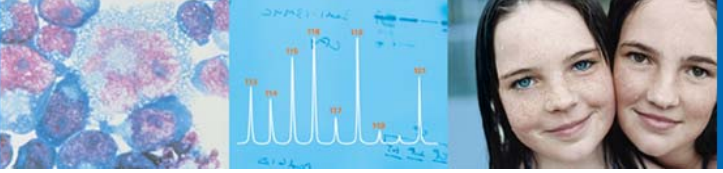

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Chemical Labeling Strategy for Generation of Internal Standards for Targeted Quantitative Proteomics

mTRAQ™ Reagents

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The utility of Multiple Reaction Monitoring (MRM) for targeted protein quantification and biomarker verification/validation studies on triple quadrupole based MS systems is an active research area[1], driven by the well known sensitivity and selectivity attributes of MRM. To generate robust and reproducible MRM assays with high analytical precision, internal standards (IS) are essential and a common strategy for this type of quantitative workflow. Within the field of targeted quantitative proteomics, there are 3 major applications areas (Pathway Profiling, Biomarker Verification and Biomarker Validation, Figure 1) all requiring internal standards. There are three main strategies for generating internal standards. These different internal standard strategies are outlined in Figure 1, and are organized by workflow to clarify which strategies are best suited for which types of experiments.

Key Features of mTRAQ™ Reagents for Quantitative MRM Assays

- Non-isobaric amine labeling reagent developed for creating internal standards for quantitative MRM assays
- Increases robustness of MRM assays, providing reproducibility in the region of 5% CV
- One step labeling procedure to create an internal standard for every protein/peptide/PTM in the sample
- Established, field-proven chemistry with >95% labeling efficiency
- Most cost effective approach when larger numbers of proteins/peptides need to be quantified
- Enables both the RIS and GIS workflows (Figure 2)
- Provides direct link to biomarker discovery with iTRAQ® Reagents

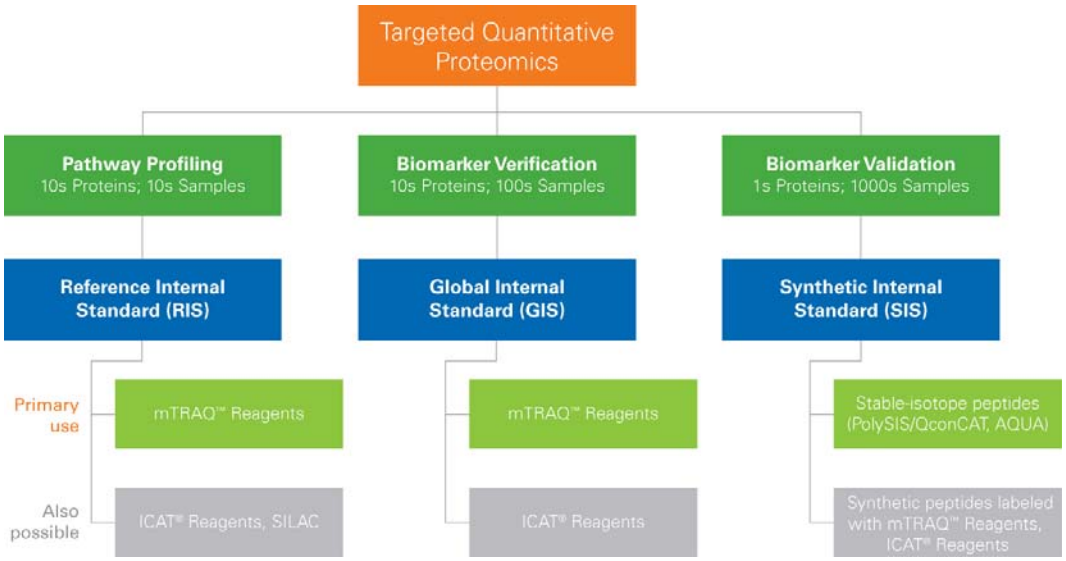

Figure 1. Internal Standards for Targeted Quantitative Proteomics. To better classify the use of internal standards in targeted quantitative proteomics, workflows have been divided into three major classes and a typical internal standard strategy for each has been associated. Absolute quantification in biomarker validation experiments is typically done with stable isotope labeled peptides, created chemically or biologically[1,2]. Relative quantification in biomarker verification or pathway profiling experiments can be done with the mTRAQ™ Reagents by creating Reference or Global Internal Standards (Figure 2). Other chemistry strategies can also be appropriate, depending on the proteins to be monitored and the specific biological question.

Creating an Internal Standard with the mTRAQ ™ Reagents

When large numbers of proteins / peptides are to be monitored in quantitative MRM assays, internal standards can be easily created through chemical labeling. Two very similar workflows are available, the Global Internal Standard (GIS) and the Reference Internal Standard (RIS). In the GIS approach, an internal standard is created from a pool of all of the biological samples to be measured (Figure 2). In the RIS approach, a single sample is chosen as the reference to which everything will be compared (for example, a Time Point Zero in a time course study or an untreated sample in a kinase assay,). In both approaches, the created internal standard is then labeled with the heavy mTRAQ™ reagent, to distinguish it from the samples of interest (which will be labeled with the light mTRAQ™ reagent). An internal standard has now been created that will have all key proteins / peptides / PTMs that will be monitored in the quantitative assay. This internal standard strategy is much more cost effective than the use of synthetic peptide standards when many proteins and peptides need to be monitored (see later section on cost comparison).

Next, the biological samples are labeled with the light mTRAQ reagent. The GIS is then mixed with each separate sample in a 1:1 ratio. MRM transitions are developed to both forms of all peptides of interest which can be done easily using MRMPilot™ Software[4]. Using the *Scheduled* MRM™ Algorithm[6], a highly multiplexed MRM acquisition method is created for all peptides of interest, both light and heavy versions (Figure 2).

All labeled biological samples are analyzed with the MRM acquisition method and the resulting data are processed with MultiQuant™ Software[5]. The MRM ratio to the GIS for every pair of light and heavy MRM transitions and the average of these ratios per peptide is computed for every sample. Now this peptide ratio can be used to compare the change in expression between all the biological samples, as it is a ratio to a constant internal standard. This constant internal standard allows data to be acquired across hundreds of samples, over an extended project time, even across multiple instruments in multiple labs.

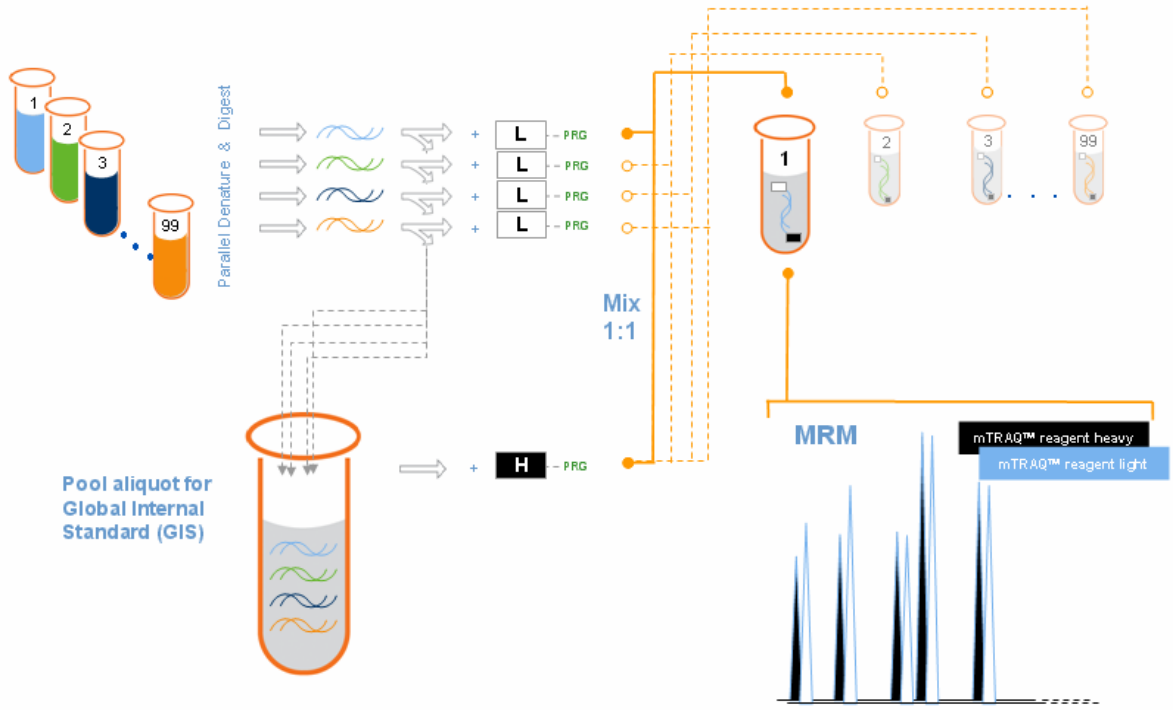

Figure 2. Global Internal Standard (GIS) Workflow. All biological samples are prepared in parallel and digested. Then, an aliquot of each sample is taken and mixed together to create the Global Internal Standard. The individual samples are labeled with mTRAQ™ Reagent light, the GIS sample is labeled with the mTRAQ™ Reagent heavy and will act as the internal standard. An aliquot of the heavy labeled GIS is then mixed into each light labeled sample at a 1:1 ratio. Finally, MRM transitions to both the light and heavy versions of the peptides are monitored and the light/heavy ratio is computed for each MRM pair. Because of the internal standard, the ratios for all MRM transitions and peptides can be compared between all samples.

Scheduled MRM™ Algorithm

As more extensive protein panels need to be monitored quantitatively across multiple samples, higher MRM multiplexing is becoming essential for throughput. With the *Scheduled* MRM™ Algorithm[6] in Analyst® Software 1.5, many more MRM transitions can be monitored in a single acquisition while maintaining the quantitative reproducibility that is essential for targeted protein quantification assays. This ability to run much higher multiplexed assays drives the need for a much more cost effective method for generating internal standards, such as the Global Internal Standard workflow using the mTRAQ™ Reagents. An MRM acquisition method was created using the *Scheduled* MRM™ Algorithm for monitoring mTRAQ™ Reagent labeled peptides in human plasma (Figure 3). Using 4 MRM transitions to light labeled and 4 to the heavy labeled peptides, 94 peptides were monitored in a single run for a total of 752 MRM transitions.

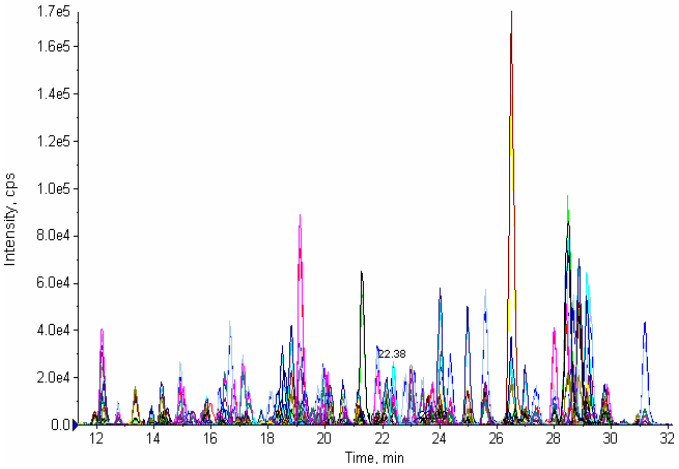

Figure 3. MRM Assay for Human Plasma. Using an acquisition method created with the Scheduled MRM™ Algorithm, 752 MRM transitions to 94 peptides labeled with mTRAQ™ Reagent were monitored in a single assay. Both labeled version of each peptide were monitored, 4 MRM transitions to the light and 4 MRM transitions to the heavy version.

Investigating Reproducibility Provided by the mTRAQ™ Reagent Labeled Internal Standard

An experiment was designed to test the ability of the mTRAQ™ Reagent labeled internal standard to enhance the reproducibility of an MRM assay. One key aspect to good quantitative assays is to have highly reproducible autosampler injections. On most autosamplers, this is achieved by using a full loop injection profile (overfill sample loop with minimum of 2x volume). In this experiment, a less reproducible method (µL pickup method) was used to challenge the workflow. Using an assay for 752 MRM transitions to 94 peptides (Figure 3) on the 4000 QTRAP® system, human plasma was analyzed and 10 replicate injections were performed. The data was processed using MultiQuant™ Software.

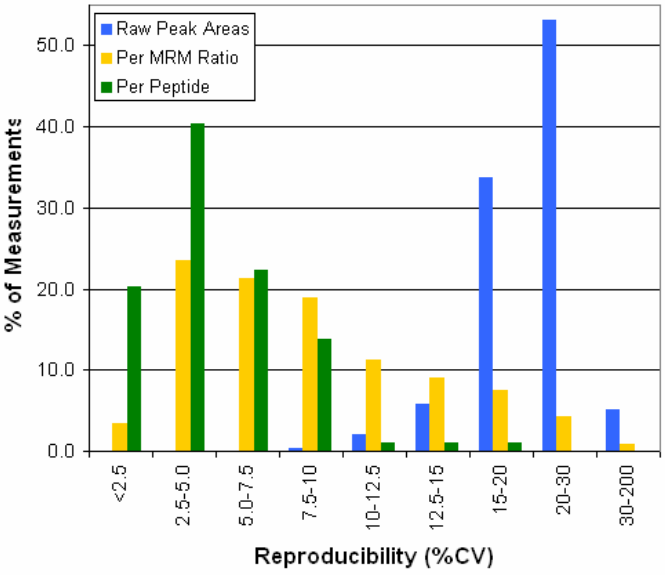

Figure 4. Use of Internal Standard Increases Reproducibility of Peptide MRM Assay. Even when raw MRM peak areas (blue) monitored across 10 replicate injections possess higher experimental variability, ratio to an internal standard (yellow) can correct for much variability and greatly improve reproducibility. An average of 4 MRM ratios per peptide (green) shows even higher reproducibility.

When monitoring the raw peak area across replicate injections, the %CVs seen for the various MRM transitions were centered at 15-25% (Figure 4, blue). This data is showing more variability than other MRM reproducibility data[1], specifically due to the added error from the injection method. However, from the exact same set of data, when the MRM ratio (light/heavy) is computed for each MRM ratio pair and the reproducibility of that MRM ratio is compared across the replicate injections, a significant improvement is observed in the reproducibility (Figure 4, yellow). This is because the Global Internal Standard (GIS) can correct for the experimental variability from the injection method and any other fluctuations in LC or MS.

In this example, 4 different fragment ions were monitored for each peptide (light and heavy) therefore 4 MRM ratios were computed per peptide. When those 4 MRM ratios are averaged together to produce a single peptide ratio, and the reproducibility of that peptide ratio is compared across replicate injections, the reproducibility improves even further (Figure 4, green). This data highlights the robustness that can be built into an MRM assay by using the mTRAQ™ Reagent to create an internal standard for every peptide and MRM.

Analytical Reproducibility of Quantitative MRM Assays using mTRAQ™ Reagents

To evaluate the ultimate reproducibility of an MRM assay using the mTRAQ™ Reagent Global Internal Standard, another experiment was performed where a full loop injection method was used to increase the reproducibility of the raw MRM peak areas. Here, an acquisition method was created using the *Scheduled* MRM™ Algorithm for 784 MRM transitions to 98 peptides and applied to the analysis of human plasma, 10 replicate injections were performed on the 5500 QTRAP® system. The data was again processed using MultiQuant™ Software.

A comparison between the raw MRM peak areas, MRM ratios and peptide ratios is shown in Figure 5. This plot is similar to the plot in Figure 4 however in this case the y-axis is cumulative number of measurements at each increasing reproducibility level. This view easily illustrates the improvement in assay robustness by plotting the amount of data contained below each increasing level to % CV. Using the 5% CV level as a cutoff, it is clear how the use of the internal standard can greatly increase the assay robustness. Greater than 90% of the peptides have reproducibility better or equal to 5% CV across replicate injections. By using mTRAQ™ Reagent to create RIS or GIS where there will be an internal standard peptide and MRM for every analyte to be monitored, highly robust reproducible MRM assays can be developed.

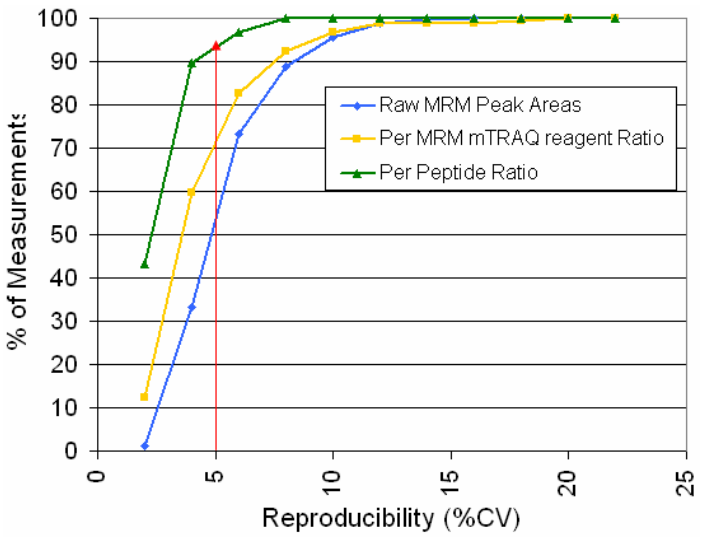

Figure 5. Reproducibility of MRM transitions in Human Plasma. An acquisition method of 784 MRM transitions was created using the *Scheduled* MRM™ algorithm and run using the 5500 QTRAP® system. A very high percentage of the peptide ratio data had %CV <5, the use of mTRAQ™ reagents as an internal standard provides extremely high reproducibility data.

mTRAQ™ Reagents Provide a Cost Effective Way to Create Internal Standards

When larger numbers of proteins / peptides need to be monitored in an MRM assay, the mTRAQ™ Reagents provide a much more cost effective way of creating internal standards for all peptides. A cost calculator has been developed to compare the cost of creating the required internal standards to be compared so that the best, most cost-effective approach can be selected[3].

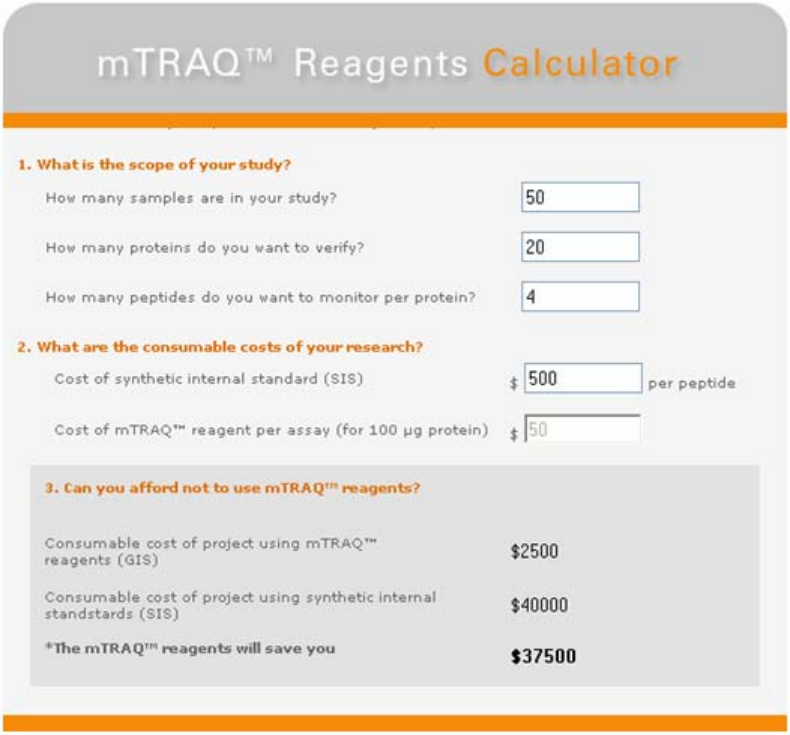

Figure 6. Cost Calculator to Determine Cost of Creating Internal Standards for MRM Assays. Enter the scope of the specific study (# of samples, # of proteins, etc) and the consumable cost of each internal standard strategy will be computed[3].

Powerful Software for MRM Assay Development and Data Processing

MRMPilot™ Software allows a user to rapidly create robust MRM assays for the quantification of proteins and peptides in biological matrices. The software enables the iterative optimization of MRM transitions by leveraging the MIDAS™ Workflow on the QTRAP® Systems[4]. A final optimized MRM assay is built using the *Scheduled* MRM™ Algorithm to use in targeted quantitative assays. MultiQuant™ Software provides a comprehensive package for processing peptide quantitation data from mTRAQ™ Reagent based MRM experiments[5].

Conclusions

- Using the mTRAQ™ Reagent to create internal standards for all peptides provides much more reproducibility in the quantitative MRM assays for many proteins/peptides across large datasets.
- MRM assays with analytical reproducibility of <10% are easily achievable with mTRAQ™ Reagents
- When higher numbers of peptides need to be monitored, mTRAQ™ Reagents provide a more cost effective strategy compared to other internal standard approaches

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