Quantitative Peptide Assays for Mass Spectrometry Applications

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Overview

Purpose: We set out to develop microplate assays for simple, reliable, and sensitive quantitation of individual peptides or complex digests. These assays are being designed to aid in the evaluation and normalization of samples before analysis by mass spectrometry.

Methods: Two methods are being examined and optimized; an novel absorbance-based colorimetric assay, based on the reduction of copper, and a fluorescence-based assay, based on the production of a fluorescent signal from the labeling of N-terminal amines. Each assay was developed and evaluated with a series of different peptides and protein digests. Finally, both assays were used to evaluate the processing of several labeling and fractionation experiments upstream of analysis by mass spectrometry.

Results: Both methods show significant sensitivity and flexibility, making them excellent tools for determining and monitoring the concentration of peptide samples. Both assays use a minimal amount of sample (10–20 µl) yet still display excellent sensitivity with a working peptide concentration range for the absorbance and fluorescence assays of 12.5–1000 µg/ml and 2.5 – >500 µg/ml, respectively. In addition, each assay displays unique features that make them useful tools for monitoring other common mass spectrometry applications. The fluorescent assay reacts with the N-terminus of peptides, which allows it to monitor protein digestions or the labeling of peptides with TMT reagents. The absorbance-based assay is peptide amide-backbone dependent allowing for the quantitation of both labeled and unlabeled peptides.

Introduction

Mass spectrometry is a sensitive method for the proteomic scale identification of proteins and for the relative quantitation of regulation differences between multiple samples. Advances in mass spectrometry continue to push our ability to characterize complex systems and identify biomarkers. However, despite the power of mass spectrometry, a majority of samples are analyzed without significant “pre-injection” characterization or normalization as many of the standard methods to monitor and measure proteins, such as UV absorption or protein assays, work poorly with peptides or they consume too much valuable sample, due to a lack of sensitivity. This lack of sample characterization and normalization leads to difficulties with the standardization and reproducibility of mass spectrometry experiments which can lead to significant amounts of under-productive instrument time. There is a need for a robust solution to accurately and easily measure the concentration of peptide samples or monitor the efficiency of digestion and labeling reactions. This need is of particular interest to researchers who want to measure recovery from peptide fractionation methods or standardize HPLC load amounts for quantitative proteomic analysis. To address these needs, we explore multiple options for the development of novel sensitive assays to quantitate peptide samples before analysis by mass spectrometry.

Methods

Materials

All peptides were purchased from AnaSpec and used without further treatment unless specified. All chemicals and proteins were purchased from Thermo Scientific, Sigma-Aldrich, or Acros. Tryptic digests, TMT® labeled samples, and fractionated samples were all produced using a variety of internal laboratory procedures.

Absorbance Assay Protocols

Standard solution, peptide samples, or protein digests were loaded at 20 µl/well into microplates. Assay working reagent was added at 180 µl/well working solution. The plate was sealed, quickly mixed and incubated for 30 min at 22–45°C. The absorbance was read at 480 nm using a SpectraMax® Plus^{384} or Thermo Scientific™ Varioskan™ Flash (Type 3001) microplate reader.

Fluorescence Assay Protocols

Standard solution, peptide samples, or protein digests were loaded at 10 µl/well into microplates. Assay buffer (70 µl) and detection reagent (20 µl) were then consecutively added and mixed. The plate was incubated for 5 min at room temperature. The fluorescence was measured with excitation at 390 nm and emission at 475 nm on a Varioskan Flash or Tecan Safire™ microplate reader.

Results

Determining Assay Sensitivity and Benchmarking

During optimization experiments, a variety of peptides were serially diluted and the limit of quantification (LOQ) was determine for the new assays and other common measurement methods. In Figure 1, the sensitivity of our new absorbance-based assay was compared to our Thermo Scientific™ Micro BCA™ assay of two peptides. The new assay shows a 3–4 fold increase in S/N when compared to the Micro BCA assay for all peptides tested. Similar results were seen for all peptides tested, and average concentration at the LOQ was ~15 µg/ml. Figure 2 shows the working range of our new fluorescence-based assay for both a digest of BSA and the peptide bacitracin. The limit of quantification was calculated at approximately 2.5 µg/ml and a linear response curve could be seen for sample concentrations ≥1.0 mg/ml. By comparison, 214 nm absorption measurements for the same sample volume (10 µl) of bacitracin diluted to 100 µl and read in a quartz 96-well microplate (100 µl minimal volume) had a limit of detection of approximately 25 µg/ml (data not shown). The fluorescent assay was approximately ten times more sensitive than UV absorption measurements, without the need for sequence data to calculate A214 nm.

FIGURE 1. Comparison of novel absorbance based peptide assay and the Micro BCA assay.

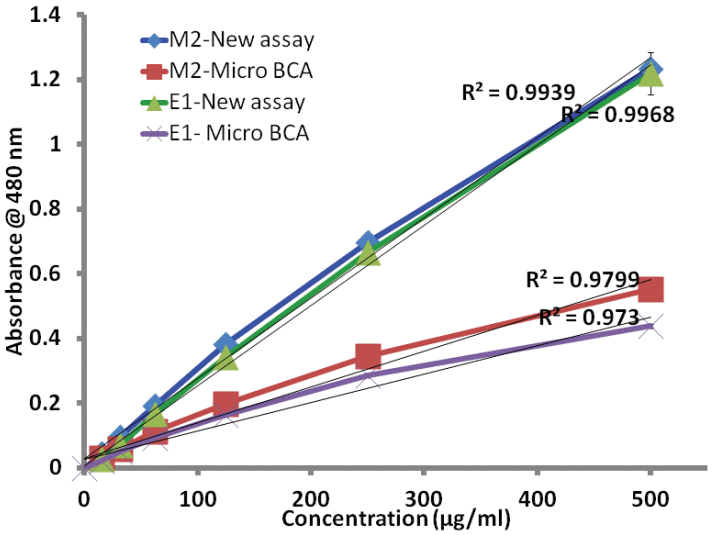
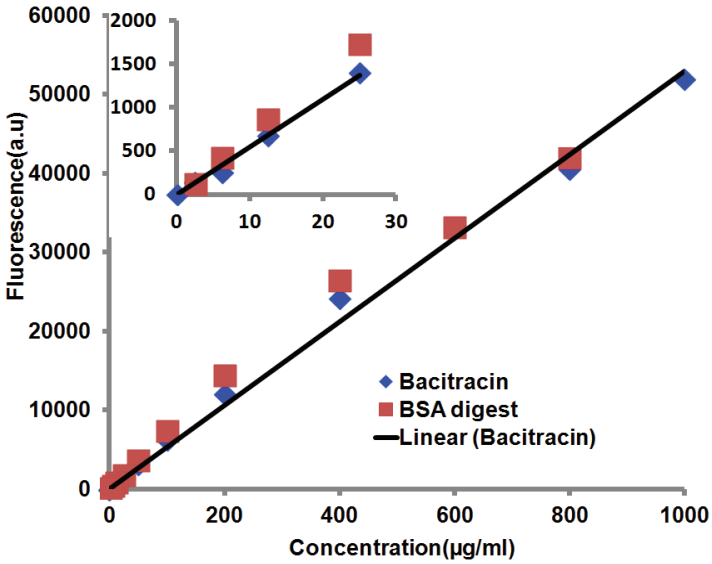


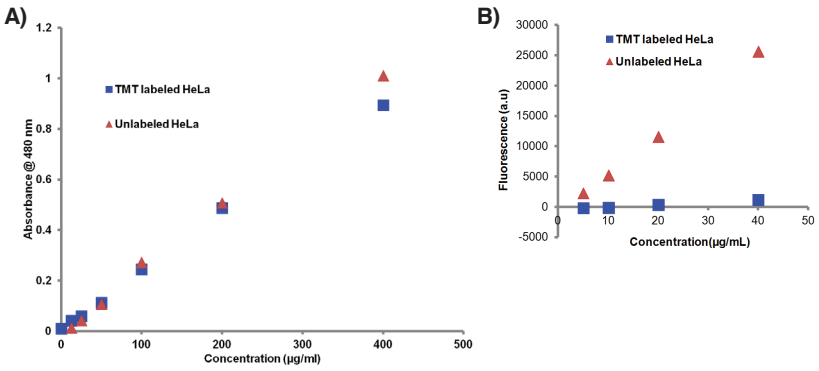
FIGURE 2. Working range for the new fluorescence based assay.



Additional Assay Applications: Use with Labeled Peptides and Digestion Monitoring

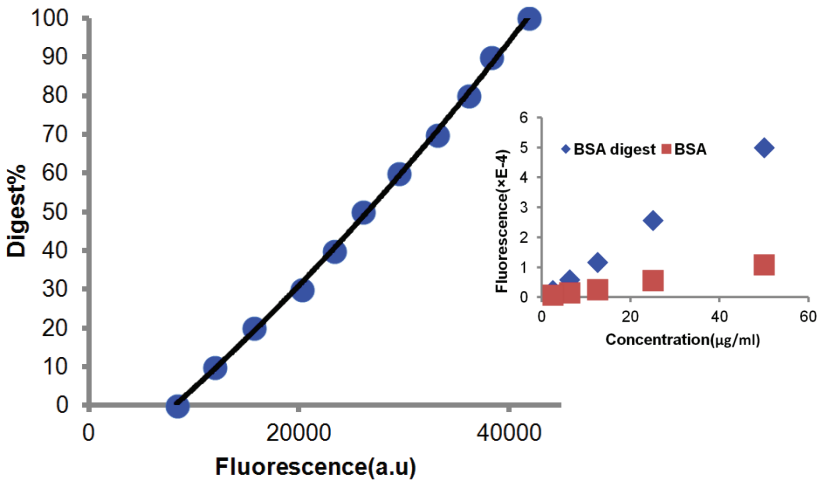
The two assays under development react with peptides via different mechanisms, which gives the assays unique properties that are complementary for a variety of applications. To demonstrate this, both assays were used to measure a TMT-labeled and unlabeled tryptic digest of a HeLa lysate. The absorbance assay is based on the reduction of copper which is largely dependent on the peptide amide-backbone; therefore, it is not effected by peptide modification such as TMT-labeling of amine groups. This can be seen in Figure 4A, which demonstrates identical responses for a TMT-labeled and unlabeled sample. This allows load/injection amounts of TMT-label samples to be standardized between MS analysis experiments improving results and consistency. The fluorescence assay, however, is dependent upon reaction with the N-terminus of peptides to produce a fluorescent signal. Therefore, TMT-labeled peptides will not produce a signal as shown in Figure 4B. This property can be exploited for monitoring the completeness of labeling reactions prior to analysis by mass spectrometry.

FIGURE 4. Assay of HeLa digests with and without TMT labeling. (A) Absorption assay. (B) Fluorescent assay.



The dependence of the fluorescent assay on N-termini also allows it to be used to monitor the tryptic digestion of protein samples. The inset of Figure 5 shows minimal signal from intact BSA. However, upon digestion, the creation of new N-termini results in a corresponding increase in signal. To demonstrate the direct response to digestion efficiency, 100 μg samples of undigested and digest BSA were mixed at a series of ratios from 0:100 to 100:0 (undigested:digested). Mixtures were assayed and the fluorescent signal was plotted in Figure 5. The fluorescence assay shows a very linear correlation between digest content and fluorescence.

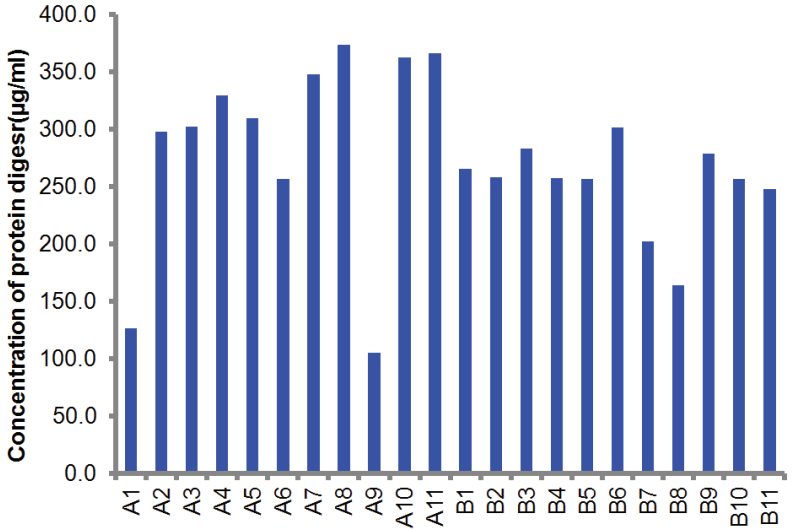
FIGURE 5. Fluorescent response to digestion efficiency.



Assessing Assay Variation

To assess the variation in fluorescent response, 22 proteins in a pure powdered form were weighed and formulated at identical protein concentrations (352 µg/ml). Samples were then digested under the identical conditions and the total peptide concentration was measured using the fluorescent assay (Figure 6). The average digest concentration determined by the fluorescent assay using a BSA digest as a standard was 274 µg/ml with a coefficient of variation between the measurement of ~26%. This demonstrates surprising little variation despite the different molecular weights, molar protein concentration, and potentially different digestion efficiencies for the proteins tested. We believe this because most proteins (at the same starting weight concentration) will produce similar molar concentrations of peptides after tryptic digestions due to a fairly consistent percentage content of lysine and arginine residues. This trait will be even more consistent across digests of complex samples such as cell lysates and serum samples allowing these peptide assays to be used and compared across a wide variety of samples types.

FIGURE 6. Variation in fluorescent assay response for a series of protein after tryptic digestion.



Key	Protein	Key	Protein
A1	Alcohol dehydrogenase	B1	Transferrin
A2	α-Cesein	B2	Rabbit IgG
A3	Holo-transferrin	B3	Human IgG
A4	Ribonuclease B	B4	Protein A
A5	B-Casein	B5	Protein G
A6	Haptoglobin	B6	Protein A/G
A7	Lysozyme-I	B7	Protein L
A8	Apo-transferrin	B8	Neutravidin
A9	B-galactosidase	B9	Ovalbumin
A10	Albumin	B10	Streptavidin
A11	Myoglobin	B11	BGG

Normalization of MS Samples for Relative Quantitation

Non-small lung carcinoma cell lines were treated with ten different sets of conditions, lysed, reduced, alkylated, and digested with trypsin before desalting using C18 columns. The concentration of each digest was then determined using both peptide assays (Figure 7). Each peptide sample concentration was then normalized before labeling individually with one of the Thermo Scientific™ TMT10plex™ isobaric labeling reagents. Labeled samples were combined before peptide identification and relative quantitation using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. After normalization, non-regulated peptides displayed ratios of 1:1:1:1:1:1:1:1:1:1 despite complex sample handling and preparation (Figure 8).

FIGURE 7. Concentration comparison and normalization across multiple samples before TMT10plex labeling normalized to sample #1.

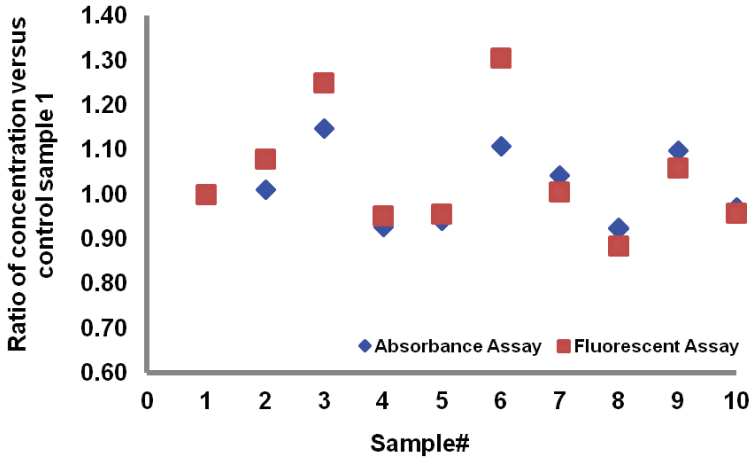
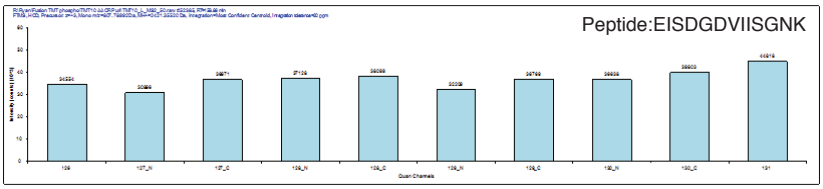


FIGURE 8. TMT10plex quantitation of non-regulated peptides show similar ratios for normalized samples.



Conclusion

- Two sensitive, rapid, and easy-to-use peptide assays display significant potential as tools for analyzing and standardizing samples before analysis by mass spectrometry.
- A colorimetric and fluorescent assay have been developed that allow the analysis of peptide concentrations using as little as 0.3 μg or 0.03 μg of sample, respectively.
- Both assays display low levels of sample-to-sample variation for digest containing a mixture of peptides. In the future, we wish to examine and optimize assay conditions to enhance sensitivity and potential minimize peptide-to-peptide variability for other applications utilizing pure individual peptides.
- Future Goals: Conduct collaborative testing to further identify potential uses and/or limitations of both assays.

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