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Recommendations for the generation, quantification, storage and handling of peptides used for mass spectrometry-based assays

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Keywords

calibrators; internal standards; lc-ms/ms; mass spectrometry; peptide; reference material

Introduction

The Clinical Proteomic Tumor Analysis Consortium (1) (CPTAC) of the National Cancer Institute (NCI) is a comprehensive and coordinated effort to accelerate the understanding of the molecular basis of cancer through the application of robust technologies and workflows for the quantitative measurements of proteins. The Assay Development Working Group of the CPTAC Program aims to foster broad uptake of targeted mass spectrometry-based assays employing isotopically labeled peptides for confident assignment and quantification, including multiple reaction monitoring (MRM; also referred to as Selected Reaction Monitoring), parallel reaction monitoring (PRM), and other targeted methods.

Guidelines for reagents and methods will ensure that targeted measurements of peptides are of high quality, distributable, and fit-for-purpose to quantify analytes in the intended matrix (plasma, serum, cells, and tissues). Towards these goals, we have: (i) coordinated a consensus approach to outline recommendations for the development of different classes of targeted mass spectrometry (MS)-based assays using a fit-for-purpose approach (2), (ii) launched the CPTAC Antibody Portal (3) (antibodies.cancer.gov) to facilitate the production, characterization and distribution of renewable affinity reagents to the community in order to support protein/peptide measurement and analysis, and (iii) launched and begun to populate the CPTAC Assay portal (4) (https://assays.cancer.gov/) to disseminate highly characterized targeted MS-based assays to the community, via access to standard operating protocols (SOP), reagents, and assay characterization data.

Within workflows designed to quantify protein-derived biomarkers by proteolytic digestion and LC-MS/MS, synthetic peptides are often used in three ways: (1) stable isotope-labeled internal standard peptides with the same sequence as the analyte of interest are spiked into the digest and help ensure that the correct peptide is being identified and quantified (i.e., they have the same retention time, same secondary structure, and similar fragmentation pattern as the endogenous analyte), (2) stable isotope-labeled internal standard peptides help

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normalize sample-specific ion suppression and are used in the calculation of a peak area ratio (i.e., the ratio of the endogenous analyte chromatographic peak area to that of the internal standard), and (3) unlabeled or labeled peptides can be used to generate calibration materials for the quantification of peptide in proteolytic digests of complex protein mixtures (i.e., determining the peak area ratio at known concentrations of analyte in a relevant matrix). Well-characterized peptides, along with detailed standard operating procedures for proteolysis and sample preparation, are necessary to harmonize peptide-based assays (4). In the clinical laboratory, calibration materials are more commonly based on intact proteins in a relevant matrix, which may be useful in further harmonizing the quantification of proteins between laboratories (5-7). Of note, in addition to isotope-labeled internal standard peptides, isotope-labeled extended peptides (also called “winged” peptides, which include a proteolytic digestion site) and recombinant proteins can be used to compensate for the additional variability due to digestion (5, 6). The latter will be increasingly important, particularly in clinical applications, as properly folded recombinant isotope-labeled proteins become more widely commercially available.

Once proteolytically digested, the quantification of peptides in the sample can be easily facilitated by using stable isotope-labeled peptides as internal standards and traditional LC-MS/MS methods. Most commonly trypsin is used for proteolysis, resulting in arginine or lysine at the C-terminus. As a result of the variability amongst peptides in their recovery and stability following proteolytic digestion, especially between samples and sample types, as well as the isoform complexity of human proteins, the peak area ratio or measured concentration of any peptide may not *accurately* reflect the concentration of any given intact protein or isoform. Importantly, the same potential limitation also applies when using isotopically-labeled proteins as internal standards. While potentially providing improved precision and less bias than labeled peptides in quantitative MS-based assays (8, 10), differences in the repertoire of modifications (e.g., phosphate, carbohydrate, ubiquitin, etc.) on amino acid residues near enzymatic cleavage sites, for example, can alter digestion and recovery of desired analyte peptides from internal standard proteins. Regardless of the internal standard chosen, with a detailed, reproducible, and robust standard operating procedure for sample preparation and digestion in place, it is possible that the peak area ratio of a liberated peptide could be precise enough to be a biomarker without calibration to a protein concentration.

Therefore, the goal of establishing recommendations for peptide-based, targeted MS measurements is to achieve *precise, relative quantification that can be harmonized across laboratories*, increasing the replicability of research and enabling the aggregation of data across experiments and laboratories, as well as enabling the robust quantification of peptides and proteins in clinical laboratories. In addition to the need for transparency in digestion methods and sample preparation (i.e., freely available standard operating procedures) (4, 11), the major challenges to achieving this analytical bar are: (i) selection of peptides that can be measured with high precision and repeatability in the matrix of interest, (ii) generating well-characterized, pure synthetic peptide internal standards and calibrators, (iii) determining the accurate concentration of pure synthetic peptide internal standards and calibrators, (iv) assurance of the quality (e.g. concentration, stability) of the peptide internal standards and calibrators in lyophilized form and in solution over time, during both storage

and handling, and (v) proper interpretation of peptide-based measurements. The purpose of this manuscript is to address these key components by aggregating recommendations, based on published studies and/or the consensus experiences of the authors. The use of “crude” peptides (that have limited or no purification or quantification but are far less expensive than purified peptides) is also discussed, and the limitations of their use for quantitative measurements are presented. The manuscript provides a thorough framework for proteomics researchers and an introduction towards clinical applications. Ideally, the recommendations included here provide a starting reference point for the production of formal guidelines and best practices in the future (e.g., from the Clinical Laboratory Standards Institute).

Criteria and process for selecting peptide analytes for targeted MS assays

Assays to measure protein concentration by proteolysis and LC-MS/MS (e.g. MRM) selectively quantify “proteotypic” (12) (typically tryptic) peptides that are unique to a single gene product or proteoforms (13) and that are observable by mass spectrometry. Because peptides vary greatly in their performance across many aspects (e.g. ease of synthesis, stability, solubility, recovery, responsiveness in the mass spectrometer) of targeted MS analysis, careful selection of peptide analytes is critical to developing the highest quality assays.

Peptide analytes can be selected using MS-based proteomic data or prediction algorithms (14-22). Selecting peptides from empirical MS data greatly increases the likelihood for success in developing a targeted MS-based assay, as the peptide analytes have been demonstrated to release from the protein of interest upon digestion and are detectable by MS. Candidate peptide analytes from proteins can be selected from either in-house or public empirical data. For the latter, numerous open source proteomic databases and data repositories exist [e.g. GPM (23), PRIDE (24), Peptide Atlas (25-27), PhosphoSitePlus (28)]. The use of spectral libraries, either generated with one's own MS/MS data or obtained from one of the online spectral library repositories, can greatly facilitate selection of the peptides (and the most intense ions) to target for quantification. Bioinformatic approaches can also be used to identify peptide sequences that can be theoretically formed upon proteolytic digestion and that may be useful for MS-based assay development. However, such tools are not as reliable as empirical MS/MS data, which remain the gold standard. In practice, a combination of the two approaches in an iterative manner is often used to hone the choice of the best peptide(s) as rapidly as possible.

A summary of criteria for peptide selection is presented in Table 1. Despite the above-mentioned considerations, peptide selection is an empirical exercise that balances ideal characteristics with practical limitations. In some cases, due to the sequence of the protein of interest, it may be unavoidable to include peptides that do not meet all of these recommended criteria. Therefore these criteria are simply meant to be guidelines for peptide selection, and some relaxation of the inclusion criteria may be necessary to develop the MRM assay. For example, it may be impossible to exclude all cysteine and methionine residues. In such cases, the precursor and product mass-to-charge ratio (*m/z)* values should account for any potential oxidation and carbamidomethylation modifications (+16 and +57

Da, respectively). Additionally, selection of peptides to develop assays for phosphosite quantification is very constrained by the position and potential clustering of phosphosites.

Specifications for production and quality assurance of peptides

Once proteotypic peptides have been selected for assay development, the synthetic peptide is generated as unlabeled and stable isotope-labeled versions and characterized to assess the performance of the peptide assay in the digested matrix of interest and to enable quantification of the endogenous analyte via isotope dilution. Thus, the quality of peptides is a major determinant of reliable quantification. The specifications presented here are intended as a guide for procuring unlabeled and stable isotope-labeled peptides suitable for evaluation and analytical validation of targeted MS assays and to quantify peptides derived from proteins in proteolyzed biological fluids (e.g. serum/plasma, CSF, urine) and cell or tissue lysates. Specifications are provided for purified peptides and for “crude” or unpurified peptides.

Purified peptides are chromatographically purified after synthesis to remove most of the residual salts, synthesis reagents (e.g. deblocking and scavenger), partially deblocked peptides, and truncated peptides (29, 30). In addition, the amount of peptide and its purity need to be specified in advance to ensure that material of sufficient quantity and quality is available for assay development (see https://assays.cancer.gov/about/faq/ for guideline document for assay development and characterization). Purified peptides are typically analyzed by amino acid analysis (AAA) (31) to determine the net peptide content. Net peptide content is a measurement, usually in the form of a percentage that represents the amount of actual peptide within a gravimetrically measured sample. The measurement excludes the weight of water and counter ions that exist in all peptides. *Crude peptides*, on the other hand, may or may not be subjected to additional purification steps after synthesis (e.g., batch solid phase extraction) to remove synthesis by-products, and neither accurate quantity nor purity are possible. The identities of crude peptides must be confirmed by mass spectrometry, and since crude peptides vary greatly in purity it is recommended that further evaluation of purity (e.g., by reversed phase-HPLC-UV) or estimated quantity be performed by the end-user. While crude peptides can help confirm the identity of endogenous peptides and improve the precision of relative quantification within a research laboratory using a single batch of peptides, well characterized, purified peptide calibrators and detailed standard operating procedures are required to distribute assays to the community and harmonize results across laboratories. Importantly, methods intended for use in clinical laboratories use only the highest purity (isotopic and chemical) peptides available.

Unfortunately, the quality and consistency of peptides obtained from commercial sources vary widely. A large number of companies that advertise custom peptides for sale do not manufacture the peptides they sell, but are simply resellers of product made elsewhere. Not only will this have an effect on the batch-to-batch consistency of peptides over time (given the inability to trace production to specific laboratories), the instruments, resins, and amino acid building blocks, as well as the methods utilized to synthesize, purify, freeze dry and package peptides will vary widely dependent upon the manufacturer. It is recommended to use vendors that manufacture their own peptides, and that provide detailed specification and

characterization data for their synthesized products. For purified peptides, this must include MS data (preferably MS/MS data as well) and HPLC-UV chromatogram profiles, preferably using shallow gradients of no more than 2% change in organic concentration/min, otherwise the presence of impurities can be masked by co-elution of contaminant synthesis byproducts. More details on characterization are provided below. Regardless of whether the quality of the peptides is assessed by the vendor, a vendor sub-contractor, or in-house, the standard operating procedures used for quality control should be made available and linked to the corresponding quality control data.

High purity, well characterized peptides

To ensure that the quality of targeted MS data meets the specifications of the assay (e.g. specificity, precision, bias, lower limits of quantification), it is necessary to communicate with the peptide vendor and supply a comprehensive list of detailed specifications (e.g. chemical and isotopic purity, amount, formulation, aliquot size, packaging, etc.), as summarized in Table 2. Critical to the long-term success of assays that rely upon peptide calibrators and internal standards is working with a vendor(s) whose methods are well-documented and whose personnel operate under SOPs to ensure consistency of production over time, and who are willing to customize their methods to meet the end-user's needs. Peptides can be ordered in any amount, from microgram to gram quantities. Peptide synthesis using current automated peptide synthesizers is typically performed at micromole scale (0.1 to 1 mmol) (32-34), which produces yields much higher than those required for targeted MS-based assays (~ 10 fmol/peptide/sample or 100 picograms/peptide/sample for a peptide of MW = 1000 Da). Synthesizing peptides at larger scales results in higher yields, but increases the costs when stable isotope-labeled ‘heavy’ amino acids are used. We have found that a synthesis scale of 1 – 5 mg of purified peptide provides a reasonable balance between cost and yield. As shown in Table 2, one milligram is formulated for amino acid analysis (AAA; see below) and used in assay development. Additional quantities of 1 mg (up to 5 mg total), if ordered, can be delivered as dry powder and stably stored at -20 °C or below until needed.

Stable isotope-labeled amino acids used to produce stable isotope-labeled standard peptides can be synthesized with various elemental compositions (e.g., single position carbon, uniformly ^{13}C labeling at all carbon atom positions, or combinations of ^{13}C and ^{15}N); see Table 2. While it is often less costly to use deuterium as the isotopic label, its use is not recommended because the presence of several deuterium atoms in a peptide can alter the peptide's retention time and prevent the desired co-elution with the unlabeled peptide (35-37). Further, deuterium atoms in reactive functional groups exchange with hydrogen atoms in aqueous solutions, which after time leads to the presence of unlabeled peptide in the internal standard. The difference in mass relative to the unlabeled peptide should be selected to be large enough to avoid interference of the natural peptide's isotopic envelope with that of the isotope-labeled internal standard, otherwise, inaccuracies in quantification can result (38). Peptide precursor *m/z* and charge state should be taken into consideration when selecting a mass difference for the heavy amino acid: the smaller the peptide and higher the charge-state, the larger the mass difference needs to be. For doubly charged precursors, a minimum separation of 6 Da is recommended, while 8-10 Da is recommended

for triply charged precursors. Lysine is available in isotope-labeled forms that are +6 and +8 Da heavier than the unlabeled form, while arginine is available in +6, +8 and +10 Da versions. When additional internal standard peptides are going to be included to provide quality control for enrichment or other steps of sample preparation, additional labeled amino acids can be added to increase the mass difference.

The isotopic enrichment of the “heavy” amino acids used in synthesis should be greater than 99% (Table 2). These percentages refer to the chance of finding the heavy isotope of an atom at each potential label site in the amino acid. Because more than one site in the amino acid is labeled (often 6-10 sites), the probability of finding a labeled atom at every labeled site is lower than the stated enrichment percentage (for a means to visualize this effect, see http://www.chemcalc.org/main). Under-labeled peptide “isotopologues” will introduce inaccuracies in the quantification and can interfere with the measurement of the endogenous unlabeled peptide. For these reasons, it is advised to only use the highest possible isotopic purity labeled amino acids available (>99% enrichment per isotope) when having labeled peptides synthesized. It is also recommended that the relative ratio of heavy-to-light peptide be kept in a reasonable range < 1:25, and preferably <1:10. In clinical laboratories, the final concentration of internal standard peptide is most commonly set near a medically relevant concentration of the endogenous analyte. Other stable isotope-labeled amino acids can be used for incorporating in the sequence when K or R is not present in the peptide (protein C-terminus).

During sample preparation, disulfide bonds in proteins are typically reduced, and cysteine residues are alkylated to prevent reformation of disulfide bonds with the aim of producing stable, denatured proteins with trypsin cleavage sites more accessible to the enzyme. When stable isotope-labeled internal standards are added post-digestion, cysteine residues in synthetic peptides need to be present in the identical alkylated form (e.g., carbamidomethylated, delta 57 amu). Synthesis of peptides containing chemical modifications such as phosphorylation or acetylation is now routine. However, it is worth noting that synthesis of multiply phosphorylated peptides have a higher failure rate than singly phosphorylated peptides. It should be noted that the production of recombinant proteins and peptides does not guarantee proper disulfide bond formation and this can impact digestion efficiency thereby causing the internal standard to behave differently from the native analyte in the assay.

The characterization of synthetic peptides during the course of synthesis and purification was described in the “Six Year Study of Peptide Synthesis” by the Association of Biomolecular Resource Facilities from 1991 -1996 (31). Reversed-phase HPLC (C18 column) with UV monitoring is the best method to assess the complexity of peptide products, although examples of co-elution with truncated peptides have been observed (39). Mass spectrometry using either electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) is essential for the identification of desired products and mass impurities. The coupling of RP-HPLC to ES tandem MS (LC-MS/MS) enables the confirmation of amino acid sequence and residue location of modifications (40). MALDI-MS provides a means for identifying the molecular ion of the desired product and presence

of byproducts, although the relative ion intensities will not accurately measure the molar content of contaminants (41).

The purity and identity of purified peptides are typically evaluated using analytical reversed-phase (RP) chromatography with UV detection (HPLC-UV; monitoring at wavelengths 214 or 220 nm and 280 nm) and by mass spectrometry. The presence of multiple significant peaks in the HPLC-UV trace suggests the presence of synthesis side products; however, a single major peak may or may not correspond to the desired product. MS of the peaks in the UV trace (either by analysis of collected fractions using MALDI or, preferably, using on-line HPLC-MS) defines which peak is the desired peptide and which ones are impurities. MS/MS data are necessary to confirm the sequence of the desired product and to determine the chemical nature of impurities that may be present (e.g., incomplete deblocking, premature termination, etc.).

For characterization by HPLC-UV, MALDI, and especially amino acid analysis (AAA; see below) the peptide must be completely dissolved in solution. Importantly, the formulation and composition of the peptide used for quality control should be identical to that used for assay development and validation. The formulation range recommended in Table 2 was chosen based on our experience handling 1000's of tryptic peptides with a wide range of sequences, lengths and hydropathy profiles. We recommend formulating peptides in 5 – 30 % acetonitrile with 0.1 % formic acid to a target concentration of 0.5-1 mg/mL (500-1000 μM) for AAA (see below) and evaluation by MS. The higher percentages of acetonitrile (up to 30%) are recommended for more hydrophobic peptides. Formulation and storage in neat DMSO is also possible, but oxidation of methionines may occur. Peptide solutions in DMSO need to be diluted prior to LC-MS analysis to avoid poor chromatography. It is also important to note that peptides stored in neat DMSO may precipitate when added to an aqueous solution, so use caution during dilution.

Crude (i.e., lower purity) peptides

Purification by preparative HPLC and quantification by AAA adds significantly to the cost of synthetic peptides. Eliminating these steps and generating “crude” peptides significantly reduces the cost for both natural abundance and stable isotope-labeled peptides. Due to this economy, a larger set of peptides can be selected and tested for development of a protein assay. Crude peptides (also referred to as partially purified peptides) are defined as the deblocked peptides that have been released from the solid phase resin and precipitated with an organic solvent (e.g. ether). Some vendors will perform an additional purification step. However, even after additional purification, preparations of lower purity “crude” peptides may still contain a wide range of impurities, such as residual salts, deblocking and scavenger reagents, and truncated and partially deblocked peptides.

The use of highly purified stable isotope-labeled internal standard peptides is recommended for the development of distributable assays and to improve between-laboratory agreement of assays that rely solely on peak area ratio for peptide concentration assignment. In addition, highly purified internal standards are vital for the successful development and deployment of clinical assays in a CLIA-regulated environment. However, crude peptides can be used for relative quantification of peptide analytes in Tier 2 assays (2), provided that the performance

of the resulting assay is carefully assessed. Crude stable isotope-labeled peptides can also be useful for identifying endogenous analyte, particularly in complex matrices such as cell and tumor digests. Figures of merit (e.g., LLOQ) of Tier 2 assays are often characterized by using various concentrations of unlabeled peptide calibrators in spike-in experiments. It is therefore important to remember that the use of lower purity peptides prevents accurate determination of assay LLOQ, hinders assay transferability, and complicates the comprehensive analysis of data and subsequent assay performance across research laboratories. Crude peptide preparations must still be analyzed by LC-MS/MS, and/or MALDI-TOF-MS to demonstrate that the correct sequence has been synthesized and that the desired product is the predominant species. Since purity varies considerably among different suppliers, crude peptides should also be analyzed by the laboratory developing peptide assays to ensure sufficient quality.

Table 3 summarizes the specifications and analyses to consider in qualifying crude peptides for assays that use a heavy-labeled peptide for each endogenous analyte (Tier 2) (2), or assays that use synthetic peptides that are not paired with each analyte (Tier 3) (2). The specifications differ from those of high purity peptides (Table 2) in the level of chemical purity and effort made to determine the exact quantity of peptide delivered. However, similar to high purity peptides, the isotopic purity should be specified to be > 99 % to obtain the highest sensitivity possible for measuring endogenous unlabeled peptide in the presence of the isotopically-labeled counterpart, as discussed above for high purity peptides. This can be assessed by using MALDI-TOF or LC-MS/MS of individual peptides or mixtures of peptides, respectively.

Figure 1 shows the MALDI-MS spectra of a high and lower purity peptide of the same amino acid sequence. These high purity and lower purity peptides were synthesized and provided from independent syntheses by the vendor using the specifications given in Tables 2 and 3, respectively. A major signal that corresponded to the $[M+1H]^{1+}$ ion (m/z = 1854.94) for the FYGAEIVSALEYLHSR peptide was observed in both the lower purity (Panel A) and high purity (Panel B) peptide sample. The signal at m/z = 1570.68 is from a spiked internal mass calibration standard. In the case of the spectrum from the lower purity peptide preparation, there are multiple lower intensity peaks, likely byproducts of the peptide synthesis. For example, a peak consistent with the desired peptide minus the N-terminal Phe residue (m/z = 1707.85) was observed (Fig. 1, Panel A).

Amino acid analysis of the high purity and lower purity peptide was performed to compare the amino acid content. Unlike the case for high purity peptides, the concentration of the desired peptide cannot be accurately quantified due to the presence of incompletely deblocked and truncated peptide species. Furthermore, in previous reports using AAA of crude peptide preparations to assess content without a desalting step were compromised by residual scavenger reagents (42). Table 4 compares the AAA of the high and lower purity peptides shown in Fig. 1. The molar content for the high purity peptide was in good agreement (within 10 %) with the expected molar content, particularly for the residues that are known to be most stable during acid hydrolysis conditions and those completely released during 24 h hydrolysis (Ala, Leu, and Phe). The presence of peptide species without the N-terminal residue is consistent with the lower content of Phe in the lower purity preparation.

Some amino acids were not detected well in either the high or lower purity samples. Assuming complete residue deblocking and no interferences in the AAA from residual synthesis reagents, 274 nmol of peptide was measured using the averaged quantity from the fiducial residues, Ala and Leu. This value was lower than the amount of peptide quantity quoted for this lot of partially purified peptides by the vendor (i.e., 400 – 700 nmols/vial). Therefore, it is not possible to determine the lower limit of quantification of LC-MS/MS assays when crude peptides are used as standards. If employed in inter-laboratory studies, the same lot of the synthetic unpurified peptide dissolved into solution would need to be used.

Quantifying Pure Peptides by Amino Acid Analysis

Reliable quantification of purified peptides across synthesis batches and amongst vendors is critical to harmonizing concentrations of peptides determined solely from the endogenous peak area ratio with internal standard or determined with external peptide calibration materials across the community and over time. For biomolecule quantification, calibrators with accurate concentrations are frequently prepared by gravimetric methods [if analyte standards are available in sufficient quantity and of known purity (43)]. However, for peptides, preparation of calibrators with accurate concentrations using gravimetric preparation alone is often not feasible because of the limited available quantities and/or uncertain purity. In these cases, the concentrations of peptide calibration solutions can be determined through quantitative analysis of their constituent amino acids after hydrolysis of the peptide's amide bonds (44) (i.e., amino acid analysis; AAA). As mentioned above, AAA of pure peptide internal standards and calibrators is vital to the transferability of assays and aggregation of results amongst research laboratories. However, to improve the similarity of peptide concentration measurements, AAA of different batches of peptides must be accurate and precise, which fundamentally depends on the reliability of the methods, accurate calibration, and quality control of the AAA assays used to quantify amino acids in peptides. Clinical laboratories are much more likely to use purified proteins as internal standards and in external calibration materials. Proper quality control of the AAA assays used to assign the concentration of those proteins is equally important.

Vendors and service laboratories providing AAA analyses vary greatly in their processes and quality controls (QC). Further, peptide vendors offer different levels of assay quality (e.g., ± 5-10 % CV, ± 10-25 % CV), therefore the accuracy of their AAA assays needs to be specified. Given the critical importance of AAA to the harmonization of peptide and protein concentrations over time within a laboratory and across laboratories, it is imperative to ensure that AAA determinations of assay internal standards and calibrators are performed with a high level of rigor. Before selecting an AAA service provider, it is strongly recommended to understand the workflow, standardization, and quality control measures that are in place. This critical information is summarized in Table 5 and discussed in more detail below.

Accurate peptide quantification by AAA does not require the measurement of all constituent amino acids; for most peptides, quantification can be achieved through measurement of one or more stable amino acids (see Figure 2 for a summary of amino acid characteristics). For

example, the most stable amino acids under the conditions of hydrolysis are Ala, Arg, Gly, His, Ile, Leu, Lys, Phe, Pro, and Val, whose side chains are not acid labile. These amino acids are arguably the best targets for quantification. Serendipitously, these amino acids are among the highest frequencies found in nature, and will be present in most peptides. There are some caveats to this list: (1) hydrophobic amino acids such as Ile, Leu and Val can be problematic due to their slow hydrolysis rate, (2) in the presence of phenol (e.g., 0.2%), Tyr is stable during acid hydrolysis and plays an important role in peptide quantification by UV spectroscopy, and (3) the basic amino acids Arg, His, and Lys have longer retention times on reversed phase-ion exchange HPLC columns compared with other amino acids. Under typical acid hydrolysis conditions, Trp is readily destroyed by oxidation. If the measurement of Trp is important, this hydrophobic amino acid can be instead hydrolyzed using 4 mol/L methanesulfonic acid containing 0.2 % (v/v) 3-(2-aminoethyl)indole for AAA quantification (45). Using only stable amino acids for quantitative AAA allows for the use of hydrolysis conditions which are optimized primarily for completeness of hydrolysis.

Prior to quantification of a peptide by AAA, there are preliminary considerations and experimental optimizations that should be addressed to achieve accuracy. First, the purity of the peptide should be evaluated (46). Specifically, it should be determined whether the peptide contains any impurities that could contribute to amino acids, biasing the quantitative AAA. Errors during peptide synthesis and degradation products of the peptide are potential impurities. An LC-MS/MS analysis of the peptide should be performed to determine if peptide impurities are present and to provide a rough estimate of their amounts relative to the analyte peptide. If relative content of peptide impurities are high, a purification of the analyte peptide should be performed prior to AAA.

It is important to verify that peptides are *completely* dissolved prior to AAA to ensure that the measured concentrations of peptide in solution are relevant to the lyophilized peptide stock. Further, it is important that the protocol for solubilization of peptides prior to AAA is identical to the protocol used to solubilize the peptide internal standards and calibrators prior to use in the quantitative assay. Best practice would use UV-spectroscopy to confirm calibrator solution concentration prior to use.

AAA assays should be considered as three key steps (discussed below) with an optional derivatization stage commonly employed either pre- or post-column for increased signal response. In general, AAA assays include: 1) peptide hydrolysis, 2) separation of amino acids, and 3) detection with quantitative analysis.

Peptides are commonly hydrolyzed at elevated temperatures (110° C, but can range from 90 to 130° C) in a low pH environment using concentrated acids (6 mol/L HCl, 4 mol/L CH3SO3H, 2 mol/L TFA, etc.). Alkaline hydrolyses are also possible using concentrated KOH or NaOH, although this approach is not as commonplace. Acid hydrolysis can be performed in two ways: in concentrated acid solution or with acid in the gas-phase. Elevated temperatures used for hydrolysis are obtained using a conventional oven, heating block approach, or in a specifically designed microwave oven to control energy and temperature.

Separation of amino acids prior to detection can be achieved by several formats, including HPLC [ion-exchange (IEX) and reversed phase (RP)], gas chromatography, or electrophoresis. RP-HPLC and GC typically require chemical derivatization prior to detection, although newer HPLC column chemistries are facilitating improved retention characteristics and enable baseline separations of non-derivatized amino acids, limiting biases and imprecision associated with a derivatization step (47). High resolution amino acid separations are also possible using capillary electrophoresis (48).

Detection of amino acids is most commonly performed using one of several types of detectors: 1) a spectroscopic detector as used for Vis/UV or fluorescence, 2) measurement of electric current from redox reactions using amperometric electrochemical detection, or 3) mass spectrometry. Spectroscopic and electrochemical detections offer higher sensitivity, while mass spectrometry offers better selectivity in complex matrices and the capability of isotope-dilution quantification techniques. Non-chromatographic MS-based methods for amino acid analysis have also been developed (49).

The National Institute of Standards and Technology (NIST) has developed a AAA method based on isotope dilution (ID) LC-MS/MS analysis, which uses a stable isotope-labeled analog of each amino acid measurand that is spiked both into samples and calibrants in an equivalent manner. This “double isotope dilution” technique is beyond what routine laboratories would normally use, but is routinely used at NIST to certify concentrations for a wide variety of analytes (50). Amino acid calibration solutions are prepared gravimetrically from reference materials spiked with ^{13}C- and ^{15}N-labeled amino acids. The amino acids used in the calibration solutions are rigorously characterized for purity using elemental analysis, Karl Fischer analysis (for water content), NMR, and HPLC-UV. Calibration curves are generated from experimental peak area ratios and gravimetric mass ratios for unlabeled/labeled amino acid pairs. Internal standards used for quantification are spiked into the samples prior to hydrolysis to limit biases associated with the sample preparation. For accurate quantification, exact-matched internal standards are individually diluted and added at concentrations that more closely match the concentrations of each amino acid in the sample, and the quantification is repeated. This AAA method has been used to measure total (purified) protein concentrations or concentrations of free, unbound amino acids (51) in several NIST Standard Reference Materials (SRM®) (http://www.nist.gov/srm). Peptide or protein concentration is determined independently for each target amino acid and then the concentrations from each amino acid are compared to assess measurement quality. Measurement uncertainties of peptide or protein concentrations are calculated based on this propagated error and expanded uncertainties are determined through advanced statistical analysis. Typical coefficients of variation for AAA measurements using the NIST method are within 3 %.

The NIST double isotope dilution LC-MS/MS method for AAA is intended to value-assign peptide and protein reference materials(51) with high accuracy and low uncertainty. For routine AAA, this approach may be unnecessarily labor-intensive and time-consuming, and the added cost could factor into the cost of the production of large numbers of peptides. As an example of the way routine AAA assays are simplified, norleucine is often used as the internal standard at a single concentration because it is relatively inexpensive and easier to

use than multiple exact-matched, isotopically-labeled amino acids, it is chromatographically resolved from other measurands, and it is stable under acid hydrolysis. Although less complicated measurements are possible, adequate quality control steps, such as those described below, are needed to achieve the required measurement goals.

The peptide hydrolysis and amino acid quantification steps are most challenging during AAA, and, therefore, the most prone to bias. As such, quality control efforts in routine AAA should focus on these steps. The completeness of hydrolysis can be impacted by the amino acid sequence of the peptide, as neighboring effects of adjacent amino acid side chains alter the efficiency of the hydrolysis reaction. Therefore, both the time and temperature needed to achieve complete hydrolysis could be peptide-specific. To achieve the highest accuracy in peptide quantification, the AAA hydrolysis time and temperature should be optimized for each peptide (amino acid) using a timecourse assessment of amino acid stability and completeness of hydrolysis. A comparison of the peptide concentrations derived from each amino acid monitored can be a useful way to identify problems with hydrolysis completeness. If statistically equivalent peptide concentrations are not observed for all the stable amino acids measured, it is likely that either peptide hydrolysis was not complete, there are unknown, significant impurities remaining, or there is a problem with the quantitative amino acid measurement.

For the hydrolysis step, a peptide solution with a known concentration should be used as a trueness control. In the absence of an appropriate peptide solution reference material, labs performing AAA should consider preparing an in-house peptide standard. An appropriate in-house peptide solution standard can be prepared from a high-purity peptide and be value-assigned using a double isotope dilution LC-MS/MS method. Sufficient aliquots of the in-house peptide standard should be prepared and stored frozen so that an aliquot will be measured as a trueness control with every routine AAA measurement. A discrepancy from the expected peptide concentration of the in-house standard during routine AAA analysis could indicate either a problem with peptide hydrolysis or with accuracy of amino acids measurement, or both. It would be possible to rule out problems with amino acid measurement by using an amino acid solution reference material of known concentration, such as NIST SRM 2389a (52). Through the combined use of a peptide and amino acid standards, sufficient accuracy in routine AAA can be achieved.

Amino acid calibrants and peptide/protein QC materials should be selected carefully to ensure accuracy of the measurements. Calibrants should mimic the measurand(s) as identically as possible in both concentration and structure/form. Calibrants should be characterized for purity, both organic and inorganic contaminants, as well as for water content. Both calibrants and QC materials should be measured in a buffer that most closely resembles that of the target measurand. Similarly, QC materials should ideally consist of pure proteins or peptides with known, accurate, and stable concentrations; the calibrators and QC material should be well characterized with respect to purity, storage stability and accuracy of the aliquot. Because AAA of peptides is limited by which amino acids are available for targeted quantification, it is necessary to ensure that the QC material contains the same set of amino acids in roughly (if not identically) the same molar ratio. For isotope-dilution measurements, stable isotope-labeled internal standards must consist of matrix- and

exact-matched analogs of the target measurand. They should be added to the samples and calibrants at the beginning of the sample preparation; accuracy of the amount of the sample taken for the analysis must be assured, and replicate analysis of the measurand is preferred. To ensure coelution of the targets with the corresponding internal standards during chromatographic separation, deuterated internal standards should be avoided (53). Finally, labeled amino acid internal standards should contain a number of isotopic atoms which would provide sufficient mass difference from the isotopic envelope of their “light” analogs in order to be detectable without bias contingent on the resolution of the chosen mass spectrometer.

Need for reference materials for harmonization of AAA measurements

To help facilitate accurate and precise AAA measurements by service providers, the field would greatly benefit from a new set of reference materials for harmonization. Ideally, the new standard peptide(s) would lack specific amino acids that are degraded during hydrolysis (Trp, Met, Cys, Ser, and Thr), lack amino acids that have limited stability during long-term storage (Trp, Met, Cys, Asn, and Gln), and lack amino acid pairs that often don't hydrolyze completely (e.g., Ile-Val, Ile-Ile, and Val-Val). Including a tyrosine in the peptide would allow UV-absorption to be used to quantify the peptide using alternative methodology (UV-absorption) and including at least one of the most reliable amino acids (sometimes called fiducial residues) ensures greater confidence in the final results [i.e., Tyr (with phenol present), Ala, Arg, Gly, His, Ile, Leu, Lys, Phe, Pro, and Val]. The optimal peptide length to minimize secondary structure and ensure complete hydrolysis is 12-18 residues.

Whether standard peptides are provided in solution or as lyophilized peptides, the peptides would ideally be stable in solution for at least 30 days at 4 °C and for 3 years at -20 °C. Peptides in solution should also be stable to multiple freeze-thaw cycles and to at least one lyophilization-resolubilization step (in aqueous/organic/acid solvent) without significant loss of peptide (i.e., < 3 %) or modification of residues. While it is not possible to know how soluble and stable a peptide will be prior to synthesis, an example peptide that might be a useful standard peptide for AAA is DAKAGIHPLELRVARYR. This artificial, non-tryptic peptide that is not present in any gene sequence is 17 residues in length, 15 of its residues are taken from the list of most reliable amino acids, it contains at least half of the natural amino acids including Tyr, it lacks unstable side chains and amino acid pairs that hydrolyze irreproducibly, and it is relatively charge balanced (2 acidic and 5 basic residues) making it readily soluble for use in other assays.

Although it is not possible to produce reference materials suitable for every potential peptide application, availability of general reference materials will have a large impact on quality of the measurements. Due to the issues associated with using weighed amounts of peptide (e.g., salt and water content of lyophilized peptides), it is very desirable for the field to have available one or more standard peptides already in solution, ready for use in amino acid analysis. For example, the NIST peptide standards SRM® 8327, which were provided as a reference material without a certified concentration measurement, were aliquotted gravimetrically (target of 1 mg of peptide per vial) and then distributed as lyophilized peptides. The data in Table 6 demonstrate that the actual amount of peptide added to the vial

was much lower than 1 mg for each peptide (determined using amino acid analysis), due to salts and water associated with the lyophilized peptide.

In summary, the field of proteomics would greatly benefit from new reference materials developed and carefully characterized using a double isotope dilution technique. The new reference materials would include 1 to 3 peptides in solution that are soluble, stable, contain the most reliable amino acids for AAA, and be representative of the proteotypic peptides that are used in targeted proteomics experiments. Service providers would use these reference materials to ensure the accuracy of their assay during the methods development and in routine use of the methods.

Peptide storage and handling

Quantitative mass spectrometry-based assays are negatively impacted by a lack of proper procedures for storing and handling peptides. The following section highlights several of the most common considerations and makes recommendations for storing and handling peptide internal standards and calibrators (for a summary of the general recommendations see Table 7).

Peptide Storage

The primary detriments of extended storage of peptides are the loss of solubility and/or change in concentration due to evaporation, adsorption, microbial degradation, secondary structure formation, and chemical modification. Storage in buffers can render peptides susceptible to microbial growth and degradation. Specific amino acids are associated with several common chemical modifications. For example, cysteine, methionine, and tryptophan are prone to reversible and irreversible oxidation, and this conversion is accelerated during freeze-thaw cycles and at high pH (54). Glutamine and asparagine are prone to deamidation (55), frequently when drying solutions under acidic conditions. Certain positions are more susceptible to deamidation, including the N-terminus and N-terminal to glycine (i.e. Asn-Gly, Gln-Gly). Aspartic acid is sensitive to hydrolysis, and amino acids containing aromatic rings are susceptible to photochemical degradation (e.g., phenylalanine and tryptophan).

To minimize detrimental effects, the long-term storage of peptides (> 6 months) is most effective when peptides are lyophilized and stored at temperatures -20 to -80 °C (33, 56). Upon reconstitution, the primary concern is variability in the dissolution of peptides. Generally, AAA constitutes the best practice for concentration determination; however, for peptides with well-characterized solubility, UV absorbance using pre-defined extinction coefficients may be a suitable alternative. Once re-solubilized, the peptide calibrator solutions (0.5-2 nmol/μL) are best stored frozen at temperatures ≤ -70 °C in sealed tubes. Although stability in solution is peptide-dependent, generally working solutions are prepared from the stocks at concentrations 1-100 pmol/μL and are used for short term storage of peptide calibrators (≤ 3 months). To minimize peptide degradation, multiple freeze-thaw cycles should be avoided. Using good laboratory practice (preferably using gravimetric addition), calibrators should be made from the stock solution diluted as close to the time of use as possible. Peptides in solution should also be limited in their exposure to air. For particularly sensitive sequences, inert gases (e.g. argon, nitrogen) are recommended as a

blanket gas in storage tubes, and amber or dark storage tubes should be used for photochemically sensitive sequences.

Reconstituting Peptides

As mentioned above, solubility can be a significant factor affecting the accuracy of peptide quantification. Peptide solubility in a given solvent depends greatly on the specific amino acids in the peptide. The diversity of peptide sequences makes it difficult to apply broad recommendations to optimize solubility; however, general guidelines can be followed. Acidic peptides that contain more Asp and Glu residues than His, Lys and Arg residues are most soluble in basic solutions. Basic peptides containing more His, Lys and Arg residues than Asp and Glu residues are most soluble in acidic solutions. Peptides with neutral or < 25 % charged residues are most soluble in solutions to which an organic solvent has been added. Solutions with strong organic solvents such as acetonitrile or DMF (dimethylformamide) can be used to efficiently solubilize peptides with a high percentage of hydrophobic residues (> 50% Ala, Val, Leu, Ile, Met, Phe, Trp, Pro) and < 25 % charged residues.

Prior to reconstituting peptides, lyophilized powder should be brought to room temperature in a desiccator to avoid water absorption in the unused peptide, thus minimizing variations in concentration of lyophilized aliquots. If reconstituting a peptide for the first time, and whenever possible, a small amount of the peptide should be reconstituted before committing the entire lot by weighing out a small aliquot. As discussed above, the pH is an important parameter for peptide solubilization. Initial reconstitution is best performed in water by adjusting the pH based upon the primary amino acid sequence with a small amount of organic solvent added to aid solubilization. Buffers such as PBS should not be used for reconstitution because salts hinder solubility. If salt solutions are desired for the final formulation, they are best added once the peptides are fully solubilized.

Peptides should initially be reconstituted at a concentration that is higher than the desired final working concentration (typically between 10-1000 times more concentrated; see Table 7 for specific recommendations). Solutions of completely solubilized peptides are completely clear and are devoid of any “flecks” or cloudiness. Solubilization can be confirmed by light scattering analysis or by comparing absorbance in a series of dilutions with and without centrifugation to pellet undissolved material. A general recommended starting point for a reconstitution solution is 5 % acetonitrile with 0.1-1 % formic acid. The inclusion of organic solvent and acid in the reconstitution solution not only aids solubility, but also serves to retard microbial growth (biologically active buffers should contain 0.1 % sodium azide to prevent microbial growth). If this reconstitution solution is not successful in completely solubilizing the peptide, the amount of organic solvent can be increased or the organic solvent can be altered (e.g., try methanol instead of acetonitrile). If increasing organic solvent is not effective in solubilizing the peptide, the pH can be adjusted by adding acid (up to 1 % formic acid or TFA), or by using 1 % ammonium bicarbonate, 1% DIPEA (N,N-diisopropylethylamine), or ammonium hydroxide. Another option is to re-dry the peptide and re-dissolve it in DMSO.

Non-specific Adsorption of Peptides

Variable recovery due to non-specific adsorption is one of the major consequences of improper handling of peptides and can lead to imprecision and bias (i.e., loss of peptide to surfaces or contamination/carryover). The extent of non-specific peptide adsorption to the walls of peptide storage vessels, pipette tips, autosampler vials, and HPLC components varies based on the primary sequence, the materials used, and the concentration of the peptide solution. Complete characterization of peptide stability includes the evaluation of losses due to adsorption in all steps of the analytical method. This can be accomplished by several experimental designs, including measuring peptide amounts in serial dilutions by UV absorbance (e.g. to evaluate potential loss in tubes and/or pipet tips) or repeated injections by LC-MS (e.g. to evaluate potential loss or carryover in vials and the HPLC system). The use of carrier or chaperone molecules can minimize adsorption effects for particularly difficult peptides (57); however, choice of a suitable carrier is highly dependent on the peptide sequence, the analytical method, and the desired matrix for analysis. Thus, there is currently no consensus related to the best carrier molecules or the optimum concentration for use with peptide internal standard and calibrators. When evaluating carrier molecules, caution should be taken to choose components that do not interfere with detection of the target peptide or excessively contribute to sample complexity or instrument contamination.

The relative loss of peptides by non-specific adsorption in low concentration solutions is greater than in more concentrated solutions because of the limited binding capacity of the wetted solid surface area (58). To demonstrate the loss of peptides in solution and the effect of storage concentration, two peptide mixtures (200 and 1000 fmol/μL) were prepared in non-deactivated glass vials and analyzed by injecting 1 μL of each sample each h for 15 h. Of the 50 peptide targets in each mixture, 48 and 50 peptides were detected in the 200 and 1000 fmol/μL samples, respectively. Nine and zero peptides, respectively, showed noticeable signal decay over time under the above two conditions. This effect is seen by plotting total peak areas of two representative peptide sequences: YLGYLEQLLR (SSRC Relative Hydrophobicity 41.55) and IYEGSILEVDCDILIPAASEK (SSRC Relative Hydrophobicity 43.98), both of which are quite hydrophobic (Figure 3). In contrast to the 200 fmol/μL sample, all peptides in the 1000 fmol/μL mixture showed constant signals over the time period analyzed, consistent with improved stability and reduced adsorption at higher concentration.

Non-specific adsorption contributes to carryover, which increases variability and bias due to residual signal in sample runs (59). Carryover in sample preparation can originate from re-using pipette tips to transfer peptide solutions between vials or in dispensing aliquots. Carryover in sample preparation or analysis can negatively impact results through ion suppression of low abundance peptides (when co-elution occurs with high abundance carryover from the previous run or sample) or by producing a ‘false positive’ in sample analysis by the detection of contaminating analyte peptide. One can determine the extent of non-specific adsorption by transferring a solution of the analyte sequentially from one vial to another and analyzing a small aliquot after each transfer step to assess for losses (60). Despite the diverse physicochemical properties of peptides, various strategies can be generically applied to reduce adsorption and cross-contamination phenomena (57, 61)

leading to carryover. When preparing dilution series, one should never re-use pipette tips to avoid cross-contamination. Pipette tips should be pre-rinsed several times with the peptide solution prior to aspirating the final volume. To minimize non-specific adsorption to the walls of storage vessels, standards of peptides should be added directly to the diluent fluid instead of the sides of the tubes or vials. Finally, peptide adsorption also contributes to carryover in chromatographic systems through incomplete removal of analyte from the analytical system from the previous injection (e.g. insufficient wash of the injection valve or syringe of the autosampler). Chromatographic carryover can be evaluated by injecting a blank sample following a sample or calibrator. Complete system wash runs (e.g. rinsing all HPLC components, including autosampler, delay volumes, and columns) can be used to reduce or eliminate carryover using a series of different elution buffers/solvents. It should be noted that some peptides, especially those containing hydrophobic residues, can be retained on HPLC columns despite the use of high concentrations of organic solvents when washing. Most HPLC column manufacturers have published methods for washing/cleaning the HPLC flow-path and columns.

Different types of vials can introduce significant variability in LC-MS analyses (62). The interaction of peptides with various surfaces is greatly influenced by the specific side chains of the amino acids of the peptide. Glass and polypropylene are the materials that are most commonly used to manufacture vials, inserts, and plates. Although a single type of vial might not be optimal in terms of minimizing the non-specific interaction of all of the peptides in an analytical mixture, it should be noted that basic amino acids can form electrostatic interactions with the residual silanol groups on glass vials, and nonpolar amino acids can interact with the hydrophobic surface of polypropylene vials (63). To minimize these adverse interactions, several manufacturers of chromatography consumables offer silanized glass vials in which the silanol groups have been chemically inactivated. Similarly, polypropylene vials with modified plastic surfaces are commercially available.

To demonstrate the variability that can arise from various container materials, we investigated the signal from repeated injections of a digested protein sample stored in three types of autosampler sample vials: non-deactivated glass, deactivated glass, and polypropylene vials. Peptide stability was tested by performing 15 repeated LC-MS/MS analyses of the 50 fmol/μL sample each h for 15 h. We manually assessed the signal intensities of the replicate runs for each peptide to determine the amount of signal enhancement or decay. The results are summarized in Figure 4. Peptides were categorized as Stable, Slow decay, or Fast decay by using a cut-off of < 5%, 5-50%, or > 50% peptide loss based on signal intensity over the 15 h. We found that all three vial types enable the recovery of 43 peptides, which accounts for 86% of the monitored peptides. Twenty-nine of the detected peptides were very stable across all analyses for all vials. In this study, the polypropylene vial outperformed the two glass vials, as only 1 “unstable” peptide with significantly lower recovery was detected, while 13 and 14 “unstable” peptides were detected in non-deactivated and deactivated glass vials, respectively.

Effects of Freeze-thaw on Peptide Stability

To demonstrate the effects of freeze-thaw on peptide stability, we compared the signal intensity observed when injecting a peptide mixture stored at 4 °C, a sample undergoing a single freeze-thaw, and a sample undergoing multiple (n=10) freeze-thaw cycles. Twelve 1 pmol/μL sample aliquots prepared in solution (3% acetonitrile, 0.1% formic acid, in H_2O) were stored in polypropylene tubes at -80 °C. One sample was thawed and kept at 4 °C over 10 days, and one sample was subjected to 10 freeze-thaw cycles. The remaining ten samples underwent a single freeze/thaw. Each sample was analyzed in triplicate by LC-MS/MS by injecting 50 fmol on column (diluted from the stock solution immediately prior to the analysis) from each sample over ten consecutive days. The results are summarized in Figure 5. Figure 5A shows the mean total peak areas of all detected peptides over 10 days. Because there is a wide distribution of the peptide MRM intensities, three plots were made to show peptides with low signal abundance (peptides with poor ionization), medium signal abundance (middle box), and high signal abundance, respectively. Regardless of how the samples were handled, comparable peak areas of the peptide were observed (Figure 5A), and there was no significant difference (paired t-test, $p<0.05$) among the average peak areas for the freeze-thaw experimental conditions.

We next assessed the reproducibility under the three conditions by comparing imprecision of the replicate analyses (Figure 5B) and found a number of important observations: 1) the variability of peptide peak area (%CV) is sequence-dependent and closely related to the peptides' hydrophobicity, 2) no obvious correlation was observed between peptide peak area and variability (within the limits tested), 3) for most peptides, variability of peptide peak area (%CV) was lower for freezer storage (∼1.5-3.5 fold) than for other storage conditions, and 4) the variability of peptide peak area (%CV) was highest after ten freeze-thaw cycles. These results clearly suggest that frequent freeze-thaw cycles should be minimized, and best results are obtained from analysis of samples that are stored frozen and defrosted immediately prior to the analysis.

Peptide Storage in the Autosampler

After peptide calibrators have been properly prepared and added to the most appropriate type of vial, the amount of time that the peptide calibrators are stored in the autosampler must be carefully controlled. Ideally, stability studies should be conducted to determine whether peptide calibrators can be prepared and left in an autosampler with thermostatic temperature control for the duration of the analysis without decreased mass spectrometer signal. Peptides should be conditioned to the autosampler tray temperature prior to injection. Temperature-related differences in peak area have been observed when peptides were not equilibrated to the autosampler tray temperature before injection (64). Further detailed guidelines for conducting a study to determine the stability of peptides are provided in the Assay Development Guidelines document that is available on the CPTAC Assay Portal (4) (https://assays.cancer.gov/) and published recommendations (11). Briefly, the guidelines recommend the analysis of peptide peak area variability for six temperature- and time-related conditions (6 h at 4 °C, 24 h at 4 °C, 4 weeks at -70 °C, one freeze-thaw, and two freeze-thaws) compared to the time zero condition wherein the peptides are injected directly without being left on the autosampler tray for a prolonged period of time.

In summary, the best storage and handling conditions will depend on peptide sequence. However, despite the individuality of peptide sequences, some general recommendations for peptide handling can be made (summarized in Table 7).

Assuring specificity and reliability of quantitative data based on peptide internal standards and calibrators

When analyzing the data from LC-MS/MS assays of peptides and proteins, it is critical to confirm the specificity of the assay for the intended analyte, as well as to ensure reliable quantification of the analyte. The use of the stable isotope-labeled internal standard peptides facilitates both of these goals, as described below.

Well developed and validated targeted LC-MS/MS assays are able to provide highly specific measurements, since the *actual peptide analyte* (i.e. not an indirect measurement of the analyte, such as a chromogenic or chemiluminescent substrate used with most immunoassays) is directly detected by the mass spectrometer, and every peptide has characteristic physiochemical properties (e.g., HPLC elution time, precursor and product ion masses, and product ion ratios). Nonetheless, interferences are common in highly complex biological matrices, and they must be recognized and avoided to ensure assay specificity. For example, in complex matrices (e.g., plasma, serum, cell or tissue lysates) combinations of several precursor/product ion pairs (i.e., “transitions”) are often insufficient to accurately pinpoint the location of a given target peptide (especially for low-abundance analytes). This is because multiple “peak groups” (i.e., “clusters” of co-eluting or closely eluting chromatographic peaks in the retention time window of each measured transition) are likely to be present. However, with the use of internal standards, the endogenous peptide signals can be easily located in the HPLC elution profile, because the stable isotope-labeled peptides and their endogenous counterparts have very similar fragmentation patterns and HPLC retention times. The LC-MS peak characteristics of the light and stable isotope-labeled peptides can be manually inspected using Skyline (15) or analyzed using automated data processing (e.g., peak picking, quantification and false discovery rate calculation) with software tools such as mProphet (65). Observing the same fragmentation patterns [i.e., the same transition and the same relative peak intensity ratios across multiple transitions(66)] between the endogenous and stable isotope-labeled peptide signal patterns is used to qualitatively confirm confident detection of the endogenous peptide, as well as to determine potential interferences in specific transition(s) either by manual inspection or using the software tool AuDIT (67). In general, if ≥ 3 transition ion pairs (heavy and light) for a given peptide show identical LC elution time profiles and the relative intensity of the product ions is within the tolerance established during the assay validation, the assay can be considered to be specific.

To achieve reliable relative quantification of peptides in complex matrices, the targeted proteomic assay must be analytically characterized with respect to its specificity, LLOQ, linear range, precision, and repeatability. Guidelines for targeted proteomic assay characterization have been proposed (2, 11) and a summary document can be downloaded from the CPTAC assay portal (https://assays.cancer.gov/)(4). Open source software tools (15, 68) are available to facilitate analyses and data sharing. Validation of quantitative assays

of proteins in complex mixtures requires additional experiments (11) and analytical validation in a clinical laboratory should adhere to appropriate guidelines (i.e., Clinical Laboratory Standards Institute and CLIA).

It is generally recommended that the most intense ion that is free of interference be used to quantify the peptide, and that the next two most abundant fragment ions are monitored to evaluate specificity of the assay. It is also acceptable to sum transitions for quantification if there is a signal-to-noise benefit (and if all these transitions are demonstrated to be free of interference in each sample tested). If the target peptide contains a specific post-translational modification, then there must be at least one fragment containing the modified residue (69). In PRM experiments, all product ions are detected and could be quantified simultaneously, with a subset of those product ions used to evaluate selectivity. A summary of the recommended steps for analyzing targeted quantification results using isotopically-labeled peptide internal standards is presented below:

1. Check the extracted peaks for both heavy standard and endogenous light peptides using software tools such as Skyline (15), making sure that both peaks co-elute.
2. Confirm the transition pattern (rank and relative intensity, typically requiring at least 3 transitions) is consistent between the isotope-labeled internal standard and endogenous peptide. The confidence of detection of endogenous peptide is assured by comparison of the acquired transitions between the isotope-labeled internal standard and endogenous peptide.
3. Eliminate transitions with potential interference. The problematic transition could be determined either by visual inspection or software tools such as AuDIT (67).
4. Select the appropriate peak boundary for the labeled and endogenous peptides, and then calculate their peak areas. Either all transitions or the best transition (i.e., highest intensity, lowest limit of quantification, or best signal-to-noise) without evidence of interference can be used for quantification.
5. Calculate the peak area ratio of endogenous peptide over stable isotope-labeled peptide. Based on the peak area ratio and the known concentration of the spiked stable isotope-labeled peptide, the concentration of endogenous peptide in the unknown sample can be determined.
6. Ensure that the detected concentration of the peptide is above the LOQ of the assay and within the linear range of the assay.

As mentioned above, there are several reasons why this approach may not accurately reflect the amount of endogenous peptide or protein present in the undigested sample: (1) liberated peptides are lost or non-specifically degraded during digestion, especially with high concentrations of trypsin and long digestion times (70), (2) proteins in complex mixtures are often not digested to completion when trypsin is added in lower concentrations, (3) proteins are most often heterogeneous mixtures of related macromolecules that differ in primary sequence and post-translational modifications, which can affect digestion efficiency, (4) proteins in macromolecular complexes that are not completely denatured will digest less efficiently, and (5) there is substantial variability in digestion from day-to-day. Even if

inaccurate, the quantification of peptides in LC-MS/MS experiments can be relatively precise from day-to-day when the peak area ratio is calibrated using external calibration materials. These materials can include unlabeled or labeled peptides spiked into a relevant digest (e.g., pooled cell lysates or human serum) at various concentrations, which are prepared in parallel with other samples (i.e., internal standard is added at the same concentration). This minimizes bias due to variable amounts of internal standard added each day. Alternatively, purified protein spiked into a relevant matrix or native protein present in an unadulterated sample can be used. This approach can normalize between-day and between-laboratory variability in digestion (5-7, 71), but it must be realized that the measurement may still not accurately reflect the amount of *intact protein* in the sample.

Reference materials: Improving the harmonization of protein measurements

To avoid pitfalls in interpreting targeted LC-MS/MS peptide and protein measurements, it is critical to properly identify the measurand (i.e., the quantity intending to be measured). For the most part, this document has focused mainly on the approaches and techniques needed to effectively utilize stable isotope-labeled internal standard peptides in the quantification of an endogenous peptide in a proteolytic digest of a complex sample. In most instances, the goal of MRM assays is to measure the concentration of a target protein in a complex mixture. As discussed above, the concentration of a proteotypic peptide liberated in a protein digest may not fully reflect the concentration of an intact protein, particularly due to the heterogeneity of protein isoforms in biology. However, if the measurand is defined as the concentration of protein isoforms that contain the peptide(s) analyzed in the experiment, then with proper calibration and quality control, LC-MS/MS may be capable of providing this concentration.

Reference materials can be used to harmonize and standardize measurements of protein measurands. For quantitative methods in clinical laboratories, the accurate measurement of proteins is important for patient care, particularly when accurate diagnosis, treatment guidelines, or prognosis are based on the numeric results of laboratory tests. Relative accuracy in these assays is established using reference materials whenever possible (72, 73). The reference materials are used as calibrators in the assay or to assign the concentration of a protein in the assay calibrators, regardless of whether the assays are immunoassays or mass spectrometric assays. Assays that demonstrate good agreement on a population of samples are said to be harmonized. Reference materials can be used to harmonize assays. When an assay reports concentrations on a population of samples that have very little bias when compared with a reference measurement procedure (i.e., a robust assay with rigorous process controls that uses certified or standard reference materials in its calibration), the assay is said to be standardized. It is possible to harmonize and standardize immunoassays or mass spectrometric assays (74).

Standard or certified reference materials have had their concentration assigned by consensus or reference measurement procedure. Consensus-defined concentrations use as many different assay platforms as possible (typically immunoassays) to assign the concentration. Consensus-defined reference materials are useful for the harmonization of protein measurements. Reference materials that use a reference measurement procedure to define their concentration are rare. For purified protein reference materials, the measurement

procedure of choice is currently amino acid analysis. For proteins in a relevant matrix, there are no reference measurement procedures that use tandem mass spectrometry to assign protein concentration. The most commonly used methodology is immunoassays using polyclonal antibodies, which have well-known issues in analysis of human biological fluids (75). In these cases the measurand is difficult to define with any specificity.

Guidelines have been developed by the International Organization for Standardization (ISO 15194), which ensure the quality of manufactured reference materials. The World Health Organization (WHO) has developed complimentary guidelines for the development and value assignment of their international standards. Table 9 lists possible sources and distributors of protein reference materials that may be useful to investigators and clinical laboratories.

In the future, it is expected that amino acid analysis will be used to certify the concentration of purified protein reference materials and that trypsin digestion-isotope dilution-mass spectrometry with well-characterized isotope-labeled protein or peptide internal standards (as described in this document) will become the reference method procedures used to establish the concentration of proteins in matrix-matched reference materials. Once the field establishes assays using enzymatic digestion-isotope dilution-mass spectrometry as reference method procedures, and once basic researchers, clinical researchers, and clinical laboratories more universally adopt quantitative targeted molecular assays such as validated MRM methods for general protein quantification, the field will have taken an important step toward the more rapid translation of replicable experiments to the care of patients.

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedures. Such identification does not imply recommendation or endorsement nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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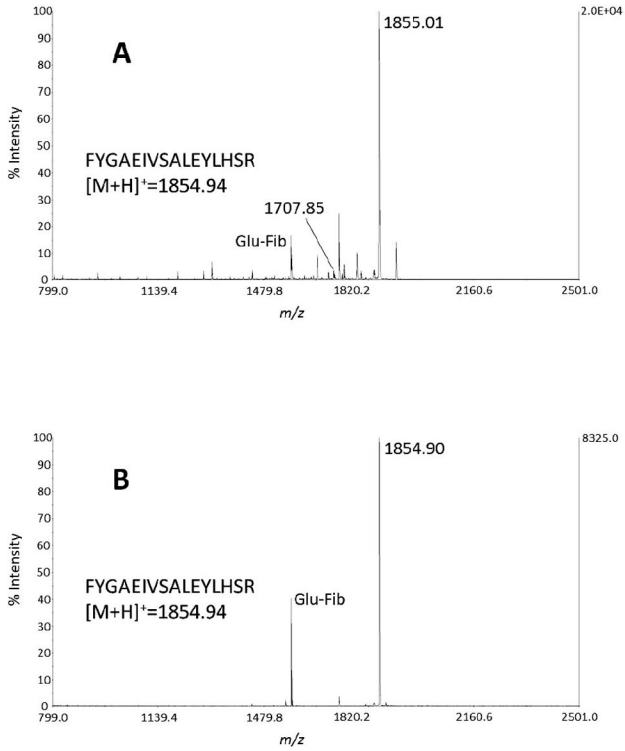

Figure 1. Example of a Recommended Assessment of Peptides

MALDI-MS spectra of lower purity (A) and high-purity (B) peptides. The internal standard was a synthetic peptide (Glu-Fib).

Amino Acid	Stable During Hydrolysis	Stable During Storage	Hydrolyze to Completion	Ideal for Quantitation
Ala	✓	✓	✓	✓
Arg	✓	✓	✓	✓
Asn	Converted to Asp	No	✓	No
Asp	✓	✓	✓	No
Cys	Destroyed	No	✓	No
Gln	Converted to Glu	No	✓	No
Glu	✓	✓	✓	No
Gly	✓	✓	✓	✓
His	✓	✓	✓	✓
Ile	✓	✓	Not when paired with I or V	Possible
Leu	✓	✓	Not when multiple L in a row	Possible
Lys	✓	✓	✓	✓
Met	Destroyed	No	✓	No
Phe	✓	✓	✓	✓
Pro	✓	✓	✓	✓
Ser	Partially destroyed	✓	✓	No
Thr	Partially destroyed	✓	✓	No
Trp	Destroyed	No	✓	No
Tyr	✓	✓	✓	✓
Val	✓	✓	Not when paired with I or V	Possible

Figure 2. Summary of the stability and efficiency of hydrolysis of the natural amino acids

Each amino acid is characterized with respect to stability in acid, stability during storage, and efficient hydrolysis. Green indicates that the amino acid is favorably stable or hydrolysable. Red indicates instability. Yellow is used to highlight three hydrophobic amino acids that can affect hydrolysis.

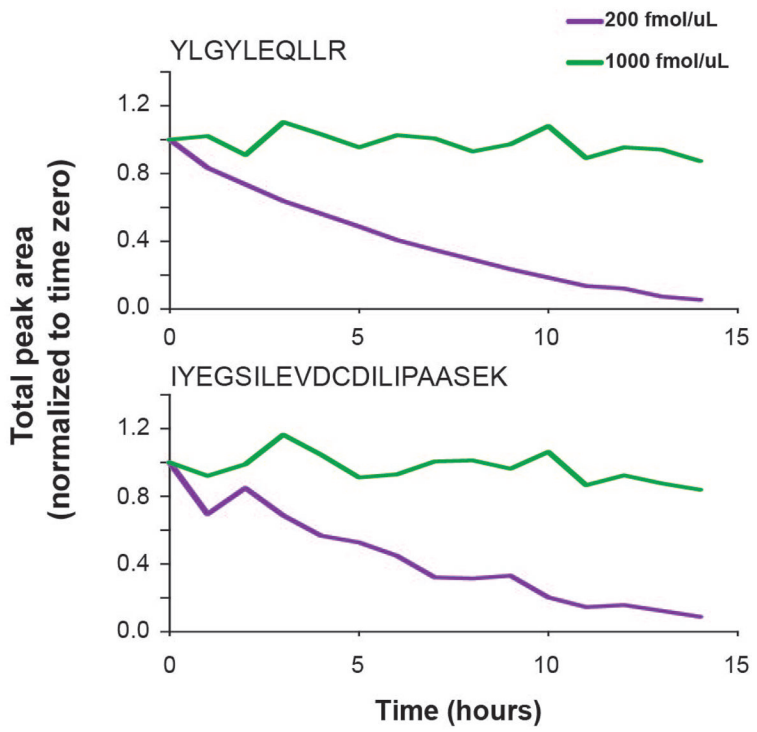

Figure 3. Evaluation of peptide concentration on stability of signal over time

The peak areas (normalized to time zero) from two representative peptides were plotted versus autosampler storage time (hours) to show that storing the peptides at higher concentration can minimize the loss of peptide signals, presumably attributed to adsorption of the peptides to vials. See Supplemental Materials and Methods for details.

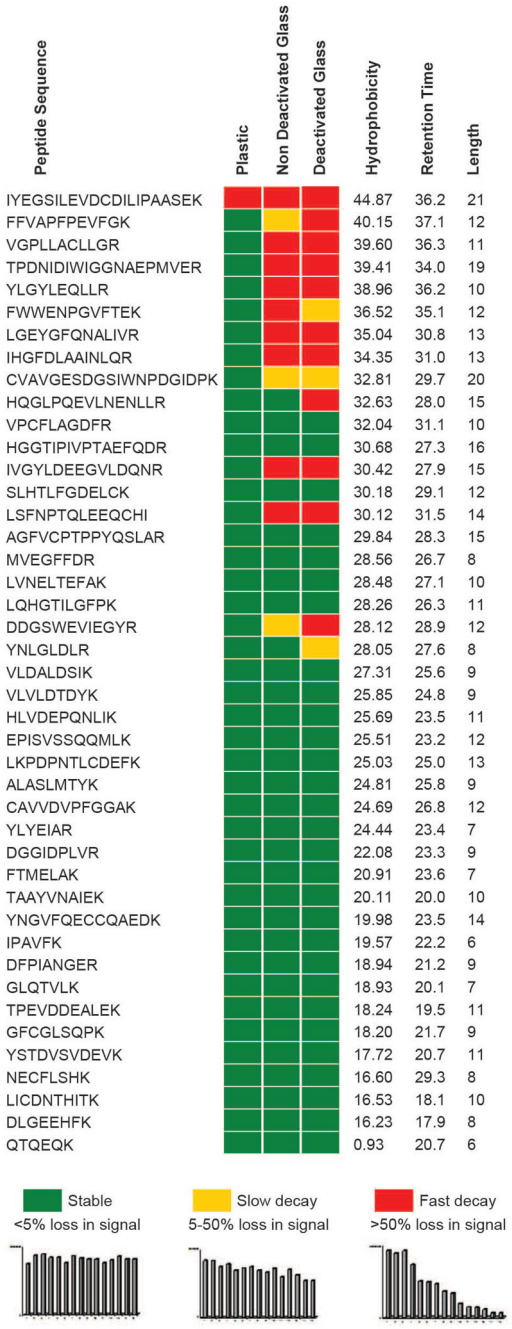

Figure 4. Effect of autosampler vial material on stability of peptides in a protein digest: plastic, non-deactivated glass, deactivated glass

The plastic vial outperforms the two glass vials, as it is associated with only one “unstable” peptide. The peptide sequences are sorted by relative hydrophobicity. See Supplemental Materials and Methods for details.

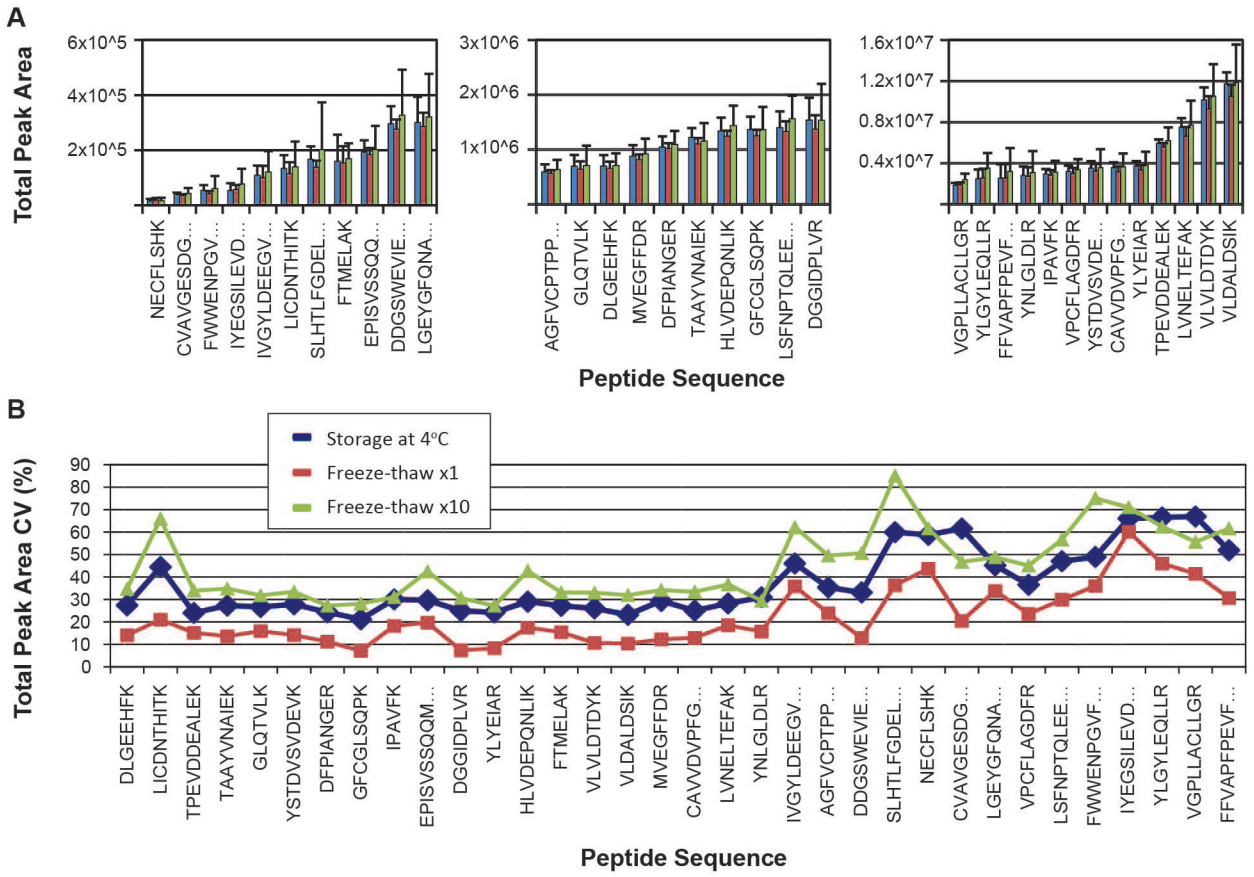

Figure 5. Evaluation of freeze-thaw effect

Mean values of total peak area from triplicate injections are plotted for all detected peptides. Error bars show standard deviation. (A) Low abundance, medium abundance, and high abundance peptide signals are plotted in separate boxes; similar peak areas of the peptide were observed after storage under three conditions: refrigerator storage (‘control’), freezer storage with one freeze-thaw cycle, and ten freeze-thaw cycles. (B) Coefficient of variation (CV) is plotted as a function of peptide sequence. Peptides are plotted on the x-axis in order of increasing retention time. See Supplemental Materials and Methods for details.

Table 1
Guidelines for peptide selection for MRM targeted assays

Filtering Criteria	Description
Uniqueness (analyte specificity)	Peptides must be unique in sequence to the gene product or proteoform[1] of interest to enable specificity of the assay.
Peptide Length	Typically 7-20 AA
Observability by MS	Ideally, peptides should be empirically identified in MS experiments using the instrument on which the method is expected to be developed. Frequency of observation, selectivity, and MS signal intensity can be used to rank order in cases where multiple peptides meet the *in silico* selection criteria.
Hydropathy	Extremely hydrophobic peptides can be problematic due to solubility issues, and extremely hydrophilic peptides can be problematic due to LC retention time instability. As a general rule, it is best to select peptides within an SSRCalc score range of 10-45, see http://hs2.proteome.ca/SSRCalc/SSRCalcX.html.
Reactive Residues (amino acid residues that may be susceptible to modifications during sample preparation)	Avoid these residues if possible, listed in decreasing priority (potential post-translational/preanalytical processing issues listed in brackets): • Cysteine (carbamidomethylation, oxidation, cyclization if N-terminal) • Methionine (oxidation) • N-terminal glutamine (pyroglutamic acid formation) • Asparagine or glutamine when followed by glycine (deamidation) • Aspartic acid followed by glycine (dehydration) or proline (peptide chain cleavage). • Tryptophan (oxidation) • Histidine (additional charge states)
Digestion Parameters	Tryptic peptides generally have an optimal length for analysis and usually form doubly or triply charged positive ions (depending on the sequence), which provide useful sequence information through MS/MS fragmentation. Peptide sequences containing inhibitory motifs for trypsin that commonly result in missed cleavages (e.g., Lys-Lys and Arg-Arg) may display variable digestion yields and should be avoided if possible[2]. Avoid ragged ends (i.e. KK, KR, RR, RK) and possible miss-cleavage sites (i.e. KP and RP).
Modification Motifs	Unless the goal is to quantify the posttranslationally modified isoform, peptides near or containing potential posttranslational modification sites [e.g. phosphorylation, N-glycosylation (NXS/T)] should be avoided where possible, since they may affect assay results by altering the recoverability and/or detection of the analyte peptide.

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Table 2
Pure Peptide Specifications and Methods for Qualification

Description	Specification
Amount	≥ 1 mg ordered; up to 5 mg net peptide content confirmed by AAA
Chemical purity	> 95%
Isotope	^{13}C, ^{15}N; for doubly charged precursors, use minimum of 6 Da mass delta to unlabeled amino acid; for triply charged precursors a minimum of 8-10 Da is recommended
Isotopic purity (atom percent)	> 99%
Heavy AA	Lys, Arg are recommended in the case of tryptic peptides; other AA may be labeled e.g. Leu, Phe)
Number of heavy AA	0 - 2, depending on sequence and label
Mass difference (relative to unlabeled peptide)	6 - 20 Da depending on AA sequence
Location of heavy AA	C-terminal Lys or Arg (unless noted otherwise)
AA's to be chemically modified as part of peptide synthesis	All Cys as carbamidomethylated Cys; incorporate posttranslational modifications if the goal is to quantify the modified peptide (e.g., phosphorylation at Ser, Thr, and/or Tyr; acetylation; epigenetic modifications on Lys/Arg)
Delivery time	4 – 6 weeks
Formulation – up to first 1mg of peptide	5 – 30 % acetonitrile/0.1-1% formic acid at approximate concentration of 0.5 - 2 nmol/μL (500-2000 μM). Aliquots of this solution are used for AAA.
Formulation - remaining mg of peptide	dry powder (preferably in 0.1 - 1 mg aliquots) stored under argon/nitrogen or in a desiccator, at -20C or lower for longer term storage (> 6 mos)
Purification method	preparative RP HPLC
QC – 1 (LC-UV)	analytical RP HPLC chromatogram (determine % purity)
QC – 2 (MS or LC-MS/MS)	MALDI, ESI spectrum (mass ID confirmation)orMS/MS (label + sequence verification)
QC – 3 (Amino acid analysis, AAA)	Concentration (pmol/μL or μM)Percent variation or percent relative error from expected AA composition (%)

Table 3
Lower Purity Peptide Specifications and Methods for Qualification

Description	Specification
Amount	≥ 100 μg, by weight or maximum possible quantity by AAA
Chemical purity	> 50%, the peptide should be the highest peak in the HPLC chromatogram
Isotopic atoms	^{13}C, ^{15}N; for doubly charged precursors, use minimum of 6 Da mass delta to unlabeled amino acid; for triply charged precursors a minimum of 8-10 Da is recommended
Isotopic purity (atom percent)	> 99%
Heavy AA	Lys, Arg are recommended in the case of tryptic peptides; other AA may be labeled e.g. Leu, Phe)
Number of heavy AA	0 - 2, depending on sequence and label
Mass difference (relative to unlabeled peptide)	7 - 20 Da depending on AA sequence
Location of heavy AA	C-terminal Lys or Arg (unless noted otherwise)
AA's to be chemically modified as part of peptide synthesis	All Cys alkylated (e.g., carbamidomethylated Cys); phosphorylation of specific Ser, Thr, and/or Tyr
Delivery time	2 – 4 weeks
Formulation – up to first 1mg of peptide	5 – 30 % acetonitrile/0.1-1% formic acid at estimated (by dry wt.) concentration of 1 mg/mL.
Formulation - remaining mg of peptide	none
Purification method	none, or SPE only
QC – 1 (LC-UV)	LC-UV chromatogram (% purity)Performed and assessed by submitting laboratory.
QC – 2 (MS or LC-MS/MS)	MALDI, ESI spectrum (mass ID confirmation)orMS/MS (label + sequence verification)
QC – 3 (AAA)	None until identification of desired peptide as major species by MS

Table 4

Amino acid composition of high and lower purity preparations of a peptide (FYGAEIVSALEYLHSR)

Amino Acid	Expected	High Purity[1]	Lower Purity[1]
Ala	2	2.0	2.0
Arg	1	1.0	1.3
Asx	0	n.d.	n.d.
Glx	2	2.0	2.1
Gly	1	1.0	0.9
His	1	1.1	1.3
Ile	1	0.9	0.9
Leu	2	2.1	2.5
Lys	0	n.d.	n.d.
Phe	1	1.0	0.7
Pro	0	n.d.	n.d.
Ser	2	1.9	2.2
Thr	0	n.d.	n.d.
Tyr	2	2.0	2.0
Val	1	1.0	1.1

[1]The average of four AAA determinations with coefficient of variation = 7.6% and 4.8% for the high and lower purity peptides, respectively.

Table 5

Important information to be obtained from commercial labs regarding their AAA methods

Assay characteristic	Questions to ask
Hydrolysis	How do you hydrolyze your peptides? What hydrolysis reagents do you use? At what temperature do you incubate the reactions and for how long?
Internal standards	What standards do you use for: **1** Hydrolysis **2** Instrument validation **3** Amino Acid Calibration When do you add your internal standards? How many isotope labeled analog compounds are included in the internal standards?
Method of amino acid separation	Do you use chromatography or electrophoresis to separate the amino acids?
Method of detection	How do you detect resolved amino acids (e.g., UV using a fluorophore or MS)?
Calibration materials	How do you calibrate your assay? How often the calibration is performed? How many calibration standards are used for the calibration?
Quality controls	How do you assess the quality of the sample hydrolysis? What quality control materials are used in your assay?
Precision	What is the precision of your assay? How did you determine the precision of your assay? How do you continue to monitor the precision of your assay?
Accuracy and traceability	How did you establish the accuracy of your method? Is your assay traceable to NIST or another reference material? How do you ensure continued traceability? Is the laboratory participating in external quality control program?

Table 6

Comparison of field methods for amino acid analysis

Peptide [a]	Mass (Da) [b]	nmol (per 1 mg)	NIST (nmol) [c]	Site A (nmol) [d]	Site B (nmol) [d]	%-difference [e]
DAEPDILELATGYR	1561.8	640.3	441.8	469	414	12.5
KAQYARSVLLEKDAEPDILELATGYR	2948.6	339.1	247.6	298	267	11.0
RQAKVLLYSGR	1289.8	775.3	519.5	586	561	4.4

[a] Peptides were developed by the Peptide Standards Project Committee (PSPC) of the Association of Biomolecular Resource Facilities (ABRF)(See JOURNAL OF BIOMOLECULAR TECHNIQUES, VOLUME 11, ISSUE 2, JUNE 2000 pages 102-105), and aliquoted, lyophilized, and distributed by the National Institute of Standards and Technologies as SRM® 8327.

[b] The monoisotopic mass for each peptide.

[c] Amount of peptide in each vial as determined by amino acid analysis per SRM® 8327 package insert.

[d] Amount of peptide in each vial as determined by amino acid analysis in two field laboratories in 2015.

[e] Percent difference between the two field laboratories (calculated as the absolute value of the difference in measurements divided by the mean of the two measurements).

Table 7
Recommended guidelines for peptide storage and handling

Description	Specification
Artifacts due to chemical modification or degradation	• Limit air exposure of peptides in solution • Use an inert blanket gas in storage tubes • Use amber or dark storage tubes for photochemically active sequences • Avoid multiple freeze-thaws
Duration of storage	• Short-term (≤ 3 months): High concentration (1 – 100 pmol/μL) liquid solution at 4 °C or frozen solution at -20 to -80 °C • Medium-term (3 months – 1 year; peptide-dependent): Frozen solution at high concentration • Long-term (> 1 year): Lyophilized at -20 to -80 °C • Concentrated stock solution (0.5-2 nmol/μL): Storage duration depends on peptide • Short-term: Working solutions (1-100 pmol/μL)
Reconstitution	• General reconstitution solution: 5 % acetonitrile/0.1-1 % formic acid • Troubleshooting: Increase organic solvent and adjust pH • Obtain AAA concentration and UV absorbance data
Minimization of non-specific adsorption	• Add peptides directly to diluent fluid instead of tube walls • Rinse pipette tip several times with peptide solution prior to aspirating final volume • Use new pipette tip for each dilution • Maintain relatively high concentrations (0.5-2 nmol/μL)
Storage vessels	• Silanized glass vials • Polypropylene vials or plates with modified plastic surfaces • Wash vials and tubes with same solution being used for peptides, and examine plastics for residual plasticizers
Evaluation of peptide stability	• Condition peptides to autosampler tray temperature prior to injection (consider temperatures above 4° C, which can improve stability) • Quantify reconstituted peptides by AAA and benchmark the concentration using UV absorbance • Characterize solubility and adsorption behavior through UV absorbance of a series of dilutions or replicates and repeated injection on the LC-MS system • Evaluate peptide stability for six temperature- and time-related conditions as outlined in Assay Development Guidelines available on CPTAC Assay Portal (https://assays.cancer.gov/)

Table 8
Sources and distributors of reference materials

Institution	URL
National Institutes of Standards and Technology	www.nist.gov/srm/index.cfm
Institute for Materials and Measurements	ec.europa.eu/jrc/en/reference-materials
Joint Committee for Traceability in Laboratory Medicine	www.bipm.org/jctlm
World Health Organization	www.who.int/bloodproducts/catalogue
LGC Standards	www.lgcstandards.com/Catalogues
National Institute for Biological Standards and Control	www.nibsc.org/products/brm_product_catalogue.aspx
Sigma-Aldrich	www.sigmaaldrich.com