IROA TruQuant Internal Standard Kit

Cat No. IROA-IS

Kit contents

Materials and tools for the analysis of 90 experimental samples; for use with the TruQuant Workflow

- IROA Internal Standard (IROA-IS): 3 vials of lyophilized unique 95% U-^{13}C fully-labeled *S. cerevisiae* Yeast Extract*
- ClusterFinder™ software, including the library of IROA peaks and their physical characteristics identified in the Long-Term Reference Standard (LTRS)**
- User Manual

Storage: -80ºC, protected from light

Summary of Benefits of the TruQuant Internal Standard (IS)

1. **No false data. Identifies only compounds of biological origin; excludes artifactual peaks.**
2. **Accurate compound formula ID for MS alone; complete ID with addition of SWATH, or IM.**
3. **Fragments have the IROA ratio pattern derived from their parent peaks and can be identified using the "peak correlation" ClusterFinder module.**
4. **Suppression-corrected measurements for significantly better quantitation.**
5. **Reproducibility. Sample normalization to a universal standard for complete comparability.**
6. **ClusterFinder software solution builds libraries, IDs/quantitates compounds, corrects for ion-suppression and normalizes data.**
7. **Mass-spec vendor agnostic.**
8. **Economical. $100 for 90 samples.**

*Prepared from proprietary U-95% ^{13}C IROA-labeled glucose specially produced for IROA Technologies by Cambridge Isotope Laboratories, Inc. (CIL).
** Long-Term Reference Standard (LTRS) Yeast Extract provided in the TruQuant Yeast Extract Semi-Targeted Workflow Kit

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The IROA Internal Standard (IROA-IS)

The **IROA-IS** is created to be uniformly labeled at approximately 5% for the ^{12}C isotope, and approximately 95% for the second isotope, ^{13}C. Molecules labeled at 95% ^{13}C have a strongly enhanced M-1 (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 minor peaks can be easily lost. Where the ^{13}C is increased 95%, the 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 natural abundance, namely 1.1%, the height of the M+1 is only approximately 6% of the height of monoisotopic peak.

The 95% U-^{13}C labeled peaks are mathematically calculable and readily identified by the IROA ClusterFinder software. Plus, the 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.

The IROA-IS is added to experimental samples to identify and quantitate metabolites using the LTRS "dictionary". (The IROA-LTRS, provided in the Workflow Kit, 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.) 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.

The unique IROA labeling pattern (Figure 1A) 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 1B) and is used to identify compounds of interest and exclude artifacts that may look otherwise similar. 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 arginine labeled at 95% ^{13}C in Figure 1A, the base peak is still the C13 monoisotopic peak but represents only about 70% of the molecules in the internal standard.

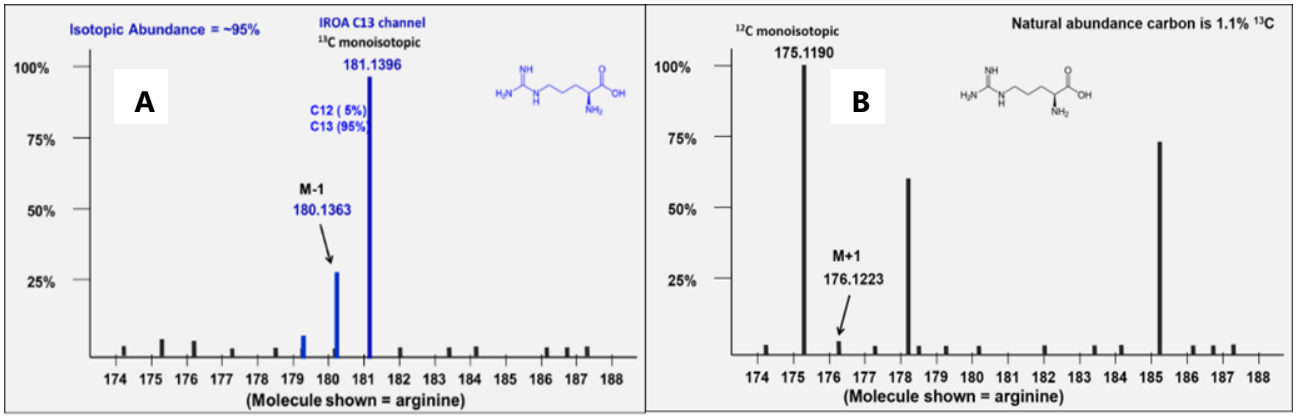

Figure 1 A. Representation of the IROA-IS (U-95% ^{13}C envelope) for arginine, blue peaks; black peaks represent noise/artefacts. B. Natural abundance arginine, monoisotopic peak shown at 175.1190 and M+1 peak at 173.1223; remaining black peaks represent noise/artifacts.

The IROA-IS together with ClusterFinder software are used to co-locate, identify, and quantitate 400 to 600[1] biochemicals in experimental samples, depending upon chromatographic mode(s) employed.

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.

- 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.

[1] 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 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[2]" 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.

The Workflow protocol (Figure 2) delivers approximately 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.**

To prepare experimental samples with IROA-IS

<u>For use as an injection standard</u>

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 IROA-IS 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.

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

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.

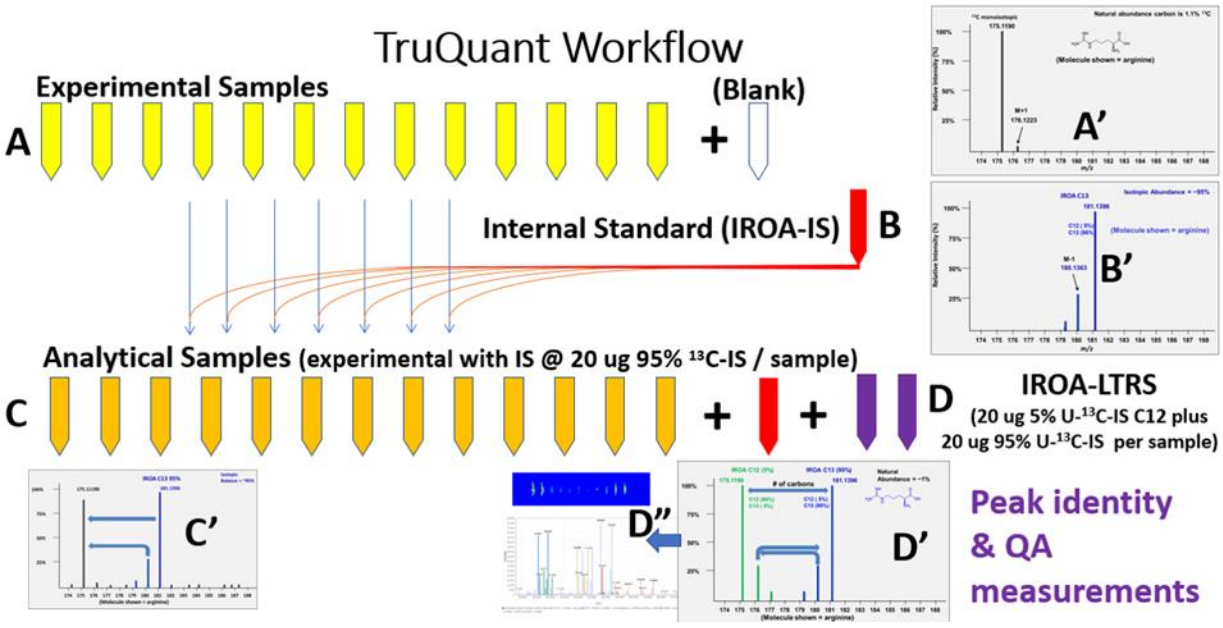

Figure 2. The IROA TruQuant measurement system is based on a well characterized Long-Term Reference Standard (D = LTRS) and a carefully matched Internal Standard (B = IS) to provide instrument and process QA/QC, validated compound identification and quantitation.

(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.

In the TruQuant Workflow (see Figure 2), 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.

For further background information and a full description of the Workflow protocol, please refer to the TruQuant Yeast Extract Semi-Targeted QC Workflow User Manual.

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, run only 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 3 below.

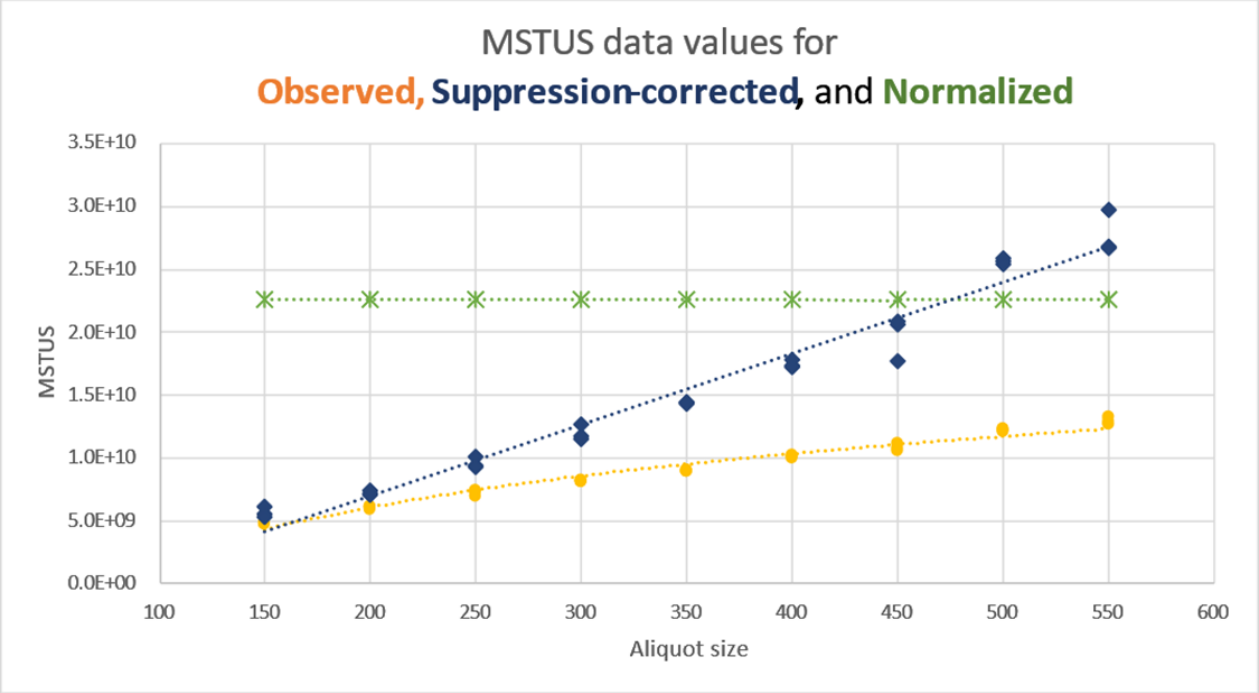

Figure 3. Suppression-correction and normalization for IS related peaks. The intersection of the suppression corrected MSTUS values (dark blue values), and the MSTUS C13 values for normalized data (green values) represents the aliquot size that will have optimal quantitation.