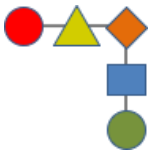
TruQuant Yeast Extract Semi-targeted QC Workflow Kit



Measurement and Normali*zation System for semi-targeted MS analysis*

Identification*, *quantitation*, *suppression-correction*, *normalization* and *Platform QC

of metabolites across time and instruments

Kit contents

Materials and tools for the analysis of 90 experimental samples

Unique fully-labeled *S. Cerevisiae* Yeast Extract*

- 3 vials of lyophilized IROA-IS (Internal Standard); U-^{13}C, 95%
- 3 vials of lyophilized IROA-LTRS (Long-Term Reference Standard); paired U-^{13}C, 95% and 5%; mixed 1:1
- ClusterFinder™ software (including library of IROA peaks in the LTRS and their physical characteristics)
- User manual

Storage: -80ºC, protected from light

Summary of Benefits of the TruQuant Measurement System

1. **Daily instrument QA/QC.**
2. **No false data. Reports only compounds of biological origin; excludes artifactual peaks.**
3. **Accurate compound formula ID for MS alone; complete ID with addition of SWATH, or IM.**
4. **Fragments** have the IROA ratio pattern derived from their parent peaks and can be **identified** using the **"peak correlation"** ClusterFinder module.
5. **Semi-targeted.** Software searches for the highly characterized 1000+ known IROA LTRS peaks and also will search for unlabeled compound peaks using user generated natural abundance libraries.
6. **Suppression-corrected measurements for significantly better quantitation.**
7. **Reproducibility. Sample normalization to a universal standard for complete comparability.**
8. **ClusterFinder software solution** builds libraries, IDs/quantitates compounds, corrects for ion-suppression and normalizes data.
9. **Economical. $200 for 90 samples.**

*Prepared from proprietary U-5%/U-95% ^{13}C IROA-labeled glucoses specially produced for IROA Technologies by Cambridge Isotope Laboratories, Inc.

Standards included in the Kit

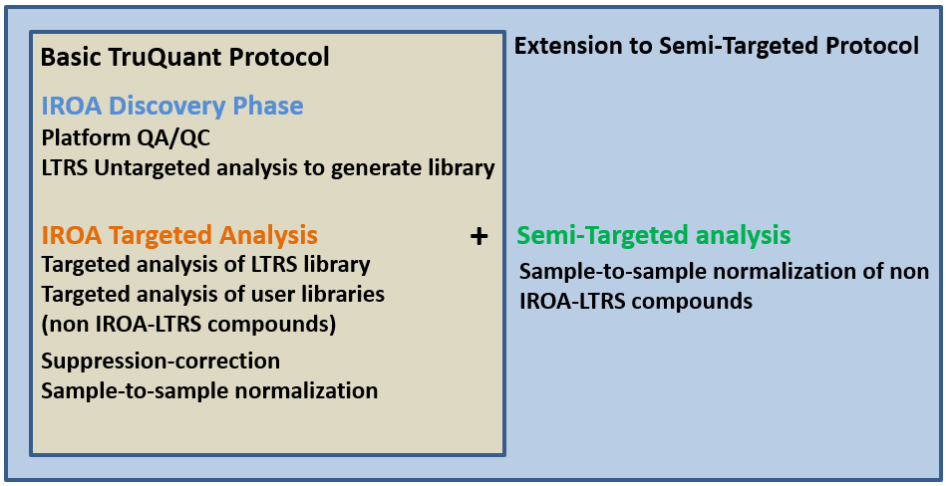
IROA Long-Term Reference Standard – IROA-LTRS (complex mixture of fully labeled 5% and 95% U-^{13}C metabolites, mixed 1:1) is used to build a daily, verified-identity "dictionary" of Retention Time (RT), *m/z* and physical characteristics for all IROA peaks. Identity of isobaric compounds is verified using fragmentation and/or Collisional Cross Section (CCS) data (ClusterFinder version 4.3). Provides complete daily QA/QC on instrument performance. IROA-LTRS data are collected randomly within every day's injection sequences.

IROA Internal Standard – IROA-IS (equivalent to the 95% U-^{13}C component of IROA-LTRS) is added to experimental samples to identify and quantitate metabolites using the co-incidentally-run LTRS "dictionary". The chemical makeup and chromatographic behavior of the IROA-LTRS is identical to that of the IROA-IS; so the IROA-LTRS "dictionary" is completely applicable to the IROA-IS. Isobaric compounds distinguished in the IROA-LTRS based on fragmentation or CCS are distinguished in the IROA-IS by reference to the IROA-LTRS and these same IROA peaks will be found in the experimental samples through the use of the IROA-IS. Even if analyzed on different chromatographic systems, results can be equated using the dictionary because of comparability of the IROA-LTRS and IROA-IS, by querying the same mass and secondary physical characteristics across systems.

Why 5% and 95% U-^{13}C

- Mathematically calculable and readily identified by the IROA ClusterFinder software.
- Unique IROA patterns discriminate peaks of biological origin from artifactual peaks allowing the removal of false data.
- Fragments have the IROA ratio pattern derived from their parent peaks and can be identified using the "peak correlation" ClusterFinder module.

We are pleased to extend the TruQuant Protocol to a Semi-Targeted Protocol



- **In the Discovery phase, the LTRS is analyzed using msms untargeted analysis. The characteristics of parent, fragment and adduct ions are collected in a library format.**
- **The LTRS library is used to identify experimental samples spiked with the same compounds (IROA-IS) in a Targeted analysis to achieve a non-sparse data set.**
- **ClusterFinder uses both the LTRS library plus other natural abundance user libraries of choice to perform targeted searches in the experimental samples.**

3 STEP PROCESS:

1. LC-MS analysis of experimental samples resolvated with IROA-IS (experimental sample + IROA IS = analytical sample) are randomly analyzed in quantitative mode (without msms).

   Within the random analytical injections the IROA-LTRS is injected approx. once for every 10 analytical samples. The LTRS is analyzed in qualitative mode using msms untargeted analysis to collect characteristics of parent, adduct and fragment ions which are collected in a library format. The LTRS is also used as a QA/QC Standard to account for fluctuations in mass and RT drift.

2. Generate "dictionary" of RT, m/z, formula and physical characteristics from the analysis of IROA-LTRS using ClusterFinder software to validly identify all compounds in the IROA-LTRS.

3. Use ClusterFinder and the LTRS dictionary to identify experimental samples spiked with the same compounds (IROA-IS) in a Targeted analysis to achieve a non-sparse data set and quantitate, suppression-correct and normalize all compounds in original experimental samples. ClusterFinder also identifies and quantitates all user defined additional metabolites of interest in a Targeted search. These metabolites can be normalized but cannot be suppression-corrected.

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The IROA Peaks and Envelopes

IROA is used to create distinct signatures in the molecules of a biological sample for identification and quantitation.

The key to understanding the IROA methodology is that we create both ^{12}C and ^{13}C isotopes to be uniformly present at approximately 5% for one isotope and approximately 95% for the second isotope. The molecules labeled at 5% ^{13}C have a strongly enhanced M+1 and the molecules labeled at 95% ^{13}C a strongly enhanced M-1, creating a mirror-image of one another (see Figure 1). Using traditional comprehensive (>98%) labeling, the monoisotopic peak of most compounds can usually be detected even if its intensity is low, but the M+1/M-1 minor peaks can be easily lost. Where the ^{13}C is increased to 5% or 95%, the M+1 and M-1 peaks for a six-carbon molecule such as arginine in Figure 1 become significantly larger, namely 32% of the height of the monoisotopic peak. Whereas if the ^{13}C is present at only 1.1%, the height of the M+1 is only approximately 6% of the height of monoisotopic peak.

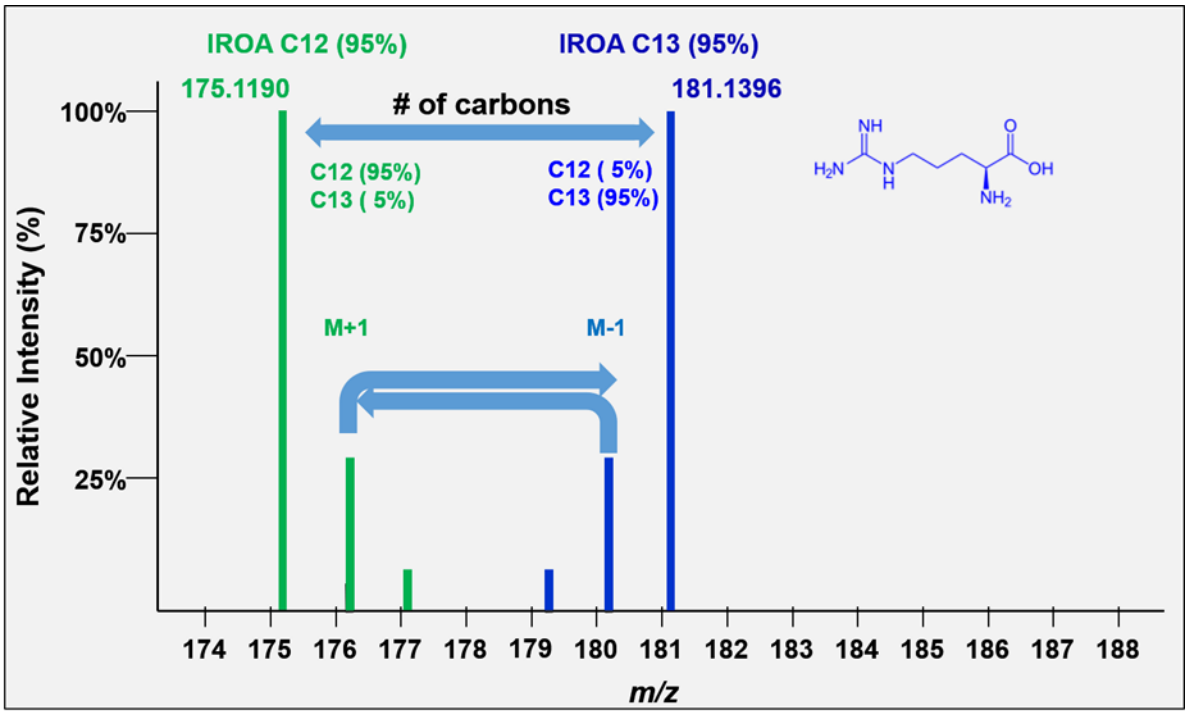
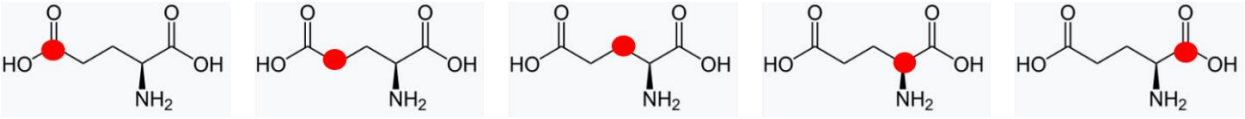


Figure 1. The IROA peaks. Molecule shown is the 6-carbon molecule arginine.
Green: Arginine C12 envelope peaks labeled with U-5% 13C.
Blue: Arginine C13 envelope peaks labeled with U-95% 13C.

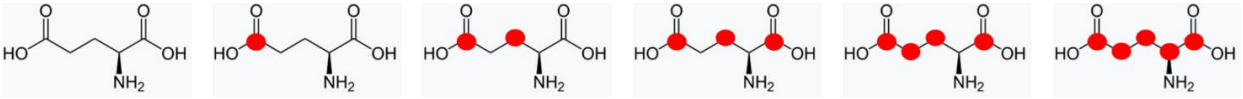
A short tutorial on isotopomers and Isotopologs

- **Isotopomers** are molecules with the same number of isotopes (regardless of position). There are 5 isotopomers of glutamic acid ($C_5H_9NO_4$) containing one ^{13}C.



Therefore, all isotopomers have the same mass and will appear in a single Mass Spectral peak.

- **Isotopologs** are molecules with the different numbers of isotopes. There are at least 6 isotopologs of glutamic acid, and aside from the first and last (the two monoisotopic isotopologs), they are not positionally defined.



Therefore, all isotopologs have different masses and will appear in multiple Mass Spectral peaks. Furthermore, most isotopolog peaks likely contain multiple isotopomers. Every chemical formula has N+1 carbon isotopologs where N is the number of carbons in its formula, beginning with its C12 monoisotopic and ending with its C13 monoisotopic.

The isotopolog ladder for all isomers of glutamic acid is shown in the Figure 2 below.

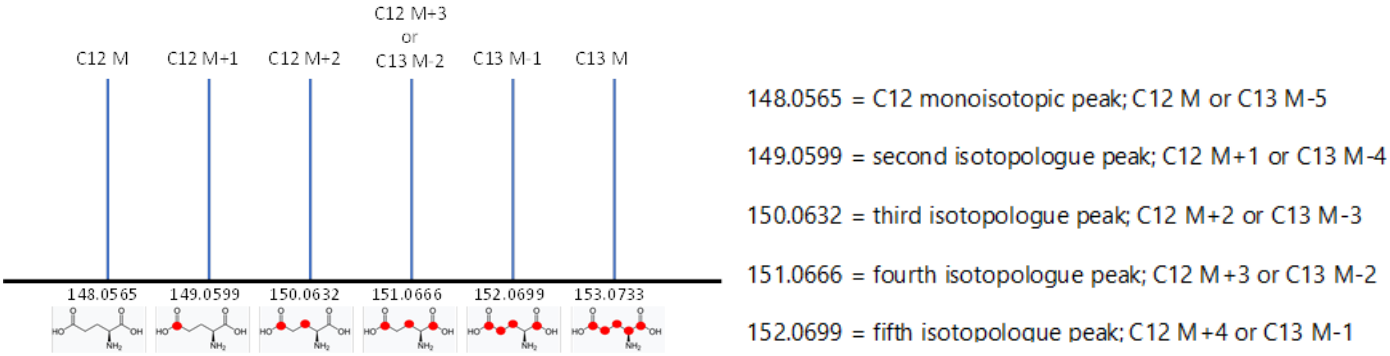


Figure 2. The Isotopomeric ladder for Glutamic acid (C11) as seen in RP pos LC-MS. Each isotopolog represents a specific mass by a molecular formula. In the case of carbon isotopologs, these are seen as a collected ladder of peaks extending from the C12 monoisotopic peak to the C13 monoisotopic peaks that differ in mass by 1.00335 amu. All molecules with six carbons will share this ladder but because each formula has a different mass each will begin and end at different masses. Therefore, each isotopolog ladder is unique to and representative of a single formula. (This is true for masses below 800, at a minimum.)

1) The isotopolog ladders for any formula are unique to that formula, but are common to all its isomers, e.g., Stereo, D/L, Structural, etc. However, the shape of the isotopolog peaks is defined by the relative percentages of the isotopes.

2) The Isotopomeric ladder for Glutamic acid is shared by all molecules that have the same formula (C5H9NO4), including Glutamic acid, O-Actetyl serine, Threo-3-methyl aspartate, and N-Carboxymethyl alanine.

3) It is the job of chromatography (LC, GC, etc.) to separate these, because techniques like Ion Mobility will not optimally do so.

Isotopolog patterns are isotopic envelopes

Figure 3 shows the isotopolog ladder for tryptophan, which contains an equal concentration of "molecules" (1:1) for the right and left side set of peaks; natural abundance on the left, 95% ^{13}C on the right.

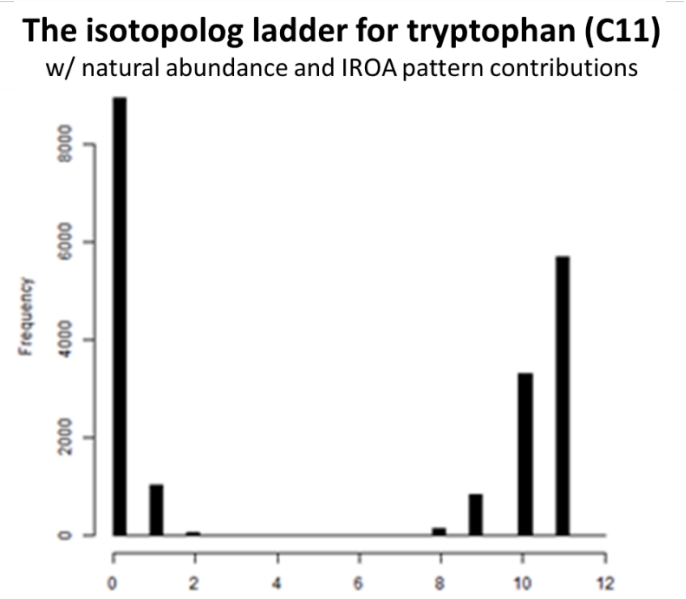


Figure 3. The Isotopomeric ladder for Tryptophan (C11).

The relative heights of each isotopolog peak in a ladder is determined by the isotopic balances of the source materials. The height of the peaks of isotopically defined compounds (enriched in a single element such as carbon) may be effectively calculated by the binomial expansion[1] of the expression ($^{12}C\%$ + $^{13}C\%)^N$ where N equals the number of carbons, and $^{12}C\%$ and $^{13}C\%$ equals the relative isotopic abundance.

In this image we see a situation common in IROA, namely a ladder that has the isotopic signatures contributed from two sources; the first source on the left is the natural abundance compound, and the second source labeled at 95% ^{13}C on the right is its internal standard. These are presented at exactly equal concentrations of molecules from both sources however they are distributed very differently.

[1] This is technically a polynomial expansion in which the dominance of carbon makes the remaining terms less important. See "Addressing the current bottlenecks of metabolomics: Isotopic Ratio Outlier Analysis, an Isotopic labeling technique for accurate biochemical profiling" page 7.

- Note that the height of the base peak is never indicative of concentration; rather **the sum of all peaks from each collection must be considered**.
- In the case of tryptophan, the base peak is still the C13 monoisotopic peak and represents only about half molecules in the internal standard.
- The base peak for an IROA compound with more than 20 carbons will no longer be the C13 monoisotopic peak. Instead, depending on the number of carbons, it will become one of the lower mass isotopologs. This is because as the number of atoms in a molecule increases, the probability that the entire molecule contains at least one heavy isotope also increases.

For this reason we need to consider another new concept, the **isotopic envelope**.

Isotopic Envelopes

All molecules with the same number of carbons will show the same pattern of peaks but will differ in the mass of their monoisotopic peaks according to the remainder of the formula. We refer to these peak height patterns as the peak "*isotopic envelopes*". These envelopes are diagnostic for each formula.

The IROA carbon envelope shapes are readily and exactly calculable. The defining feature of the IROA carbon envelope is the mass of both monoisotopic peaks and the mass difference between them. The mass difference between the monoisotopic peaks is always a multiple of the mass of a neutron (~1.00335 amu). The additional peaks discussed above contribute to the extended isotopic envelope (M+2, M+3 etc., M-2, M-3 etc., see Figure 4) and the IROA ClusterFinder software can easily identify these peaks by their mass difference (the mass difference between a ^{13}C and ^{12}C isotope).

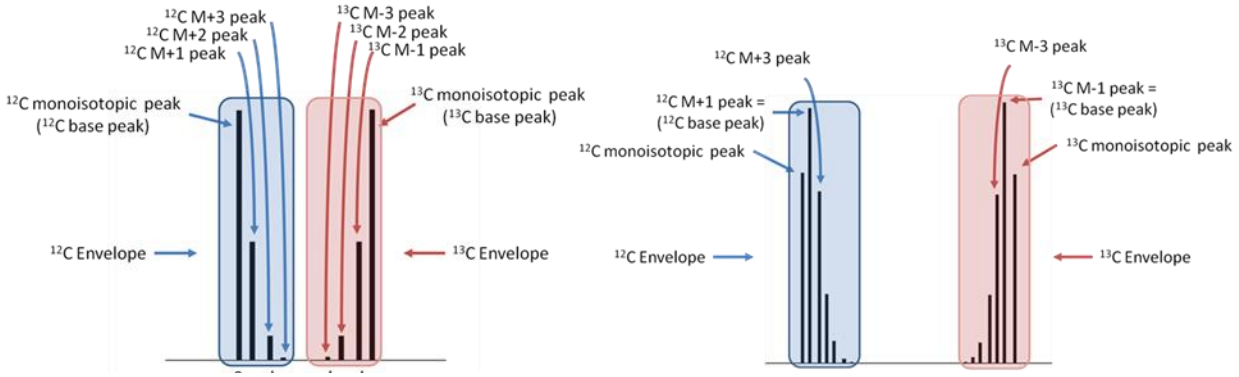


Figure 4. IROA isotopic envelopes (clusters) illustrating parts for a 9 carbon and 27 carbon molecule labeled with 5%13C:95%13C. Note the base peak shift. The monoisotopic peak for the 27-carbon molecule (right side of figure) is not the most abundant peak in the envelope.

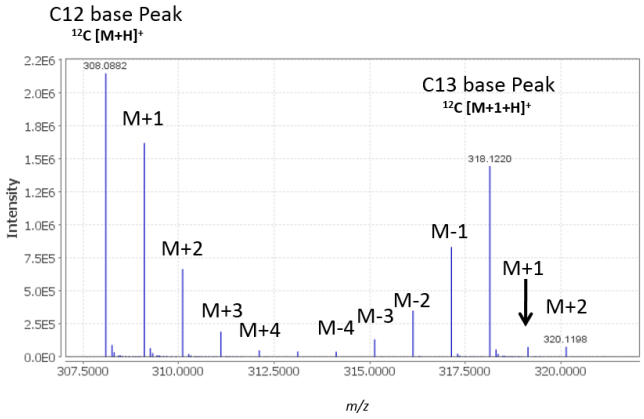
- Isomers, isotopomers, and isotopolog patterns become complex very quickly. No two isotopologs are the same, and any isotopolog will generally contain more than one isotopomer and each contains different collections of isotopomers. We find this exact language to be useful however cumbersome.
- For IROA, we generally use two different isotopic distributions creating patterns that represent different isotopic balances, for example 1.1%13C:95%13C or 5%13C:95%13C. One of these, usually based on the C13 monoisotopic peak, will be the internal standard.

- For each formula all these isotopic patterns must use the same isotopolog ladders, but they will fill them differently.
- We believe it is easier to combine the embodiments of the isotopomers and isotopologues under the rubric of an "isotopic envelope".
- There are two isotopic envelopes in any IROA sample.

Carbon is the only element that has an exact unit mass. This is because carbon is defined to be exactly 12.000000 amu. One outcome of this is that the exact unit masses of all other elements have slightly different "defects", i.e., the fractional number beyond the decimal point. The mass "defect" is the difference between nominal mass (mass of the most abundant elemental isotope; for a molecule, the sum of the nominal masses of the constituent elements; i.e. H_20=18) and the monoisotopic mass (exact mass) of an atom or molecule. These defects can only be added together a certain number of ways to get a specific mass as seen by a mass spectrometer.

Figure 5 shows an example of using mass defect to determine molecular formula. Using IROA we can calculate residual mass of all the other elements using both C12 and C13 base peaks. Since both the C12 and C13 base peaks share the same formula, the error on this residual value can be minimized by averaging the two residual masses.

With reasonably high-resolution mass MS (mass accuracy of 50 ppm or grater) and the knowledge of the number of carbons in a molecule (the distance between the C12 and C13 monoisotopic peaks), generally only one formula exists for molecules with masses below 500 amu. In the few cases where more than a single formula is possible, the use of mass defect residual tables can often resolve any ambiguity.



Glutathione Formula $C_{10}H_{17}N_3O_6S$

	C12	C13
Base Mass	308.0862	318.1220
Less mass of proton =	1.0078	1.0078
Less mass of carbon =	120.0000	130.0335
Residual mass =	187.0784	187.0807

Average residual = 187.0796

Only H17N3O6S corresponds to 187.0796

Figure 5. Calculation of average residual mass (mass defect) for glutathione.

Fundamental to the IROA concepts (and inherent in the name Isotopic Ratio Outlier Analysis) is the fact that the ratio of the C-12 envelope to the C-13 envelope is **unaffected by suppression** even though both the C-12 and C-13 isotopomeric sets may be strongly suppressed. This has afforded a mechanism for **suppression correction** that has been built into ClusterFinder. Once suppression is corrected, a Dual MSTUS[3] algorithm is employed to provide a very accurate mechanism for the **normalization of samples** against sample-to-sample variances. This version of ClusterFinder outputs three values: 1) the raw (suppressed) values observed; 2) a suppression-corrected value; and 3) a normalized (suppression-corrected and normalized) value.

Summary

- All **isotopomers** are isotopic isomers that share a single mass. There is no positional constraint.
- All **isotopologs** contain different numbers of isotopes but otherwise share a formula.
- In **IROA** there are always contributions from two different sources, usually an analyte and an internal standard. The peaks from both sources will superimpose onto the same ladder.
- An **isotopolog ladder** exists between the two monoisotopic peaks (from 2 different sources), and this ladder is diagnostic for the molecular formula that it is derived from.
- The collection of peaks donated by each source is grouped into a collection of isotopologs that is most easily identified as an **isotopic envelope**.
- **Accurate quantitation** requires summing all of the peaks in each envelope.

[3] Warrack BM, Hnatyshyn S, Ott KH, Reily MD, Sanders M, et al. (2009) "Normalization strategies for metabonomic analysis of urine samples.", J Chromatogr B Analyt Technol Biomed Life Sci 877: 547–552.

The IROA Standards

In the TruQuant Workflow two standards are employed: the **IROA-LTRS** and the **IROA-IS** which is added to the experimental samples (the combined IS/experimental sample is called the analytical sample). There is one IROA-LTRS injection for approximately every 10 analytical (experimental/IS) samples.

The IROA Long Term Reference Standard (IROA-LTRS)

The IROA-LTRS, a Long-Term Reference Standard, is a fully labeled *S. cerevisiae* yeast cell extract IROA separately labeled at both 5% and 95% U-^{13}C and mixed 1:1. The result is a collection of over five hundred metabolites* that exhibit unique labeling patterns as represented by the peak pairs of the molecule arginine in Figure 1.

The monoisotopic IROA peaks (often the base peak) and the associated "carbon envelope" isotopic peaks can be clearly detected during MS analysis. In all IROA peak pairs the selection of the correct monoisotopic peaks is always confirmed by either the M+1 (shown in Figure 1 as green peaks and containing approximately 5% universal and random ^{13}C; defining the C-12 envelope) or the M-1 (shown in Figure 1 as blue peaks and containing approximately 95% universal and random ^{13}C; defining the C-13 envelope).

The number of carbons in the molecule may be determined in three ways; by the mass difference between the C12 and C13 monoisotopic peaks, the height of the C13 M-1, and the height of the C12 M+1. In all IROA peak clusters or carbon envelopes these three values will be identical and therefore achieve triply redundancy with the added restriction that the peaks will exhibit symmetry (Figure 1). It is therefore very easy to computationally find all IROA peak pairs in the IROA LTRS and characterize them. For masses below 500 amu the isotopic mass plus the number of carbons uniquely identifies the molecular formula. Isobaric formulae are then distinguished using secondary MS scans collecting msms fragmentation, or Collisional Cross Section (CSS, with ClusterFinder Version 4.3); thus, all IROA-LTRS peaks have verified identities that correlate directly with their associated IS containing analytical samples.

The IROA-LTRS supports three major functions every day:

1) ***Library Building Standard*** – The IROA-LTRS (injected randomly within the analytical sample set) and IROA-IS (spiked into samples) have the same concentration of compounds and should have extremely reproducible RTs and peak characteristics across all samples.

The IROA-LTRS is used to build a triply redundant dictionary (library) of RT, *m/z* and physical characteristics including fragmentation data. All peaks are named according to the IROA-LTRS database. The adducts and fragments for each compound are indexed individually. These compound IDs are loaded into ClusterFinder's internal databases as a separate editable IROA-LTRS database.

*IROA-LTRS contains a broad spectrum of metabolites including amino acids, organic acids, peptides, vitamins, carbohydrates, and co-enzymes. Over four hundred metabolites have been characterized in the IROA-LTRS Standard using ClusterFinder software. The final number is expected to reach between 700-800 as different analytical approaches enable the identity of more compounds.

A library of IROA peaks in the LTRS and their physical characteristics is provided in the ClusterFinder™ software and distributed with the TruQuant kits. This library is distributed without retention time information; however, it is updateable by the user to assure that every compound in it will be reproducibly named according to the library.

2) **Validated Compound Identification** – The IROA-LTRS sample and ClusterFinder software and databases are used to create a daily RT library of all the compounds to be found in the IROA-IS so that their identification is reproducible and assured. The daily library is used as the basis of a targeted search of the IS in the analytical samples, and to quantify the natural abundance isotopologues of each experimental compound. For each compound, once the IROA-IS is found in any sample, a value, even <LOD, will be returned; therefore, the targeted search should yield a non-sparse dataset.

Since the chemical makeup and chromatographic behavior of the IROA-LTRS sample is identical to the IROA-IS, it is possible to use the in-depth and informationally strong, triply redundant chemical information obtained from the IROA-LTRS sample and apply it to identify compounds in the experimental sample. The dictionary catalog of all peak pairs, their RT, number of carbons, and IM and fragmentation characteristics provides information where each of these same IROA peaks will be found in the experimental samples through the use of the IROA-IS. The experimental natural abundance peaks are easily located and quantitated as they will co-locate with their corresponding IROA peaks at a mass that is the mass of the IROA 13C monoisotopic peak less the number of carbons it contains times the mass of a neutron.

All compound identities are validated in the IROA-LTRS where secondary physical characteristics such as fragmentation (ms/ms), ion mobility (ims/ms – CCS) may be collected to assure identity without lessening the quantitative aspects of the IS in the analytical sample.

3) ***QC Standard*** – As the name implies, the Long-Term References Standard (LTRS) is a Reference Standard and is always the same collection of compounds at the same concentrations. Although chemically complex it is well characterized so that on a daily basis its composition will provide insights into instrument performance: 1) The total of compounds seen is a measure of instrument sensitivity, 2) The retention times for familiar compounds are a measure of chromatographic performance, 3) The relative strength of many compounds and their known fragments is an important measure of in-source fragmentation, and 4) The total found signal for all IROA peaks is a measure of injection accuracy. The characteristics ClusterFinder sees and reports today should compare with what it saw yesterday; after all, it is a Long-Term Reference Standard.

The IROA-LTRS is a pure IROA mixture with unique isotopic signatures. Following LC-MS, the paired compound IROA peaks (U-95% ^{13}C or U-5% ^{13}C) can be readily identified by the IROA ClusterFinder software. The IROA peaks represent actual compounds, fragments and adducts and can be discriminated from **unsigned artifacts and noise which can be removed from the data, eliminating false discoveries**. As a composite sample, sample-to-sample and analytical variance is removed and during MS analysis the identical compounds (labeled with either U-95% ^{13}C or U-5% ^{13}C) experience the same ionization efficiency and suppression.

Over 1000 peaks can be detected in the IROA-LTRS (see Figure 6D). The resulting IROA-LTRS dictionary of compounds is used to identify compounds in the IROA-IS saving time, effort and related costs.

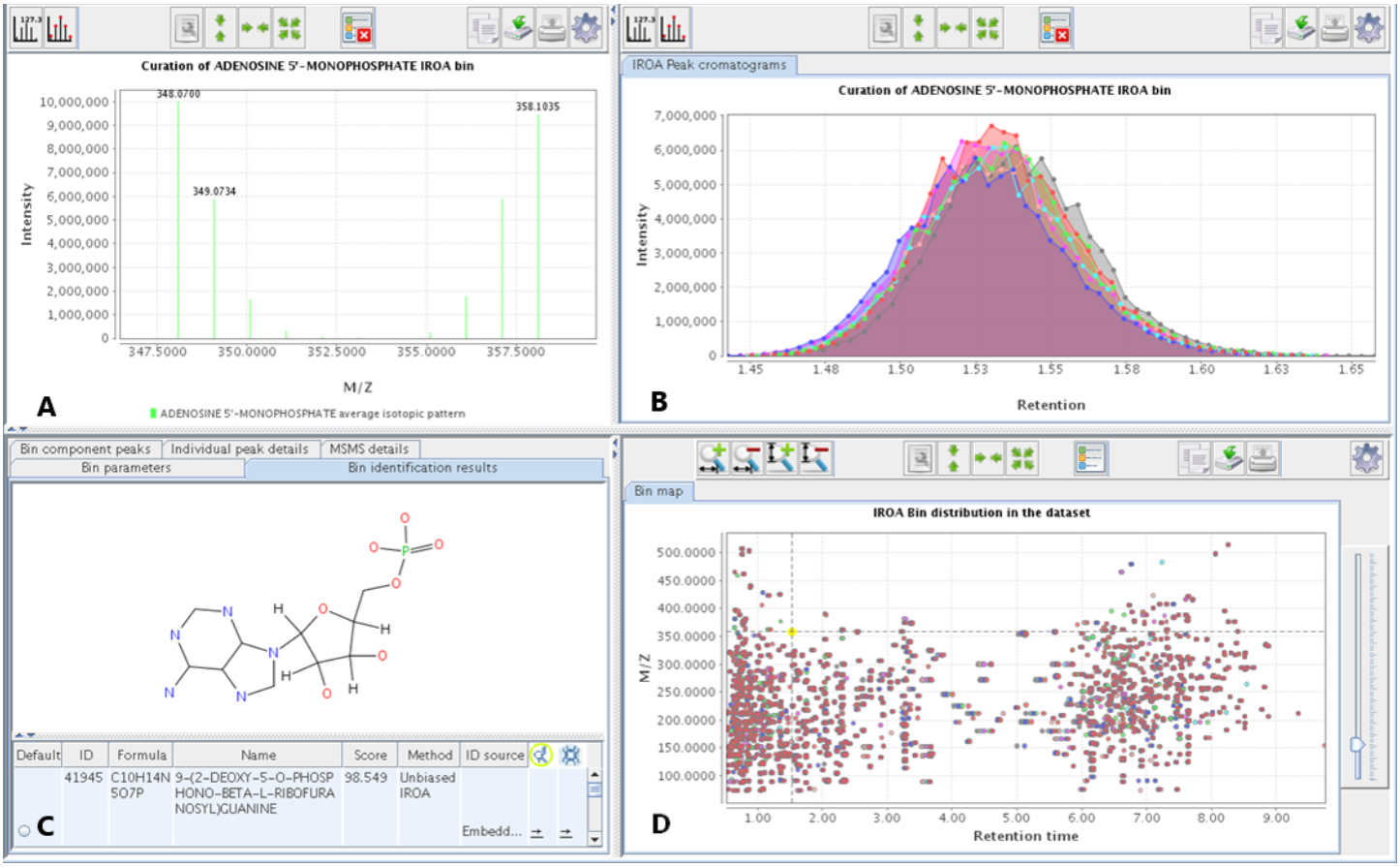


Figure 6. ClusterFinder analysis of IROA-LTRS samples. A. Average isotopic pattern of adenosine 5'monophosphate IROA-IS; B. IROA peak chromatograms of adenosine 5'monophosphate IROA-IS; C. Identification panel for adenosine 5'monophosphate; D. IROA-IS peaks identified in the dataset.

- The IROA-LTRS is injected periodically i.e. approximately 10 sample intervals
- It is analyzed qualitatively to support more accurate compound identification, i.e. with msms or other techniques.
- The IROA-LTRS is used to build a "dictionary" of RT, *m/z* and physical characteristics including fragmentation data.
- The IROA-LTRS is used as a QC Standard to account for fluctuations in chromatography, mass, or retention time drift, source, or instrument malfunctions.
- IROA-LTRS is always the same and the catalog of all IROA peaks found in each daily **IROA-IS.**
- LTRS analysis provides a way to quantitate the performance characteristics for the instrumentation for every day's analysis and a mechanism for correcting any instrumental error.

- Since there is no need to accurately quantify the compounds in the IROA-LTRS, relative quantitation is sufficient, fewer scans are needed to establish quantitative accuracy, and these scans may be better used to support compound identity verification. Thus, the IROA-LTRS may be used with alternating scans devoted to collection of secondary compound identification information, such as fragmentation, Ion Mobility, etc., while in the analysis of the analytical samples keeping all scans as primary will assure better peak shape and, thus, better quantitation.

The IROA Internal Standard (IROA-IS)

The **IROA-IS** is chemically identical to the U-95% ^{13}C component of the IROA-LTRS. **The IROA-IS together with ClusterFinder software are used to co-locate, identify, and quantitate 400 to 600[4] biochemicals in experimental samples, depending upon chromatographic mode(s) employed.**

The unique IROA labeling pattern (Figure 7A) again ensures that the monoisotopic peaks and the *carbon envelope* of the associated isotopic peaks (M-1 etc.) can be detected during LC-MS. The carbon envelope differentiates the IROA-IS from natural abundance peaks (Figure 7B) and is used to identify compounds of interest and exclude artifacts that may look otherwise similar.

The IROA-IS is a true Internal Standard and can be spiked into any natural abundance experimental sample (cells, tissue biopsy, plant material, blood, etc.) and all the IS peaks may be easily identified using the ClusterFinder software according to the presence of their characteristic M-1 peak and associated carbon envelope. It provides enough information for complete identification and quantitation of samples without the need for chromatographic base-line correction.

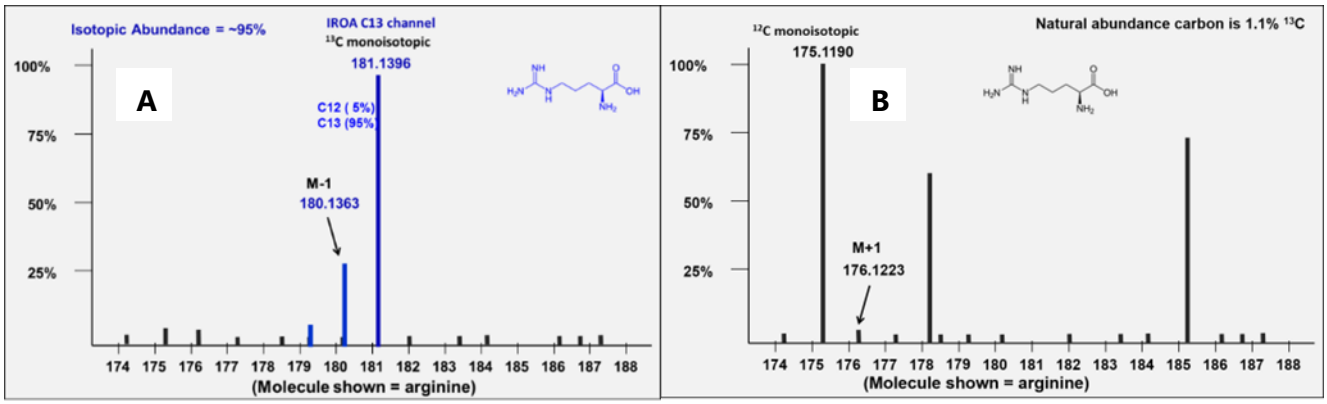


Figure 7 A. Representation of the IROA-IS (U-95% ^{13}C envelope) for arginine, blue peaks; black peaks represent noise/artefacts. B. Natural abundance arginine, monoisotopic peak shown at 175.1190 and M+1 peak at 173.1223; remaining black peaks represent noise/artifacts.

[4] As of ClusterFinder version 4.2.26 the IM CCS handling is not yet fully functional. Many peaks are fragments or adducts of a parent compound.

- The IROA-IS is used to find and align all the peaks.
- The alignment of all samples ensures reduced variability in day-to-day measurements.
- The characteristics for each compound in the IROA-IS allow the calculation of a suppression-corrected area for each compound. Suppression correction and normalization are implemented in ClusterFinder using the qualities inherent in the IS peaks. The user is always presented with raw, suppression corrected, and normalized data so the best choice may always be available according to any experimental design.
- The normalization of the total area under the curve (AUC) for all natural abundance suppression-corrected peaks to the total AUC of their corresponding IROA-IS peaks is a "Dual MSTUS[5]" algorithm that allows for normalization of samples not only within a single day's run, but since the IROA-IS is equivalent every day; all normalizations are to a true Standard, i.e. that is the same every day. Normalization overcomes sample-to-sample variation.
- The IROA-IS can be used to build a Retention Index to track "unknown" compounds not identified in the IROA-IS.

[5] MSTUS described in "Normalization strategies for metabonomic analysis of urine samples" Warrack et. al

IROA TruQuant Workflow

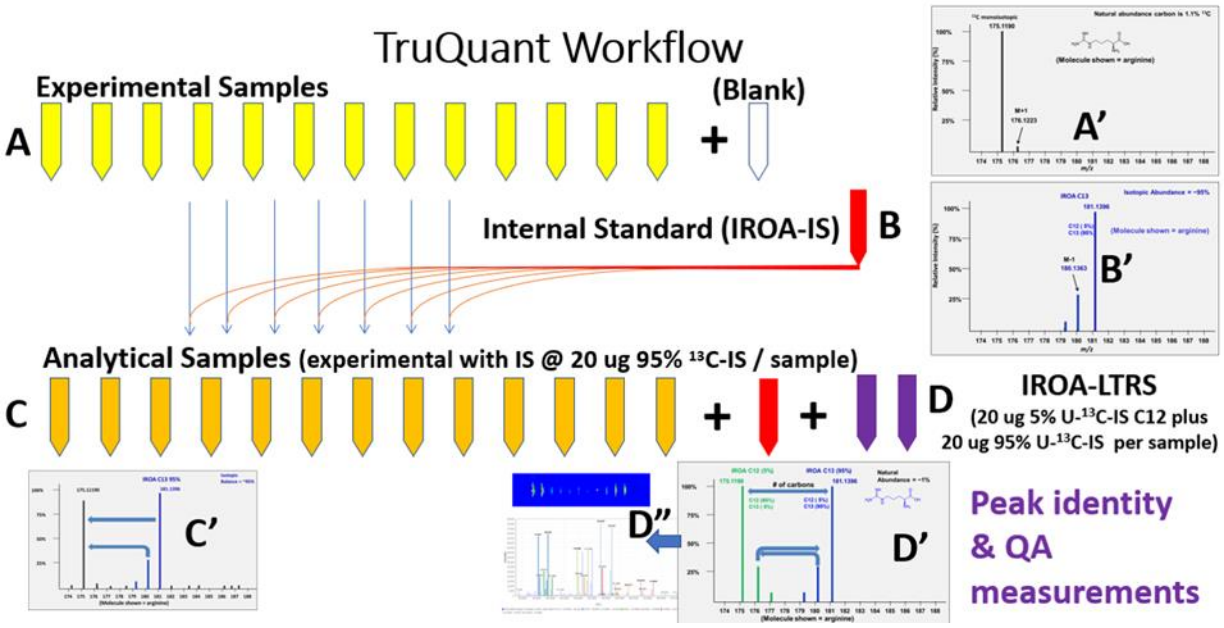


Figure 8. The IROA TruQuant measurement system is based on a well characterized Long-Term Reference Standard (D = LTRS) and a carefully matched Internal Standard (B = IS) to provide instrument and process QA/QC, validated compound identification and quantitation.

(A) Experimental samples (plasma, urine, cells, etc.) are mixed with (B) a complex 400+ compound Internal Standard (IS), fully labeled with U-95% ^{13}C (B'). Experimental/IS samples are analyzed using LC-MS, injecting the LTRS (D) intermittently, approx. every 10 samples. The LTRS has the same concentration and is chemically identical to the IS but also contains a 1:1 mixture of fully labeled U-5% and 95% U-^{13}C metabolites, producing a U-shaped pattern of carbon envelopes (D'). The relative height of the M+1, the relative height of M-1, and the mass distance between the monoisotopic peaks all provide confirmation of the number of carbons in each biological molecule resulting in a triply redundant quality control check point. The IROA peaks represent actual compounds, fragments and adducts and can be discriminated from unsigned artifacts and noise which can be removed from the data, eliminating false discoveries. As a composite sample, sample-to-sample and analytical variance is removed and during MS analysis the identical compounds (labeled with either U-95% ^{13}C or U-5% ^{13}C) experience the same ionization efficiency and suppression.

Over 400 peaks can be detected in the IROA-LTRS. Following analysis, the resulting IROA-LTRS dictionary of compounds is used to identify compounds in the IROA-IS saving time, effort and related costs. The IROA-IS together with ClusterFinder software are used to co-locate, identify, and quantitate 400 to 600 biochemicals in experimental samples, depending upon chromatographic mode(s) employed. A library of over 420 compounds (including their fragments and adducts) have been identified in the LTRS and stored in the ClusterFinder software. We expect this number to increase as more compounds are identified.

The Internal Standard (IS), fully labeled with U-95% ^{13}C (B') is also added to a blank sample. The characteristic for each compound in the IROA-IS allow the calculation of a suppression-corrected area for each compound.

Unambiguous ID and quantitation (ms2) in a single injection. Complete identification of compounds is achieved with the addition of IM or SWATH. The IROA IM peaks retain their patterns perfectly because all IROA isotopomers share the same CCS (D'). In IROA msms fragmentation, such as SWATH, the IROA peaks retain their patterns (D2') because wide windows are used. Since all fragments retain their IROA character, their formulae and the relationships between them (D3') are determinable.

A Note on Normalization and pilot experiment

We all know that sample amounts can be highly variable due to fluctuations in sample preparation and delivery. A "pre-prep" normalization should be performed on each sample prior to crash. Our recommendation is that a protein reading be done on each sample homogenate, and equal amounts of protein be delivered as the "prep sample". While this may not always be appropriate it is always better to collect all the information one can on every sample and attempt a prep-normalization.

This protocol delivers approximately 2 micrograms each of U-95% ^{13}C and U-5% ^{13}C (IROA-LTRS) and 2 micrograms U-95% ^{13}C (IS) per sample which should be appropriate for most MS platforms, **however if this is the first time you are using your chosen matrix (blood, cells, tissues, urine etc.) with this method your first experiment should be a "calibration" experiment**, i.e. test different amounts of your standard prep with the same amount of IROA-IS to figure out how much to balance with the IROA-IS. **Please see "Pilot Experiment: Internal Standard (IS) Calibration" in the Appendix.**

The Process

A) Prepare the experimental/IROA-IS and IROA-LTRS samples

To prepare experimental samples with IROA-IS

For use as an injection standard

As with the Calibration Experiment, the IROA-IS is incorporated into the re-solvation solvent. In this case there is no question that the IROA-IS is in exactly the same concentration in every sample and will be directly comparable to other samples prepared the same way. The material in each amber vial is expected to be suitable for 30 samples.

PLEASE NOTE: THE MORE POLAR COMPOUNDS IN THE IS AND LTRS WILL NOT DISSOLVE IN RELATIVELY NON-POLAR SOLVENTS. THESE REAGENTS MAY NEED TO BE DISSOLVED IN DIFFERENT SOLVENTS FOR EACH CHROMATOGRAPHIC CONDITION. THE SOLVENT EMPLOYED SHOULD BE DETERMINED IN YOUR CALIBATION PILOT EXPERIMENT.

1. As determined in the Calibration experiment, the re-solvation solvent may be either the initial chromatographic solvent (minus acid) or dH2O. (Where the chromatographic solvent contains acid then dH2O is preferable to reduce degradation prior to injection.)
2. Add 900 µL re-solvation solvent to the IS amber vial, mix, and then vortex. The material should go into solution freely. This is the Internal Standard solution (IROA-IS).
3. Hold the IROA-IS on ice until you are ready to re-solvate your samples.

4. Sample aliquots (amount determined in the calibration step) should be gently dried under a nitrogen stream. To each dried sample add 30 µL of the IROA-IS, then vortex.
5. If you have a filtration step, then filter before analysis after re-solvation.
6. Be sure to make up a blank sample, i.e. an empty tube that has no experimental material but will receive an aliquot of Internal Standard (IS). This will not only serve as a QA/QC sample but a sample which will provide important qualitative and quantitative information. This sample, referred to as the "IS-Only" sample, should be injected three or four times randomly within the collection of experimental samples.
7. Use the injection volume as determined in the Calibration experiment. The analysis may be repeated in multiple modes, i.e. positive reverse phase, negative reverse phase, positive HILIC, or negative HILIC.
8. There is no need to collect msms data for the experimental samples.

To prepare the IROA-LTRS Standard

1. Use the same re-solvation solvent as with the IROA-IS; i.e. this may be either the initial chromatographic solvent (minus acid) or dH2O.
2. Add 30 µL injection solvent (less acid) to the vial containing IROA-LTRS. Pipette up and down to ensure solubilization of IROA-LTRS material.
3. Use the same injection volume as is used for your analytical samples. Note: if you generally use different injection volumes for positive and negative modes, act accordingly. (If your injection size will require more than the original 30 µL volume then pool two LTRS prior to the initial injection.)
4. Keep on ice until use.
5. Use the injection volume as determined in the Calibration experiment. The analysis may be repeated in multiple modes, i.e. positive reverse phase, negative reverse phase, positive HILIC, or negative HILIC.
6. The IROA LTRS should be injected before and after analytical samples and should be also injected approximately every 10 samples. Make sure you also collect msms data. Since msms tends to retard the peaks, run msms on only one or two LTRS injections.

The IROA-LTRS analyses assure that the maximum number of compounds in the IROA-IS can be found, and that daily performance quality expectations are met. This procedure creates a daily library or map of the IROA-IS (Figure 9). Individual compounds can be readily identifiable and distinguished from artifacts. Figure 10 shows examples of IROA-LTRS derived amino acids.

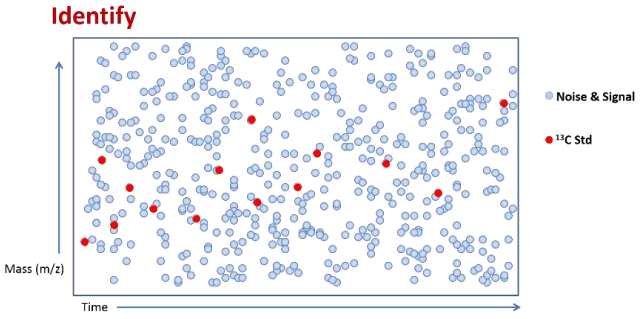


Figure 9. The IROA-LTRS (mixture of the IROA-IS and its U-5% mirror image is mapped using LC-MS to assure that the maximum number of compounds in the IROA-IS can be found.

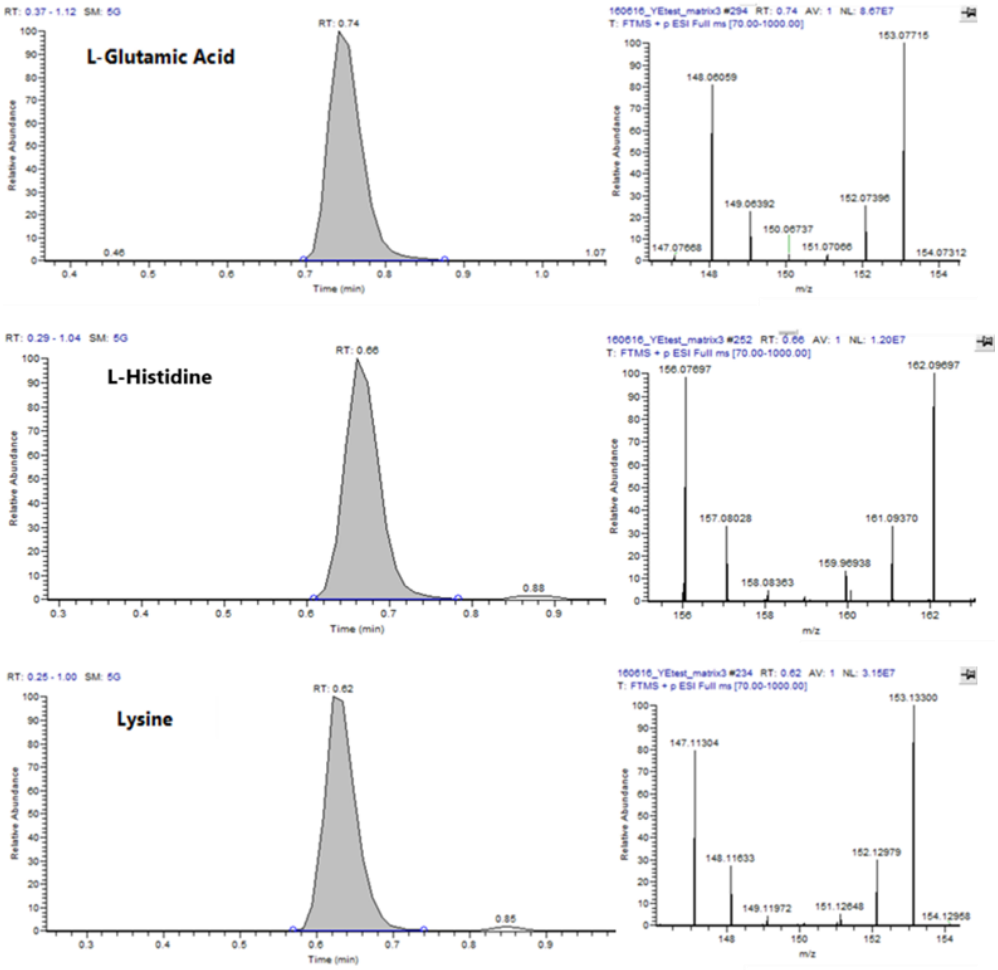


Figure 10. Examples of IROA-LTRS derived amino acid compounds after LC-MS. The compounds show the typical U-shaped pattern (mixture of the IROA-IS, U-95% ^{13}C, and its U-5% ^{13}C mirror image). The distance between the two monoisotopic peaks clearly indicates the number of carbons in the molecule.

B) Perform LC-MS analysis of the IROA-LTRS sample and experimental samples using IROA-IS

Aliquots of the IROA-LTRS are analyzed intermittently with experimental samples spiked with IROA-IS, i.e. analyzed using positive and negative ion LC-MS modes, and other analytical modes such as HILIC and orthogonal, second-stage analyses such as an Ion Mobility, SWATH fragmentation etc. that are employed in the laboratory.

C) Build a Dictionary using IROA-LTRS data files. Identify compounds, fragments and adducts using ClusterFinder software.

- Upload the IROA-LTRS LC-MS data files to IROA ClusterFinder Software. ClusterFinder uses algorithms to build, evaluate, edit and export the result of untargeted IROA search. Refer to ClusterFinder User Manual and instructional videos, provided separately.

- If you have not yet created a dictionary specific to your method connect the relevant IROA-LTRS library, likely IROA-LTRS-pos, or IROA-LTRS-neg.
- These libraries are devoid of retention times but are a good starting point. If you have created method specific libraries (see last step) please attach these instead as they will contain your previously recorded retention times.
- Compounds, fragments and adducts are identified in minutes using algorithms that search for the specific IROA signatures (See Figure 11).
- Curate the data
    - Examine spectrum bins and their constituent IROA peaks.
    - Look at the detected features in the context of underlying raw data and manually correct the results in cases where the automatic algorithm has failed.
    - The curation process takes approximately an hour to complete.
- Build a targeted library (.cflib file) from each method or LC-MS mode. Each library will be used to perform a targeted search of the clinical/experimental IROA-IS-containing files run coincidently to the IROA-LTRS.

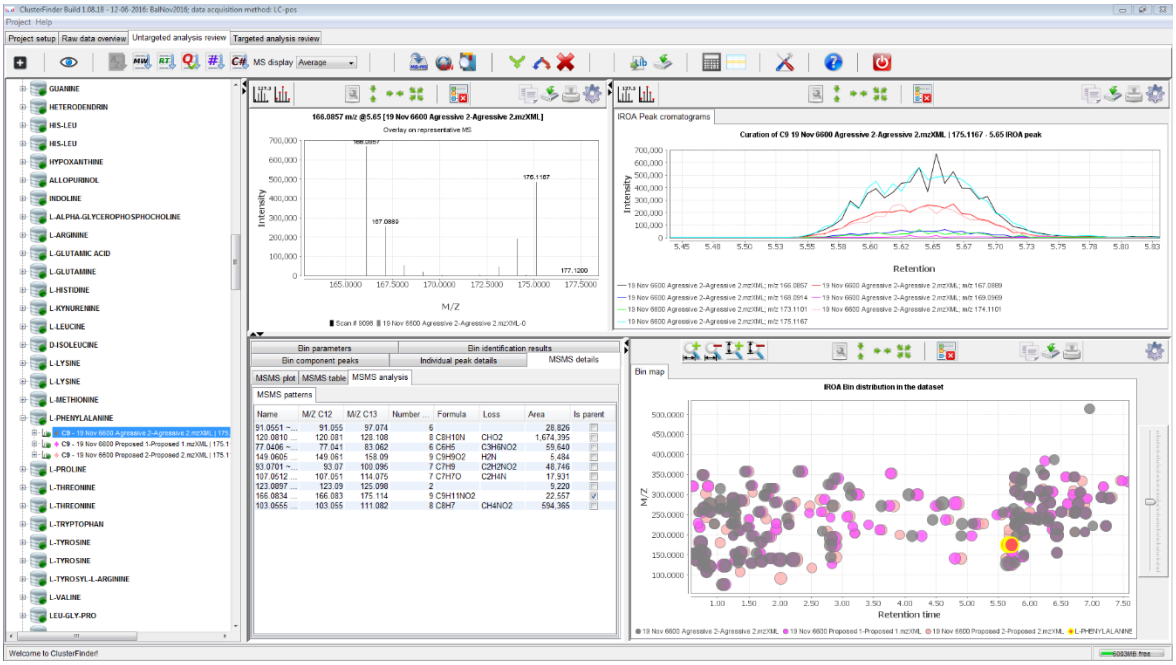


Figure 11. Example of IROA-LTRS-derived compound L-Phenylalanine identified using ClusterFinder software. The compound shows the IROA-LTRS U-shaped pattern (mixture of the IROA-IS, U-95% ^{13}C, and its U-5% ^{13}C mirror image). The distance between the two monoisotopic peaks clearly indicates that the molecule contains 9 carbons.

- Create a method specific dictionary as a ClusterFinder database:
    - Disconnect the IROA-LTRS dictionary from your search,
    - Create a new library (database), for instance, myHILICLTRSPos, and attach it to the method of analysis,

- Select all bins in your curated method,
- Right-click the selected bins, and
- Select "add to Library"
- The new library will now have retention times, spectra, and all related information, and is likely the dictionary to connect in future LTRS analyses.

- Export the resultant dictionary in comma separated value (CSV) format, or compound exchange format (CEF), or NIST (National Institute of Standards and Technology) format for storage or transport to other ClusterFinder installations.

D) **Identify compounds, fragments and adducts in the experimental samples using ClusterFinder software and the IROA-LTRS dictionary.**

- Import LC-MS analytical data files to IROA ClusterFinder Software.
- Run a targeted analysis using the targeted library (.cflib file) that was created following the curation of the IROA-LTRS files.
- Compounds, fragments and adducts are identified in minutes using algorithms that search for the specific IROA signatures.
- Curate the data
  - Examine spectrum bins and their constituent IROA peaks.
  - Look at the detected features in the context of underlying raw data and manually correct the results in cases where the automatic algorithm has failed.
  - The curation process takes approximately an hour to complete.
- Export the results as tab separated value (tsv) files.
- The quantitative output of targeted analysis, together with experiment design may serve as input for the statistical and biochemical interpretation. The User can employ their favorite statistical package.

E) **Identify compounds, fragments and adducts in the experimental samples using ClusterFinder software and user-generated libraries.**

- The TruQuant Workflow has been extended to allow the identification and quantitation of compounds in experimental samples that are not contained in the internal standard but are represented in libraries that Users may upload into the ClusterFinder software.
- Prior to running the ClusterFinder software, make sure you have uploaded any libraries that you wish to use to identify compounds in your samples.

APPENDIX: Pilot Experiment: Internal Standard (IS) Calibration

The IROA TruQuant WorkFlow Kit contains enough materials for 3 sets of experiments. For each type of experimental sample, i.e. plasma, urine, tissue etc., a **calibration experiment** should be performed to optimize the amount of experimental sample that you will use with the kit (the quantity of your experimental sample is optimized against the quantity of IROA Internal Standard used in the method). You only need to do this once for any sample type or SOP.

The calibration of IS to your SOP simply requires the generation of a single large portion of prepared "prepped" experimental sample (the sample type you plan to use for your experiment, i.e. plasma, urine or tissue, etc.), approximately 30X the normal sample size routinely used is sufficient.

PLEASE NOTE: THE MORE POLAR COMPOUNDS IN THE IS AND LTRS WILL NOT DISSOLVE IN RELATIVELY NON-POLAR SOLVENTS. THESE REAGENTS MAY NEED TO BE DISSOLVED IN DIFFERENT SOLVENTS FOR EACH CHROMATOGRAPHIC CONDITION.

Setting up the Calibration Experiment:

1. Pool and filter the prepped experimental sample to create a single homogeneous sample.
2. Deliver varying aliquots of the pooled sample, ranging from half of the normal amount delivered to 3 times the normal amount to Eppendorf capped sample tubes. (For example: 0.5X, 0.75X, 1X, 1.5X, and 3X.) Make triplicate samples.
3. Dry aliquots under a gentle nitrogen stream.
4. Be sure to make up a blank sample, i.e. an empty tube that has no experimental material but will receive an aliquot of Internal Standard (IS). This will not only serve as a QA/QC sample but a sample which will provide important qualitative and quantitative information. This sample, referred to as the "IS-Only" sample, should be injected three or four times randomly within the collection of experimental samples.
5. Resolvate a single vial of IROA LTRS with 30 µL of a solvent that will work for injections (based on the chromatographic condition) but is reasonably polar to maximize the number of compounds that will go into solution. (The compounds in the LTRS and IS are mostly polar.) Thoroughly mix to ensure complete solvation. Note: It should go into solution freely. Keep on ice until ready to use. Inject 4 and 5 µL of the LTRS to determine the best injection size. An injection size that gives you a total of several hundred peaks is the goal.
6. Add 900 µL of the same solvent to a single vial of IS. Thoroughly mix to ensure a single homogeneous solution. Note: Again, it should go into solution freely. Keep on ice until ready to use.
7. Use a 30 µL aliquot of the IS to resolvate each of the dried experimental samples. Upon addition of IS, mix thoroughly.

8. Analyze samples using the chromatographic method you would normally use using injection volumes determined in step 5. The analysis may be repeated in multiple modes, i.e. positive reverse phase, negative reverse phase, positive HILIC, or negative HILIC. The IROA LTRS should be injected before and after analytical samples and should also be injected approximately every 10th injection. (If your injection size will require more than the original 30 µL volume then pool two LTRS prior to the initial injection.)

9. The higher concentrated samples may overload the column, so we do not recommend running them in random order but rather run then from most dilute to most concentrated to reduce any carry-over effects. The randomly run LTRS and IS-Only samples will provide evidence of any carry-over.

10. There is no need for msms in the Calibration (experimental) samples, only do msms on the LTRS. Since msms tends to retard the peaks, run msms on only one or two LTRS injections.

11. Use the ClusterFinder software to find and identify all IROA peaks in the LTRS in an unbiased analysis.

12. Export the compounds found in the previous step to run a targeted analysis of the IS containing samples. Use the ClusterFinder software to export all of the quantitative data from the IS containing samples for statistical analysis. Export all of the compound identification and QA/QC data from the LTRS.

13. Analyze the samples to identify the amount of the prepped samples that yield an overall mass spectral signal that is equal to the overall mass spectral signal of the IS. This is the amount of sample that will most accurately be measured using the IS in the future, i.e. well balanced by the standard 30 µL of IS. This is illustrated in Figure 12 below.

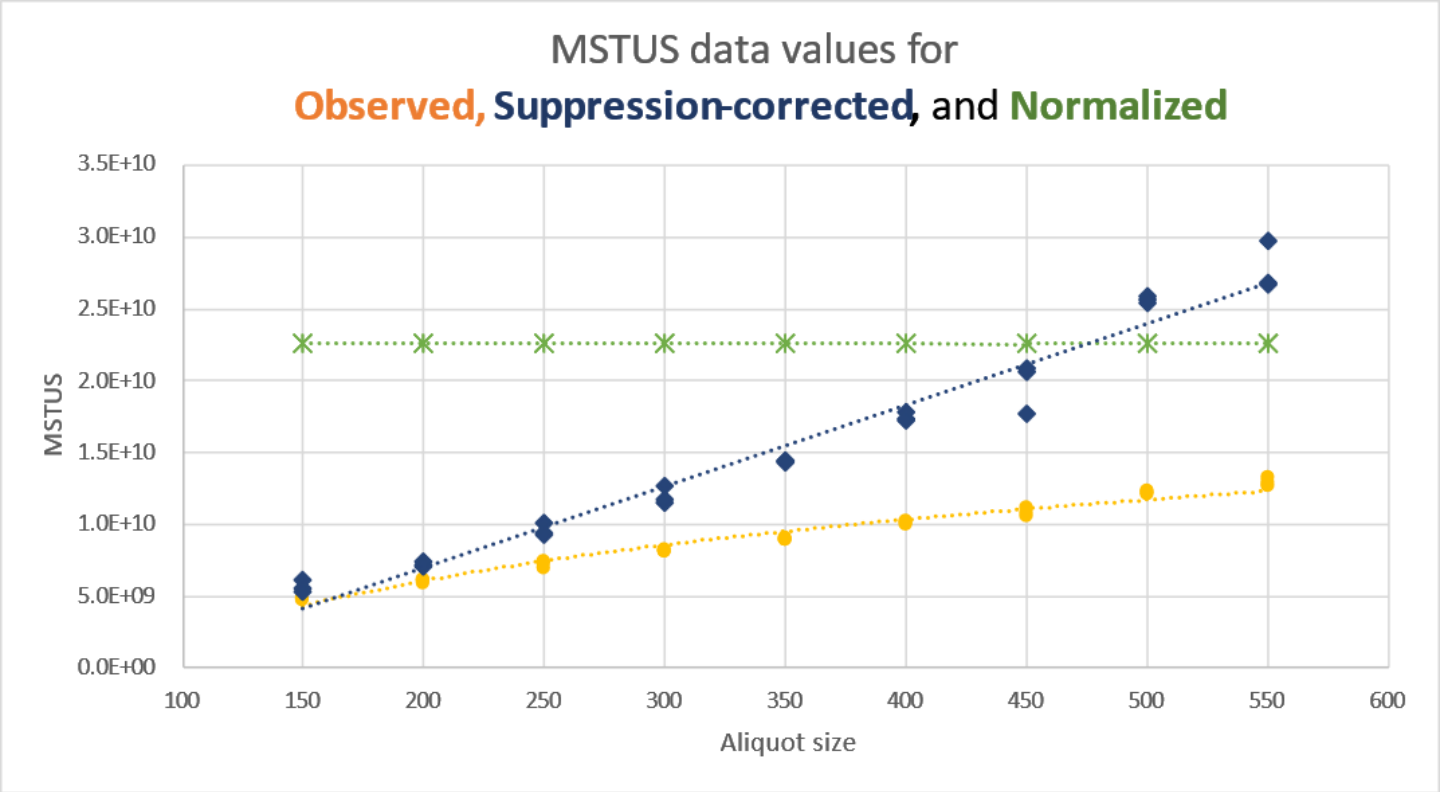


Figure 12. Suppression-correction and normalization for IS related peaks. The intersection of the suppression corrected MSTUS values (dark blue values), and the MSTUS C13 values for normalized data (green values) represents the aliquot size that will have optimal quantitation.