Reducing Sample Amounts for Isobaric Tagging Quantitative Proteomics Experiments

Thomas Abbott, Mary LoPresti, Christopher Colangelo
Yale Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven CT

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Introduction

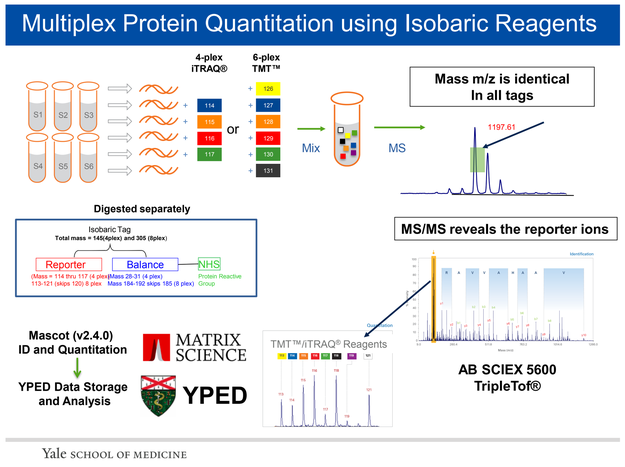
Several isobaric tagging methods have been developed for quantitative proteomics projects and are useful across mass spectrometers (MS) from various manufacturers. Isobaric tagging allows quantitative comparison across multiple conditions or replicates based on the MS/MS peak areas of reporter ions. The reporter ions are bound to peptides with a mass normalized linker region and then fragmented during the tandem MS (MS/MS) stage of acquisition. Because the individually labeled samples are pooled prior to the liquid chromatographic (LC) introduction into the mass spectrometer, peptides common across all samples add to the MS signal, and the signal contribution of individual samples can be determined by the reporter ions.

Two of the most commonly used isobaric tagging kits are the iTRAQ® labels from AB Sciex and the TMT™ from Thermo Scientific. The iTRAQ® come in 4-plex and 8-plex kits, and TMT™ are available in 2-plex and 6-plex kits. Kit instructions for both reagents recommend using 20-100 µg of digested protein per isobaric label. Both sets of reagents are supplied in excess to ensure complete peptide labeling. One notable difference between iTRAQ® and TMT™ is that the iTRAQ® reagents are supplied in excess because the tag has a 15 minute half-life in 70% organic/30% water, whereas the TMT™ reaction is quenched with 5% hydroxylamine to remove excess TMT™ reagents. Samples are then ready to be desalted or fractionated prior to LC-MS/MS.

Due to the ever increasing sensitivity and speed of new generations of mass spectrometers, more proteins and peptides can be identified and quantified with lower amounts of sample. Original experiments needed multidimensional protein identification technology (MudPIT) style workflows to increase peptide separation and decrease sample complexity in the mass spectrometer. This led to the requirements of large amounts of sample for labeling and subsequent fractionation. However, with the advances in MS cycle time and sensitivity, the required amount of sample and pre-fractionation for the same number of identified peptides has progressively shrunk. These improvements have also reduced the amount of isobaric tagging needed per sample, allowing the use of single kits for multiple experiments, thus reducing the cost per sample. In order to test the feasibility of using lesser amounts of label on small amounts of sample, we ran iTRAQ® 4-plex and TMT™ 6-plex labeling on a desalted HEK digest at 1, 5, and 10 µg aliquots per label. iTRAQ® 8-plex reagents were unavailable at this time and will be tested in the future.

Workflow

Figure 1



Materials and Methods

HEK cell lysates were prepared by RIPA lysis and sonication, and proteins were methanol-precipitated. The precipitate was resolubilized in 8 M Urea, reduced, alkylated, diluted to 2M Urea, and dual digested with Lys-C followed by trypsin. The HEK proteolytic peptide mixture was then desalted by solid-phase extraction. The iTRAQ® 4-plex kit was purchased from AB Sciex (Chicago, IL, USA) and the 6-plex TMT™ kit and 50% hydroxylamine were purchased from Fisher Scientific (Suwance, GA, USA). All additional buffers, solvents, and acids were purchased from Sigma Aldrich (St. Louis, MO, USA).

The C18 Macrospin (Nest Group, Southboro, MA) desalted HEK digest, previously quantitated by amino acid analysis at 4.3 µg/µl, was thawed and 41.28 µl was aliquotted. The sample was dried in a speedvac, then redried from 50 µl of water to remove residual acid from the C18 desalt step. The sample was dissolved in triethylammonium bicarbonate (TEAB) from the iTRAQ® kit, to a concentration of 2 µg/µl. The sample was then split into 10 aliquots of 10 µg/5µl each, 10 aliquots of 5 µg/2.5µl each, and 10 aliquots of 1 µg/0.5µl each. 4 aliquots of each concentration were set aside for iTRAQ® labeling and 6 were set aside for TMT™ labeling as outlined in **Figure 1.**

For the iTRAQ reagents, one set of 4-plex iTRAQ® reagents were allowed to come to room temperature, spun down, and 70 µl of ethanol was added to each label, vortexed, and spun down for a final volume of 72-75 µl. Because the kit instructions assume 20-100 µg of protein, lower ratios were calculated assuming a 60 µg load for a full iTRAQ® kit. So the 10 µg aliquots were assumed to be 1/6th of a label, or 12 µl, the 5 µg aliquots used 6 µl of each label, and the 1 µg aliquots used 1.2 µl of each label. Labels were added to the respective HEK aliquots, vortexed, spun down, and incubated for an hour at room temperature. Then, 100% of the samples labeled with 114 and 116 and 50% of the samples labeled with 115 and 117 were pooled, to provide known ratios. The three pooled samples, totaling 30 µg protein, 15 µg protein, and 3 µg protein, were vortexed to mix, spun down, dried in a speedvac, dissolved in 4 µl 70% formic acid, and then diluted with 46 µl 0.1% trifluoroacetic acid (TFA) in water in preparation for C18 desalting.

For the TMT™ aliquots, one 5 mg/vial 6-plex set of TMT™ reagents were allowed to come to room temperature, then dissolved in 256 µl of acetonitrile, vortexed, and let set for 5 minutes. Each label was split into 10 aliquots, and 9 were redried and stored at -80^{o}. Kit instructions say 25-100 µg will be labeled by 41µl of label. Lower ratios were calculated assuming a 60 µg load for a full TMT™ kit. The 10 µg aliquots were assumed to be 1/6th of a label, or 7 µl, the 5 µg aliquots used 3.5 µl of each label, and the 1 µg aliquots used 1 µl of each label (rounded up for pipetting). Labels were added to the respective HEK aliquots, vortexed, spun down, and incubated for an hour at room temperature. The TMT™ reaction was quenched using 4 µl of 5% hydroxylamine and incubated at room temperature for 15 minutes. Then, 100% of the 1 µg samples labeled with 126, 127, 128, 129 and 50% of the samples labeled with 130 and 131 were pooled, to provide known ratios. For the 5 µg aliquots, 100% of the samples labeled with 126, 127, 129, and 131 and 50% of the samples labeled with 128 and 130 were pooled, and for the 10 µg aliquots, 100% of the samples labeled with 126, 127, 128, and 131 and 50% of the samples labeled with 129 and 130 were pooled. The three pooled samples, totaling 5 µg protein, 25 µg protein, and 50 µg protein, were vortexed to mix, spun down, dried in a speedvac, dissolved in 4 µl 70% formic acid, and then diluted with 46 µl 0.1% trifluoroacetic acid (TFA) in water in preparation for C18 desalting.

The six pooled samples were desalted on C18 Macrospin columns if more than 30 µg of protein or C18 Microspin columns for less than 30 µg of protein. The columns were conditioned with 2 x 400 µl of 80% acetonitrile, 20% water, 0.1% TFA by centrifugation for 1 minute at 110 g, then flushed with 2 x 400 µl of 0.1% TFA in water for 1 minute at 110 g, then blotted dry. The samples were placed in the columns and spun for 1 minute at 110 g, the flow through was saved, and then the sample was washed with 2 x 450 µl 0.1% TFA in water. Samples were eluted with 360 µl 80% acetonitrile/0.1% TFA in water at 110 g for 1 minute, then again with 180 µl 80% acetonitrile/0.1% TFA in water at 110 g for 1 minute, combined, and dried in a speedvac.

The dried samples were dissolved in 4 µl 70% formic acid/water, then diluted with 13 µl 0.1% TFA in water, then quantitated by A280 absorption by NanoDrop (Thermo Scientific, Wilmington, DE, USA). Samples were either left “as is” (1 µg pools) or diluted to 0.6 µg/µl (5 and 10 µg aliquots), then loaded on a AB Sciex 5600 Triple-TOF, run with an in-line Waters (Milford, MA, USA) nanoAcquity UPLC, with 0.1% formic acid in water as Buffer A and 0.1% formic acid in acetonitrile as Buffer B, Peptides are desalted on a nanoAcquity Symmetry C18 UPLC Trap column for 1 minute at 15 ml/minute in 99% Buffer A, then on a 75 mm x 150 mm BEH C18 column, and run a 160 minute linear gradient 99%A to 65% A at 500 nanoliters/minute, then washing the column for 3 minutes with 95% B, and then reequilibrating, for an injection-to-injection time of 180 minutes. The MS conditions were 2300 V IonSpray Voltage Floating, Ion Source Gas 1 of 10, Curtain Gas of 20, Interface Heater Temperature 120, with a declustering potential of 60. MS cycle time was 250 milliseconds, with a maximum 20 MS/MS spectra taken each with a 100 millisecond accumulation time, and collision energy adjusted for iTRAQ® reagents.

Raw MS were converted to Mascot Generic File (mgf) format with AB Sciex MS Data Converter and searched with Mascot, using iTRAQ® or TMT™ quantitation, 2 missed cleavages, and peptide charge +2, +3, and +4, with fixed carbamidomethyl cysteine and variable modifications of oxidized methionine and phosphorylated serine, threonine, and tyrosine, and a decoy database, against the Swissprot Human database. Results were posted to the Yale Protein Expression Database (YPED) for comparison.

Results

Protein and peptide ID results are presented in **Figure 2**. The 5 and 10 µg runs are highly similar to each other within each isobaric tag, while the 1 µg runs show fewer proteins and peptides identified. This is likely due to losses at the final C18 cleanup step and subsequent lower amount of material loaded in the mass spectrometer. Normal practice at the Keck Lab is a 3 µg protein in a 5µl injection for isobaric experiments. Nanodrop results show that the post C18 1 µg samples are below the optimal concentration of 0.6 µg/µl, and consequently fewer proteins are identified.

Figure 2

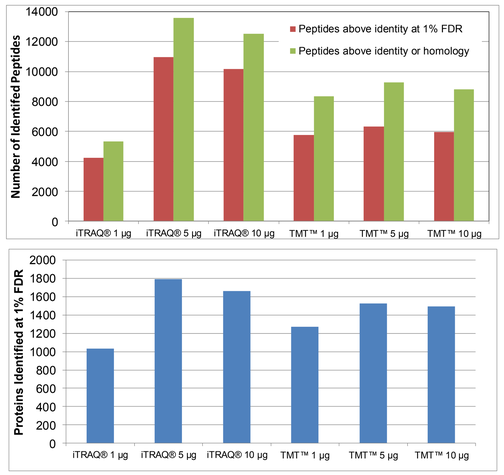


Figure 3A shows the overlaid total ion chromatograms (TIC) of the iTRAQ® samples, and **Figure 3B** shows the same for the TMT™ samples. In both cases, the 1 µg amount has a lower signal response, consistent with less material loaded, while the 5 and 10 µg loads are closely matched. Since the 1 µg/label experiment would have at most 3 µg of total protein with zero losses, it is unsurprising that lower amounts of protein are available to load onto the mass spectrometer. The nearly equivalent signal intensities for the 5 and 10 µg indicates that the losses are at the final C18 step, and that both 5 and 10 µg labeling yield high enough protein concentration even after losses during C18 desalting to give similar results.

Figure 3A

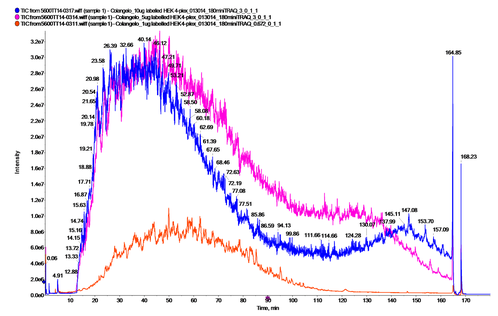


Figure 3B

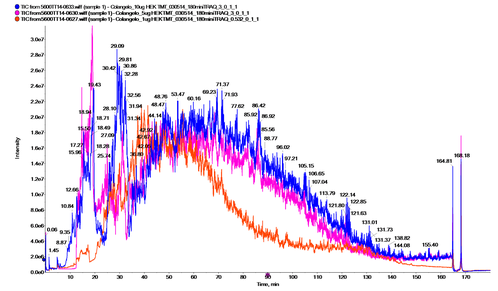


Figure 4A

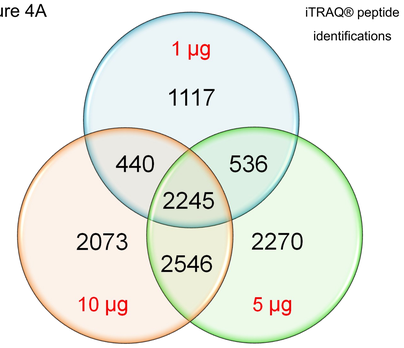


Figure 4B

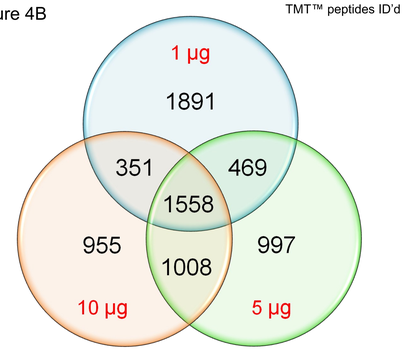


Figure 4A shows the intersection of number of peptides above the Mascot identity threshold for the three iTRAQ® samples and **Figure 4B** shows the intersection for the TMT™ samples. **Figures 5A**, **5B**, and **5C** show the number of unique and shared peptides in TMT™ versus iTRAQ® for the 1, 5 and 10 µg loads.

Figure 5A

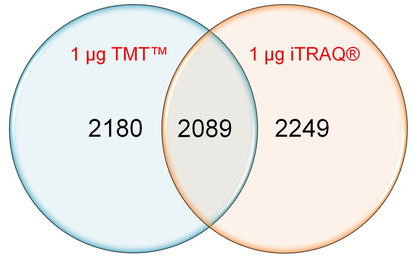


Figure 5B

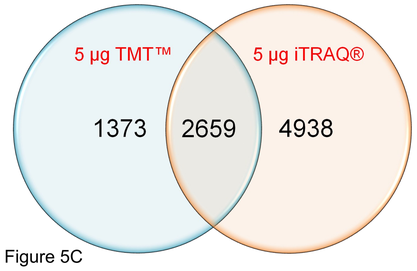
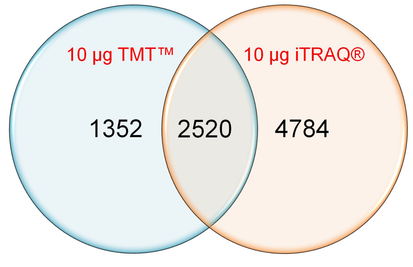


Figure 5C



Quantitation results are presented in **Table 1** and **Table 2**. **Table 1** shows the mean isobaric ratios without normalization in the iTRAQ® samples. **Table 2** shows the mean isobaric ratios without normalization in the iTRAQ® samples.

Table 1

Experiment	Unnormalized 115/114 ratio	Unnormalized 116/114 ratio	Unnormalized 117/114 ratio
iTRAQ® 1 µg	0.5484	0.9906	0.6298
iTRAQ® 5 µg	0.4684	0.9105	0.5247
iTRAQ® 10 µg	0.338	1.138	0.3673

Table 2

Experiment:	127/126	128/126	129/126	130/126	131/126
TMT™ 1 µg	0.9702	0.7416	1.325	0.9398	0.8448
TMT™ 5 µg	0.8702	0.6238	1.006	0.7988	1.168
TMT™ 10 µg	0.9708	1.0182	0.5576	0.612	1.074

Figure 6A

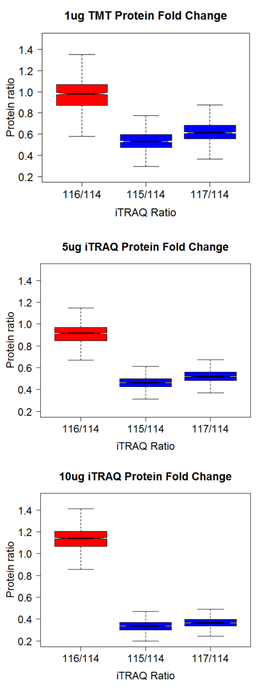


Figure 6B

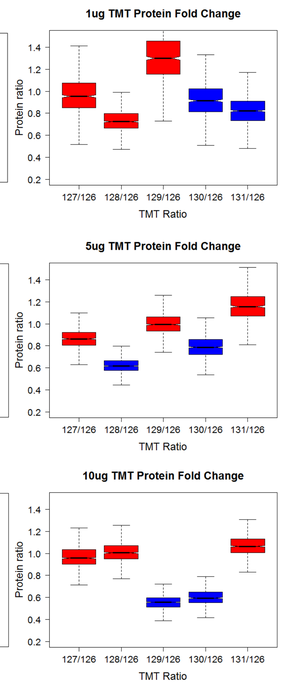


Figure 6A shows box plots of the iTRAQ® ratios for each microgram load, and **6B** shows the TMT™ label ratios. When the iTRAQ® samples were pooled, labels 115 and 117 were combined at 50% the volume to create isobaric area ratios, which is reflected in the ratios present in the sample. For the TMT™ samples, 50% of labels 130 and 131 were used in the 1 µg, 50% of 128 and 130 labels were used in the 5 µg, and 50% of the 129 and 130 labels were used in the 10 µg. Mascot ratios show the lower amounts for the labels respectively. The TMT™ reagents are dissolved in anhydrous acetonitrile, so the variation in ratios probably shows evaporation and pipetting error rather than label inefficiency.

Conclusions

Isobaric tagging reagents label completely and efficiently at lower sample amounts than recommended by the literature. High numbers of proteins and peptides can be identified with smaller amounts of material, but take losses at the lowest level due to absolute protein amount, independent of label type or efficacy. Consequently, isobaric tagging kits can be used for greater numbers of reactions.

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