture below?

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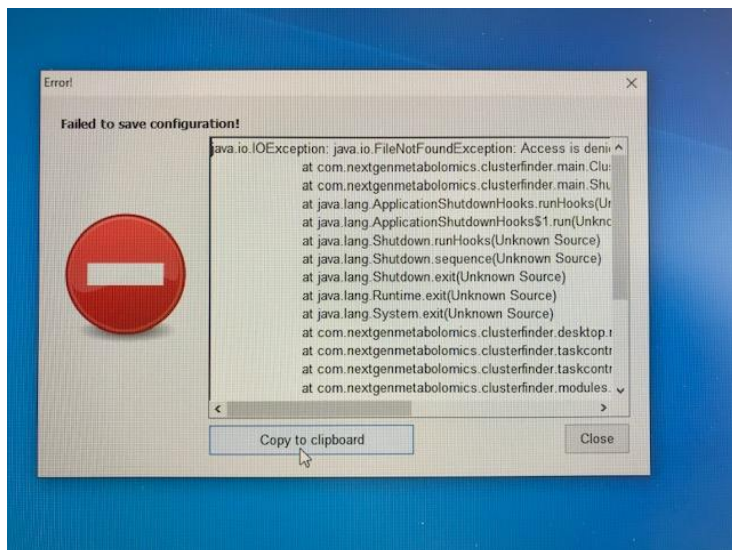
A - Uninstall the MLS program and reinstall latest version.

Q - I have exported the raw data files (from Sigma MSMLS plates) using Agilent Masshunter (exported as "mzdata" files), and successfully exported them into the MSMLS software. I am able to process the MS1 data files successfully – I am able to see which compounds have been successfully identified.

The MS2 files – acquired by autoMSMS in MassHunter (I have specified an MS2 method 'depth' for these files in the software) also show compound results, but no MS2 data is not shown for any of the compound results for these files.

MS2 appears to have been successful when looking at the raw data files in MassHunter, and I can see the MS2 spectra for the compounds when I extract them individually.

A - We suggest using ProteoWizard MSconvert to translate .D to mzML or mzXML and make sure that:

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1) data are centroided using vendor's algorithm

2) both MS1 and MS2 are translated

Q – How do you convert SCIEX wiff files?

A - Install the latest version of MSconvert and use the below wiff conversion settings, the data should now be compatible with MSMLSDiscovery.
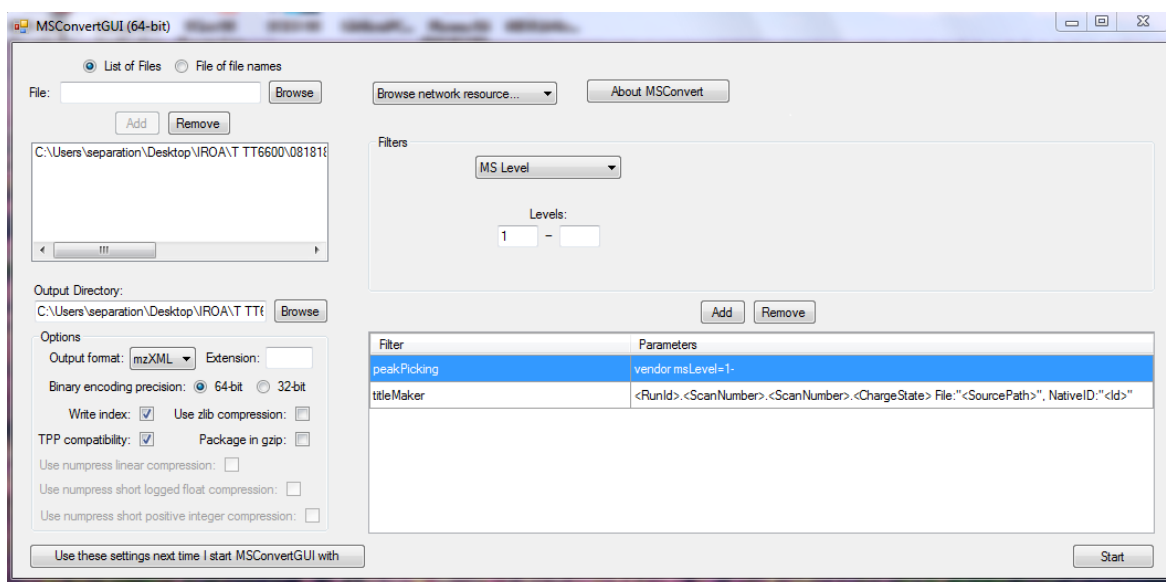
Q - What is MS depth?

A - MS depth is the number of mass spec scan levels to discover. The program supports two: 1) full scan, and 2) MS/MS.

Q - Why isn't the program picking up what looks to be a small fragment?

A - Most likely the intensity is too small and Discovery cannot find the second isotope. The program must find at least two isotopes to consider the feature real.

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Q – Does the program provide parent ion information and suggestions for possible product ion mass for MRM transition for each metabolite?

A - The Discovery software will report all parent ion, fragment and adduct information for each compound.

Preferences

Q - Is it possible to export the peak intensities and the area of the peaks?

A - When building the libraries, compounds definition is purely descriptive and has no quantitative aspects, thus height or area have no meaning (except in the ms/ms the user can set the percent of baseline that defines which fragments get written out). When using Discovery for sample analysis, the user can select the export type, either height or area (under Preferences: Data export).

Q - What does the setting "Fragment Lookup Cutoff" refer to?

A - "Fragment Lookup Cutoff" this is defined as the % intensity of the major adduct; do not set too low as you could end up with a lot of small peaks; suggest setting at between 10-25% and then adjust empirically. It should not be set too low as you could end up with a lot of small peaks; suggest setting at 25% and then adjust empirically.

Q- What is the signal limit for the software to find the peak?

A- It is user selectable and can be set in program preferences.

Building Libraries

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Q -I'm working on building out a library from our standards using the discovery software. This is only for MS data.

a) Which file type did you say to save this in so that I am able to load it into ClusterFinder?

A- Export the library in cef file format.

b) How do we stitch multiple libraries together?

 A- In the Discovery User Manual, see page 19 and click on the "merge existing libraries" icon button.

c) How are these loaded into ClusterFinder.

 A- In CF they are loaded using the "import library from file" icon button. Once the library is identified it will make a local copy in the project so that the next time the project is opened, it will know where to locate the library(ies).

Library Output

Q – I have an experimental fragmentation spectrum represented by Progenesis (which I believe is a combined/average MSMS) matched to an independent/external reference spectrum. There are several peaks in common and the match score is decent. I also have the same raw file extracted by MSMLS Discovery and the MSMS does not include any of these matching peaks, so it seems like some valuable data is being lost in the MSMS representation. Any ideas?

A - It is possible to look at the actual individual MSMS scans in Discovery and see if any of them (within the MS peak of interest) have the right fragments matching the library.

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Q – For the MS/MS shown in display window on lower right (and for intensities exported to library file), are only single scans represented? Or is the MS/MS an average of scans if there are multiple?

A - MSMS is a single scan. The way auto-msms is set up (and this is what people usually use) is one MS scan followed by several MSMS scans with different precursor ion. There is a chance that you may have your precursor of interest fragmented more than once but I limit MSMS to the single scan where precursor intensity is the highest.

Q – Is there a way to order the compound features in the data analysis tab by plate row? I uploaded 8 samples in the multiplex design (and am trying to curate row by row, adding comments to my MSMLS map that you sent awhile back) but the order of compounds seems random and I am having to bounce back and forth relative to the map to search for each compound to curate.

A - As for the sorting of the features, there is a button `on the data analysis panel toolbar (second from the right side) which toggles feature grouping between "by type" and "by file".   Activate "by file" grouping and every file includes the compounds from a single row by design.