Pilot Experiment: Internal Standard (IS) Calibration

The IROA TruQuant WorkFlow Kit contains enough materials for 3 sets of 30 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 30 µL IROA Internal Standard used in the method). You only need to do this once for any sample type or SOP.

Note: The 30 µL of IS specified here means that the amount of all compounds in the IS are exactly equal to the amount that is used for the LTRS. For accurate analytical quantitation it is optimal that the LTRS concentrations are mirrored in the IS.

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. Use the lab's extraction protocol/SOP on the sample of choice (plasma, tissue etc.), and create a large aliquot by pooling together the extracts. Mix well to create a single homogeneous sample.

2. Deliver varying aliquots of the pooled sample, ranging from a quarter of the normal amount delivered to 3 times the normal amount to Eppendorf capped sample tubes. (For example: aliquots of 0.25X, 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. Create calibration analytical samples by adding 30 µL IROA-IS to each dried calibration sample. Vortex to ensure material is dissolved.

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 requires 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 (ref 9). These IROA peaks will be named.

12. Run a Semi-targeted analysis of the IS containing calibration samples based on the peak parameters determined in the LTRS sample.

13. Use the ClusterFinder software to generate a calibration report. This report will list all of the compounds found in the calibration samples and graphically show the point (and concentration) where the calibration samples C12 MSTUS signals for each concentration are equal to the Internal Standard C13 MSTUS. This aliquot size will be used with all future samples based on the same SOP.

14. Export all of the compound identification, calibration, and QA/QC data from the LTRS.

15. 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 9 below.

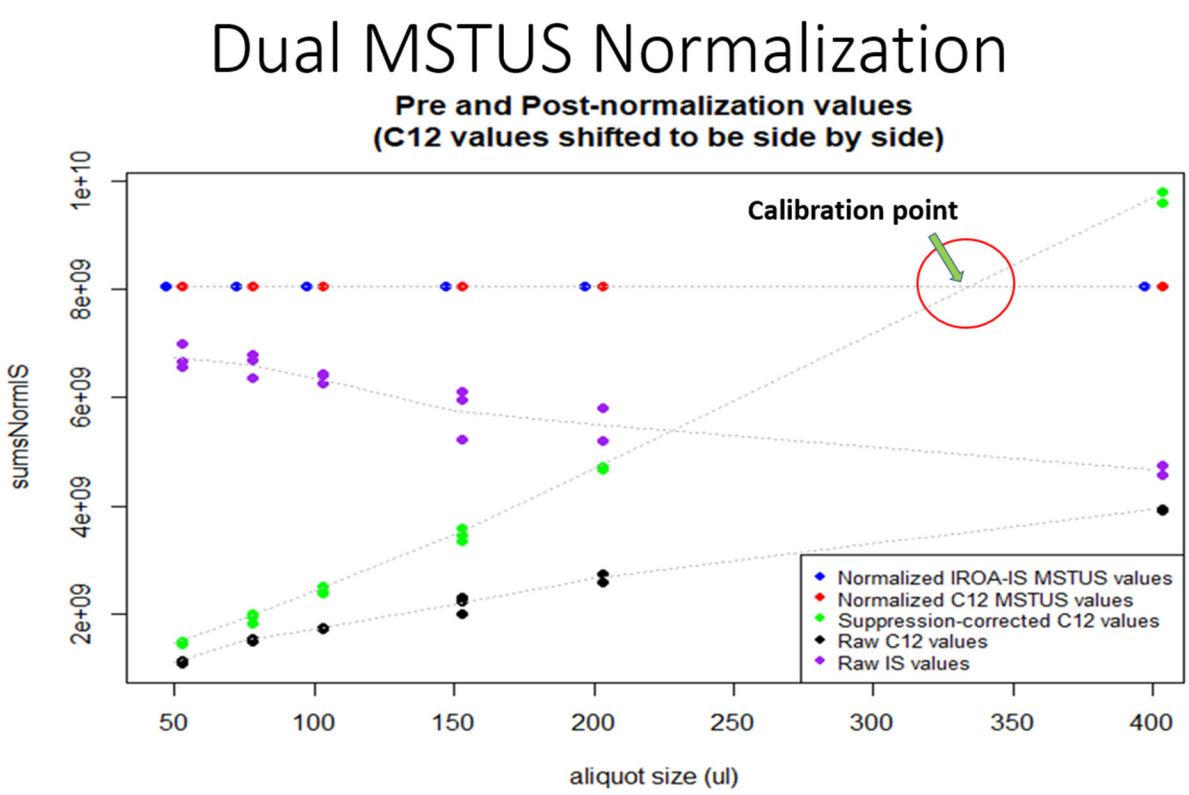

Figure 9. Suppression-correction and normalization for IS related peaks. The intersection ("calibration point") of the suppression corrected MSTUS values (dark blue values), and the MSTUS C13 values for normalized data (green values) represents the aliquot size that will have optimal quantitation.

What you will learn from a calibration experiment:

ClusterFinder (CF) software algorithms find and interpret the entire isotopic envelope for all isotopic balances, natural abundance, U-13C 95%, and U-13C 5%. Because of the nature of the labeling in these situations we now understand that all the peaks of the full isotopic envelope need to be summed to determine the actual quantity of material on either side.

However, for accurate quantitation that is not all that needs to be considered. If we have a sample and inject various aliquots, we expect that the data should reflect the aliquot size injected, but does it? For instance, by injecting varying aliquot sized samples, we would expect that the signal we see in the ms for each compound should increase by the same order, but in fact it does not! Everyone should do this very simple experiment to understand how data on their instrumentation will look and how far the datapoints deviate from the expected curve. In almost all cases, it will be an eye-opener.

What causes this discrepancy is usually the result of a number of different things which end up suppressing the signal that is seen by the detector. While a large portion of this variance is likely ion suppression, significant portions of the variances seen sample-to-sample come from other phenomena. In the following sections we will discuss how the suppression correction algorithms correct for most of the variance (i.e., instrumentation error, injector, source etc.) and then normalization algorithms correct for a large part of the remaining variance.

Consider a model Calibration experiment shown in Figure 10. The different sized aliquots discussed above (1X, 2x, 4X, etc.) would be expected to provide a graph similar to what is seen in Figure 10 A but in reality, results in a graph similar to what is seen in Figure 10 C (blue)[5]. If we put an IROA internal standard into each sample at the same concentration we expect to see a straight horizontal line (Figure 10 B), but what we see is seen in Figure 10 D (blue). Thus, in this experiment a large portion of the error is concentration dependent and therefore largely driven by ion suppression. Casual observation of Figures 10 B and 10 C suggests that the suppression seen on the natural abundance and internal standards are similar, and Figure 10 E proves that they are perfectly correlated. Thus, if we know what the unsuppressed value was for every compound we can correct the data seen in Figure 10 C (blue) to its suppression corrected values seen in Figure 10 C & D (red). It is important to note that that this correction is applied to each compound in each sample independently, i.e., suppression-correction is compound specific. Four additional features arise from this correction.

First, it is possible to examine the suppression that each compound undergoes in each injection. The result of this analysis is shown in Figure 10 F which illustrates the distribution of average suppression among all the MSTUS compounds in the samples in the most concentrated sample (here, approximately 2 times "normal" injection volume). The average compound is subjected to a 40% suppression within a range (<5% suppression to >85% suppression). Above 80% suppression the AUCs are negatively correlated to their aliquot size.

Secondly, the suppression-correction process can be applied to recover from any source of loss of ions where the loss is equally applied to all peaks within the isotopolog ladder after the time that the two envelopes are in place. This includes injector error, but does not include fragmentation error, except for the parent compound, since the breakage of C13-C13 bonds requires more energy than breaking a C12-C12 bond.

Thirdly, for the suppression-correction process to work effectively the majority of internal standard peaks need to be findable and to be sufficiently above noise so that their areas can be effectively determined. While this may seem obvious, some researchers have used the internal standard in too dilute a concentration (i.e. without calibration) and therefore their results were less than optimal. That is why we recommend that everyone who uses IROA reagents perform a calibration of the internal standard to their generic SOP samples. It only needs to be done once for each sample preparation method. The calibration determines the aliquot size of the sample that most closely matches the concentrations of internal standard that the user intends to use. This is centered around the intersection of the suppression corrected sample line with the suppression corrected internal standard line. In Figure 9 this Calibration point intersection is indicated with a red circle. Also seen in Figure 9, the suppression-correction is sensitive enough that this allows one to operate in both highly suppressed and highly dilute conditions.

Fourthly, a MSTUS normalization may be applied to this suppression-corrected data that results in more stringent normalization than current methods. This is presumably due to the removal of suppression error in the MSTUS raw data (See **Note 3**). Here, the natural abundance MSTUS values are normalized to the MSTUS suppression-corrected internal standards data. Because this normalization uses two MSTUS values we refer to this as a "Dual

[5] Because the suppressibility is a function of chemical structure each compound will be different. Graphed here are the MSTUS values of each injection, which provides a commonly-based response for all samples.

MSTUS ™" normalization method (see Figure 9). If the same internal standard is used for two different collections of samples (different experiments) and they are all normalized to the same internal standard (ideally with largely the same MSTUS collection of IROA internal standards) the samples are more mathematically comparable. In general, sample-to-sample normalizations are performed to correct for sample size (or other) differences, and for losses during sample preparation. As can be seen in Figure 10 the Dual MSTUS normalization has made vastly different sample sizes all appear to be identical (red and blue dots) and corrected raw data (black and purple) for losses (green and red).

An important distinction needs to be made between the suppression-correction processes, and the Dual MSTUS normalization processes, namely that the suppression-correction is performed compound by compound while the Dual MSTUS is performed sample by sample. This has important implications: 1) it is not possible to do a suppression-correction without an internal standard that can be used to calculate the level of suppression for a compound, but 2) the sample-to-sample Dual MSTUS normalization may be applied to any peak in the sample even if it does not have an accompanying internal standard.

As noted above, together suppression-correction and normalization correct for two of the major sources of error in most experiments, the former corrects for error introduced within the instrumentation (in the injector, source, lensing and detectors), and the latter corrects for sample preparation variances (size difference, sample losses during preparation etc.). As such these two algorithms can remove a large portion of the error in most MS based experiments (see **Note 4**).

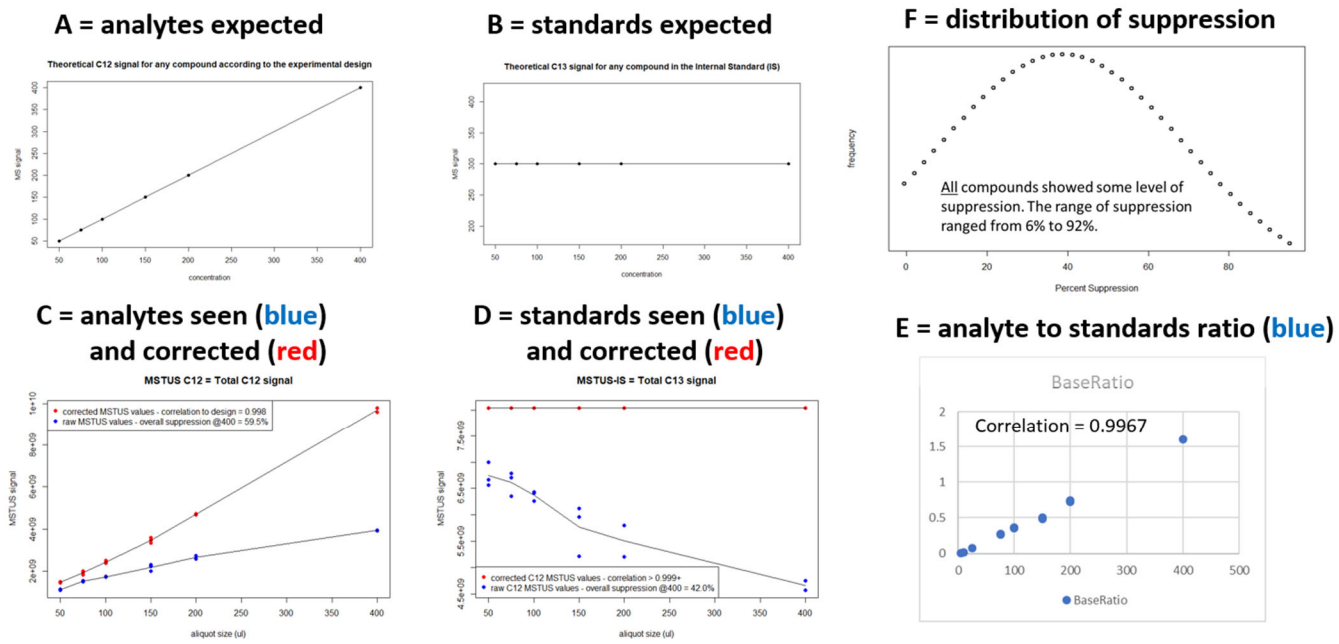

Figure 10. An experimental illustration of suppression correction. Suppression is more common that most researchers assume, but the loss is suffered equally by all isotopologs so the C12/C13 ratio is unaffected and therefore affords a route to correction.