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Special Reports

Recommendations for the Generation, Quantification, Storage, and Handling of Peptides Used for Mass Spectrometry–Based Assays

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BACKGROUND: For many years, basic and clinical researchers have taken advantage of the analytical sensitivity and specificity afforded by mass spectrometry in the measurement of proteins. Clinical laboratories are now beginning to deploy these work flows as well. For assays that use proteolysis to generate peptides for protein quantification and characterization, synthetic stable isotope–labeled internal standard peptides are of central importance. No general recommendations are currently available surrounding the use of peptides in protein mass spectrometric assays.

CONTENT: The Clinical Proteomic Tumor Analysis Consortium of the National Cancer Institute has collaborated with clinical laboratorians, peptide manufacturers, metrologists, representatives of the pharmaceutical industry, and other professionals to develop a consensus set of recommendations for peptide procurement, characterization, storage, and handling, as well as approaches to the interpretation of the data generated by mass spectrometric protein assays. Additionally, the importance of carefully characterized reference materials—in particular, peptide standards for the improved concordance of amino acid analysis methods across the industry—is highlighted. The alignment of practices around the use of peptides and the transparency of sample preparation protocols should allow for the harmonization of peptide and protein quantification in research and clinical care.
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The Clinical Proteomic Tumor Analysis Consortium *(1)* (CPTAC)[29] of the National Cancer Institute is a comprehensive and coordinated effort to accelerate the understanding of the molecular basis of cancer through the application of robust technologies and work flows for the quantitative measurements of proteins. The Assay Devel-

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[29] Nonstandard abbreviations: CPTAC, Clinical Proteomic Tumor Analysis Consortium; MS, mass spectrometry; MRM, multiple reaction monitoring; SOP, standard operating procedure; GPM, Global Proteome Machine; PRIDE, Proteomics Identifications; AAA, amino acid analysis; RP, reverse phase; LLOQ, lower limit of quantification; SRM, standard reference material; SSRC, Sequence Specific Retention Calculator; ISO, International Organization for Standardization.

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opment Working Group of the CPTAC aims to foster broad uptake of targeted mass spectrometry (MS)-based assays that use isotopically labeled peptides for confident assignment and quantification, including multiple reaction monitoring (MRM) (also referred to as selected reaction monitoring), parallel reaction monitoring, 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). Toward these goals, we have (*a*) coordinated a consensus approach to outline recommendations for the development of different classes of targeted MS-based assays with a fit-for-purpose approach *(2)*; (*b*) launched the CPTAC Antibody Portal *(3)* (https://antibodies.cancer.gov) to facilitate the production, characterization, and distribution to the community of renewable affinity reagents to support protein/peptide measurement and analysis; and (*c*) 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 procedures (SOPs), reagents, and assay characterization data.

Within work flows designed to quantify protein-derived biomarkers by proteolytic digestion and LC-MS/MS, synthetic peptides are often used in 3 ways. First, 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 and secondary structure as those of the endogenous analyte, as well as a similar fragmentation pattern). Second, stable isotope–labeled internal standard peptides help normalize sample-specific ion suppression and are used in the calculation of a peak area ratio (the ratio of the endogenous analyte chromatographic peak area to that of the internal standard). Third, 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 SOPs 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 proteolytic digestion sites, 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 peptides are proteolytically digested, their quantification in the sample can be facilitated by use of 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 among peptides in their recovery and stability after 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. Although potentially providing improved precision and less bias than labeled peptides in quantitative MS-based assays *(8, 9)*, differences in the repertoire of modifications (e.g., phosphate, carbohydrate, ubiquitin) 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 SOP 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 SOPs) *(4, 10)*, the major challenges to achieving this analytical bar are (*a*) selecting peptides that can be measured with high precision and repeatability in the matrix of interest, (*b*) generating well-characterized pure synthetic peptide internal standards and calibrators, (*c*) determining the accurate concentration of pure synthetic peptide internal standards and calibrators, (*d*) ensuring 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 (*e*) properly interpreting peptide-based measurements. The purpose of this special report is to address these key components by aggregating recommendations on the basis of published studies and the consensus experiences of the authors. The use of "crude" peptides (those that have limited or no purification or quantification but are far less expensive than purified peptides) is also

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discussed, and the limitations of their use for quantitative measurements are presented. This special report provides a thorough framework for proteomics researchers and an introduction to clinical applications. Ideally, the recommendations included here provide a starting reference point for the production of formal guidelines and future best practices (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" *(11)* (typically tryptic) peptides that are unique to a single gene product or proteoforms *(12)* and that are observable by MS. Because peptides vary greatly in their performance across many aspects of targeted MS analysis (e.g., ease of synthesis, stability, solubility, recovery, responsiveness in the mass spectrometer), careful selection of peptide analytes is critical to developing the highest-quality assays.

Peptide analytes can be selected by use of MS-based proteomic data or prediction algorithms *(13–21)*. Selecting peptides from empirical MS data greatly increases the likelihood for success in developing a targeted MS-based assay, since 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., Global Proteome Machine (GPM) *(22)*, Proteomics Identifications (PRIDE) *(23)*, PeptideAtlas *(24–26)*, PhosphoSitePlus *(27)*]. The use of spectral libraries, either generated with one's own MS/MS data or obtained from an online spectral library repository, 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 2 approaches in an iterative manner is often used to hone the choice of the best peptides as rapidly as possible.

A summary of criteria for peptide selection is presented in Table 1. Despite the above considerations, peptide selection is an empirical exercise that balances ideal characteristics with practical limitations. In some cases, because of 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 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 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 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 proteolysed 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 AND CRUDE 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 *(28, 29)*. 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 a guideline document on assay development and characterization). Purified peptides are typically analyzed by amino acid analysis (AAA) *(30)* 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 byproducts, and neither accurate quantity nor purity are possible. The identities of crude peptides must be confirmed by MS, and because crude peptides vary greatly in purity, we recommended that further evaluation of purity [e.g., by reverse-phase (RP)-HPLC-UV] or estimated quantity be performed by the end user. Although crude

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Table 1. Peptide selection guidelines for MRM targeted assays.

Filtering criterion	Description
Uniqueness (analyte specificity)	Peptides must be unique in sequence to the gene product or proteoform[a] of interest to enable specificity of the assay.
Peptide length	Typically 7–20 amino acids.
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 owing to solubility issues, and extremely hydrophilic peptides can be problematic owing to LC retention time instability. As a general rule, it is best to select peptides within an SSRC 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 the following residues if possible, listed in decreasing priority (potential posttranslational/preanalytical processing issue): • 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.[b] Avoid ragged ends (i.e., KK, KR, RR, RK) and possible miscleavage 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 if possible, since they may affect assay results by altering the recoverability or detection of the analyte peptide.

[a] Smith et al. *(12)*.
[b] Riviere and Tempst *(75)*.

peptides can help confirm the identity of endogenous peptides and improve the precision of relative quantification within a research laboratory with a single batch of peptides, well-characterized, purified peptide calibrators and detailed SOPs are required to distribute assays to the community and harmonize results across laboratories. Importantly, methods intended for 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 manufacturers), but also the instruments, resins, and amino acid building blocks, as well as the methods used to synthesize, purify, freeze dry, and package peptides, will vary widely depending on the manufacturer. We recommend the use of 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 with shallow gradients of ≤2% change in organic concentration per minute; otherwise, the presence of impurities can be masked by coelution of contaminant synthesis byproducts. More details on characterization are provided

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Table 2. Pure peptide specifications and methods for qualification.

Description	Specification
Amount	≥1 mg ordered; ≤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 amino acids	Lys, Arg are recommended in the case of tryptic peptides; other amino acids may be labeled (e.g., Leu, Phe)
Number of heavy amino acids	0–2, depending on sequence and label
Mass difference (relative to unlabeled peptide)	6–20 Da depending on amino acid sequence
Location of heavy amino acids	C-terminal Lys or Arg (unless noted otherwise)
Amino acids 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 1 mg peptide	5%–30% acetonitrile/0.1%–1% formic acid at approximate concentration of 0.5–2 nmol/μL (500–2000 μmol/L); aliquots of this solution are used for AAA
Remaining peptide	Dry powder (preferably in 0.1- to 1-mg aliquots) stored under argon/nitrogen or in a desiccator at −20 °C or lower for longer-term storage (>6 months)
Purification method	Preparative RP-HPLC
QC	
Step 1 (LC-UV)	Analytical RP-HPLC chromatogram (determine percentage purity)
Step 2 (MS or LC-MS/MS)	MALDI, electrospray ionization spectrum (mass ID confirmation), or MS/MS (label plus sequence verification)
Step 3 (AAA)	Concentration (pmol/μL or μM); percent variation or percent relative error from expected amino acid composition

below. Regardless of whether the quality of the peptides is assessed by the vendor, a vendor subcontractor, or in-house, the SOPs used for QC should be made available and linked to the corresponding QC 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 limit of quantification (LLOQ)], 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), as summarized in Table 2. Critical to the long-term success of assays that rely on peptide calibrators and internal standards is working with vendors whose methods are well documented, 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 with current automated peptide synthesizers is typically performed at a micromole scale (0.1 to 1 mmol) *(31–33)*, which produces yields much higher than those required for targeted MS-based assays (approximately 10 fmol/peptide/sample or 100 pg/peptide/sample for a peptide of molecular weight 1000 Da). Synthesizing peptides at larger scales results in higher yields but increases the costs when stable isotope–labeled heavy amino acids are used. A synthesis scale of 1–5 mg purified peptide provides a reasonable balance between cost and yield. As shown in Table 2, 1 mg is formulated for 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, uniform ^{13}C labeling at all carbon atom positions, or combinations of ^{13}C and ^{15}N). Whereas it is often less costly to use deuterium as the isotopic label, its use is not recommended because the presence of several

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deuterium atoms in a peptide can alter the peptide's retention time and prevent the desired coelution with the unlabeled peptide *(34–36)*. 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 *(37)*. 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, we recommend a minimum separation of 6 Da, and 8–10 Da for triply charged precursors. Lysine is available in isotope-labeled forms that are 6 and 8 Da heavier than the unlabeled form, whereas arginine is available in +6, +8, and +10 Da versions. When additional internal standard peptides are going to be included to provide QC 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 >99% (Table 2). This percentage refers to the chance of finding the heavy isotope of an atom at each potential label site in the amino acid. Because >1 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). Underlabeled peptide "isotopologues" will introduce inaccuracies in the quantification and can interfere with the measurement of the endogenous unlabeled peptide. For these reasons, we advise using only the highest possible isotopic purity labeled amino acids available (>99% enrichment per isotope) when having labeled peptides synthesized. We also recommend 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 Lys or Arg is not present in the peptide (protein C-terminus).

During sample preparation, disulfide bonds in proteins are typically reduced and the cysteine residues 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 postdigestion, 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, the synthesis of multiply phosphorylated peptides has a higher failure rate than that of singly phosphorylated peptides. Furthermore, the production of recombinant proteins and peptides does not guarantee proper disulfide bond formation, which 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 Angeletti et al. *(30)*. RP-HPLC (C18 column) with UV monitoring is the best method to assess the complexity of peptide products, although examples of coelution with truncated peptides have been observed *(38)*. MS with either electrospray ionization or MALDI is essential for the identification of desired products and mass impurities. The coupling of RP-HPLC to electrospray MS/MS enables the confirmation of amino acid sequence and residue location of modifications *(39)*. 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 *(40)*.

The purity and identity of purified peptides are typically evaluated by use of analytical RP chromatography with UV detection (HPLC-UV, monitoring at wavelengths 214 or 220 nm and 280 nm) and MS. 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 (by analysis of collected fractions with MALDI or, preferably, on-line LC-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 determine the chemical nature of impurities that may be present (e.g., incomplete deblocking, premature termination).

For characterization by HPLC-UV, MALDI, and especially AAA, the peptide must be completely dissolved in solution. Importantly, the formulation and composition of the peptide used for QC should be identical to that used for assay development and validation. The formulation range recommended in Table 2 was chosen on the basis of our experience handling thousands 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 μmol/L) for AAA (see below) and evaluation by MS. The higher percentages of acetonitrile (≤30%) are recommended for more hydrophobic peptides. Formulation and storage in neat DMSO is also possible, but oxidation

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of methionines may occur. Peptide solutions in DMSO need to be diluted before 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 caution should be used during dilution.

CRUDE (LOWER-PURITY) PEPTIDES

For crude (lower-purity) peptides, purification by preparative HPLC and quantification by AAA adds substantially to the cost of synthetic peptides. Eliminating these steps and generating crude peptides markedly reduces the cost for both natural abundance and stable isotope–labeled peptides. Because of 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. 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 to develop distributable assays and improve interlaboratory 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 use of 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. Because 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. As with high-purity peptides, however, 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 use of MALDI-TOF or LC-MS/MS of individual peptides or mixtures of peptides, respectively.

Fig. 1 shows the MALDI-MS spectra of a high-purity and lower-purity peptide of the same amino acid sequence. These peptides were provided from independent syntheses by the vendor with 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 (Fig. 1A) and high-purity (Fig. 1B) peptide samples. 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. 1A).

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 could not be accurately quantified owing to the presence of incompletely deblocked and truncated peptide species. Furthermore, in previous reports, AAA of crude peptide preparations to assess peptide content without a preceding desalting step was compromised by residual scavenger reagents *(41)*. 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 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 peptide was measured by use of the mean 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 (400–700 nmol/vial). Therefore, it is not possible to determine the LLOQ of LC-

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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 amino acids	Lys, Arg are recommended in the case of tryptic peptides; other amino acids may be labeled (e.g., Leu, Phe)
Number of heavy amino acids	0–2, depending on sequence and label
Mass difference (relative to unlabeled peptide)	7–20 Da depending on amino acid sequence
Location of heavy amino acids	C-terminal Lys or Arg (unless noted otherwise)
Amino acids 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 1 mg peptide	5%–30% acetonitrile/0.1%–1% formic acid at estimated (by dry wt) concentration of 1 mg/mL
Remaining peptide	None
Purification method	None or SPE only
QC	
Step 1 (LC-UV)	LC-UV chromatogram (% purity) performed and assessed by submitting laboratory
Step 2 (MS or LC-MS/MS)	MALDI, electrospray ionization spectrum (mass ID confirmation), or MS/MS (label plus sequence verification)
Step 3 (AAA)	None until identification by MS of desired peptide as major species

MS/MS assays when crude peptides are used as standards. If used in interlaboratory 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 among vendors is critical to harmonizing concentrations of peptides determined solely from the endogenous peak area ratio with internal standard or 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 *(42)*]. For peptides, however, preparation of calibrators with accurate concentrations by use of gravimetric preparation alone is often not feasible because of limited available quantities or uncertain purity. In these cases, the concentrations of peptide calibration solutions can be measured through quantitative analysis of their constituent amino acids after hydrolysis of the peptide's amide bonds *(43)* (i.e., AAA). As mentioned above, AAA of pure peptide internal standards and calibrators is vital to the transferability of assays and aggregation of results among 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 QC 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 QC 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 QC. Further, peptide vendors offer different levels of assay quality (e.g., within 5%–10% CV, within 10%–25% CV); therefore, the accuracy of their AAA assays needs to be specified.

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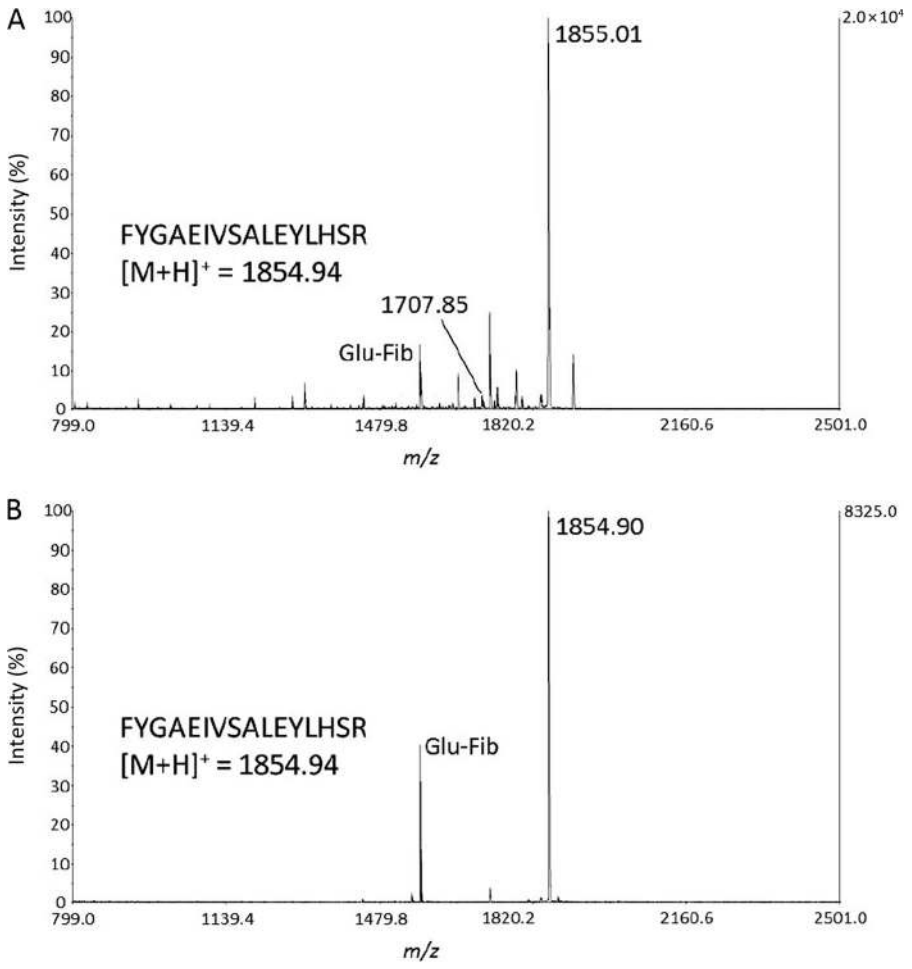

Fig. 1. Example of a recommended assessment of peptides.
MALDI-MS spectra of lower-purity (A) and high-purity (B) peptides. The internal standard was a synthetic peptide (Glu-Fib).

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 work flow, standardization, and QC 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 ≥1 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 those with the highest frequencies found in nature and will be present in most peptides. There are some caveats to this list. First, hydrophobic amino acids such as Ile, Leu, and Val can be problematic due to their slow hydrolysis rate. Second, 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. Third, the underivatized basic amino acids Arg, His, and Lys have longer retention times on RP–ion exchange HPLC columns than 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 with 4 mol/L methanesulfonic acid containing 0.2% (vol/vol)

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Table 4. Amino acid composition of high- and lower-purity preparations of a peptide (FYGAEIVSALEYLHSR).

AA	Expected	High purity[a]	Lower purity[a]
Ala	2	2.0	2.0
Arg	1	1.0	1.3
Asx	0	ND[b]	ND
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	ND	ND
Phe	1	1.0	0.7
Pro	0	ND	ND
Ser	2	1.9	2.2
Thr	0	ND	ND
Tyr	2	2.0	2.0
Val	1	1.0	1.1

[a] Mean of 4 AAA determinations; CV = 7.6% and 4.8% for high- and lower-purity peptides, respectively.
[a] ND, not detected.

3-(2-aminoethyl)indole for AAA quantification *(44)*. The use of only stable amino acids for quantitative AAA allows for the use of hydrolysis conditions that are optimized primarily for completeness of hydrolysis.

Before 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 *(45)*. 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. LC-MS/MS analysis of the peptide should be performed to determine whether peptide impurities are present and provide a rough estimate of their amounts relative to the analyte peptide. If relative content of peptide impurities is high, purification of the analyte peptide should be performed before AAA.

It is important to verify that peptides are completely dissolved before 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 before AAA is identical to the protocol used to solubilize the peptide internal standards and calibrators before use in the quantitative assay. Best practice would use UV-spectroscopy to confirm calibrator solution concentration before use.

AAA assays should be considered as 3 key steps, with an optional derivatization stage commonly used either pre- or postcolumn 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 increased temperatures (110 °C, but can range from 90 °C to 130 °C) in a low-pH environment with concentrated acids (6 mol/L HCl, 4 mol/L methanesulfonic acid, 2 mol/L trifluoroacetic acid, etc.). Alkaline hydrolyses are also possible with concentrated KOH or NaOH, al-

Table 5. Important information to be obtained from commercial laboratories regarding 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 (*a*) hydrolysis; (*b*) instrument validation; and (*c*) AA 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 is the calibration performed? How many calibrators are used?
QC	How do you assess the quality of the sample hydrolysis? What QC 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 an external QC program?

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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

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

Each amino acid was characterized with respect to stability in acid, stability during storage, and efficient hydrolysis. Green indicates that the amino acid is favorably stable or hydrolyzable. Red indicates instability. Yellow is used to highlight 3 hydrophobic amino acids that can affect hydrolysis.

though this approach is not as commonplace. Acid hydrolysis can be performed in 2 ways: in concentrated acid solution or with acid in the gas phase. Increased temperatures used for hydrolysis are obtained with a conventional oven, a heating block approach, or a specifically designed microwave oven to control energy and temperature.

Separation of amino acids before detection can be achieved by several methods, including HPLC (ion-exchange and RP), gas chromatography, and electrophoresis. RP-HPLC and gas chromatography typically require chemical derivatization before detection, although newer HPLC column chemistries are facilitating improved retention characteristics and enable baseline separations of nonderivatized amino acids, thus limiting bias and imprecision associated with a derivatization step *(46)*. High-resolution amino acid separations are also possible with capillary electrophoresis *(47)*.

Detection of amino acids is most commonly performed with 1 of several types of detectors. These include (*a*) a spectroscopic detector as used for visible/UV or fluorescence, (*b*) measurement of electric current from redox reactions with amperometric electrochemical detection, and (*c*) MS. Spectroscopic and electrochemical detections offer higher analytical sensitivity, whereas MS offers better analytical selectivity in complex matrices and the capability of isotope-dilution quantification techniques. Nonchromatographic MS-based methods for AAA have also been developed *(48)*.

NIST has developed an AAA method on the basis of isotope-dilution LC-MS/MS analysis that uses a stable isotope–labeled analog of each amino acid measurand spiked both into samples and calibrators in an equivalent manner. This "double isotope dilution" technique is beyond what routine laboratories would typically use, but is routinely used at NIST to certify concentrations for a wide variety of analytes *(49)*. 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 with elemental analysis, Karl Fischer titration (for water content), nuclear magnetic resonance, 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 before 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 *(50)* in several NIST standard reference materials (SRMs) (http://www.nist.gov/srm). Peptide or protein concentration is determined inde-

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pendently 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 on the basis of this propagated error, and expanded uncertainties are determined through advanced statistical analysis. Typical CVs for AAA measurements by 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 *(50)* 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 QC 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, QC efforts in routine AAA should focus on these steps. The completeness of hydrolysis can be affected by the amino acid sequence of the peptide, as 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) by use of a time-course 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 peptide hydrolysis was not complete, there are unknown 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, laboratories 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 by use of 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 a problem with peptide hydrolysis or accuracy of amino acid measurement or both. It would be possible to rule out problems with AAA measurement by use of an amino acid solution reference material of known concentration, such as NIST SRM 2389a *(51)*. Through the combined use of peptide and amino acid standards, sufficient accuracy in routine AAA can be achieved.

Amino acid calibrators and peptide/protein QC materials should be selected carefully to ensure accuracy of the measurements. Calibrators should mimic the measurands as identically as possible in both concentration and structure/form. Calibrators should be characterized for purity, both organic and inorganic contaminants, and water content. Both calibrators 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 calibrators at the beginning of the sample preparation; accuracy of the amount of the sample taken for the analysis must be ensured, 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 *(52)*. Finally, labeled amino acid internal standards should contain a number of isotopic atoms that would provide sufficient mass difference from the isotopic envelope of their "light" analogs 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 peptides would lack specific amino acids that are degraded during hydrolysis (Trp, Met, Cys, Ser, and Thr), amino acids that have limited

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Table 6. Comparison of field methods for AAA.

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 of the Association of Biomolecular Resource Facilities [see *(76)*] and aliquoted, lyophilized, and distributed by NIST as SRM 8327.
[b] Monoisotopic mass for each peptide.
[c] Amount of peptide in each vial as determined by AAA per SRM 8327 package insert.
[d] Amount of peptide in each vial as determined by AAA in 2 field laboratories in 2015.
[e] Percent difference between the 2 field laboratories (calculated as the absolute value of the difference in measurements divided by the mean of the 2 measurements).

stability during long-term storage (Trp, Met, Cys, Asn, and Gln), and amino acid pairs that often do not 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 with alternative methodology, and including ≥1 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 ≥30 days at 4 °C and 3 years at −20 °C. Peptides in solution should also be stable to multiple freeze–thaw cycles and to ≥1 lyophilization-resolubilization step (in aqueous/organic/acid solvent) without significant loss of peptide (<3%) or modification of residues. Although it is not possible to know how soluble and stable a peptide will be before synthesis, an example that might be a useful standard peptide for AAA is DAKAGIHPLELRVARYR. This artificial, nontryptic peptide that is not present in any gene sequence is 17 residues in length, 15 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 nonreproducibly; 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. Because of the issues associated with using weighed amounts of peptide (e.g., salt and water content of lyophilized peptides), it is desirable for the field to have available ≥1 standard peptides already in solution, ready for use in amino acid analysis. For example, the NIST peptide standards SRM 8327, provided as a reference material without a certified concentration measurement, were aliquotted gravimetrically (target of 1 mg peptide per vial) and then distributed as lyophilized peptides. The actual amount of peptide added to the vial was much lower than 1 mg for each peptide (determined with AAA), owing to salts and water associated with the lyophilized peptide (Table 6).

To summarize, the field of proteomics would greatly benefit from new reference materials developed and carefully characterized with a double isotope dilution technique. The new reference materials would include 1–3 peptides in solution that are soluble and stable, would contain the most reliable amino acids for AAA, and would 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 method development and routine use of the method.

Peptide Storage and Handling

Quantitative MS-based assays are negatively affected by a lack of proper procedures for storing and handling peptides. This 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, Cys, Met, and Trp are prone to reversible and irreversible oxidation, and this conversion is accelerated during freeze–thaw cycles and at high pH

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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	• Concentrated stock solution (0.5–2 nmol/μL): storage duration depends on peptide • Short-term (≤3 months): high concentration (1–100 pmol/μL) liquid solution at 4 °C or frozen solution at −20 °C to −80 °C • Medium-term (3 months to 1 year; peptide-dependent): frozen solution at high concentration • Long-term (>1 year): lyophilized at −20 °C to −80 °C
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 nonspecific adsorption	• Add peptides directly to diluent fluid instead of tube walls • Rinse pipette tip several times with peptide solution before 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 before injection (consider temperatures >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 LC-MS system • Evaluate peptide stability for 6 temperature- and time-related conditions as outlined in Assay Development Guidelines, available on CPTAC Assay Portal (https://assays.cancer.gov/)

(53). Gln and Asn are prone to deamidation *(54)*, 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., Phe and Trp).

To minimize detrimental effects, the long-term storage of peptides (>6 months) is most effective when peptides are lyophilized and stored at temperatures −20 °C to −80°C *(32, 55)*. 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 by use of predefined extinction coefficients may be a suitable alternative. Once resolubilized, the peptide calibrator solutions (0.5–2 nmol/μL) are best stored frozen at temperatures below −70 °C in sealed tubes. Although stability in solution is peptide dependent, generally working solutions are prepared from the stocks at concentrations of 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. According to good laboratory practice (preferably by use of 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

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dark storage tubes should be used for photochemically sensitive sequences.

RECONSTITUTING PEPTIDES

As mentioned above, solubility can be a major 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 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.

Before 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 on 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 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., methanol instead of acetonitrile). If increasing organic solvent is not effective in solubilizing the peptide, the pH can be adjusted by addition of acid (≤1% formic acid or trifluoroacetic acid) or by use of 1% ammonium bicarbonate, 1% *N*,*N*-diisopropylethylamine, or ammonium hydroxide. Another option is to redry the peptide and redissolve it in DMSO.

NONSPECIFIC ADSORPTION OF PEPTIDES

Variable recovery because of nonspecific adsorption is 1 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 nonspecific peptide adsorption to the walls of peptide storage vessels, pipette tips, autosampler vials, and HPLC components varies on the basis of 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 pipette 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 *(56)*; 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 nonspecific adsorption in low-concentration solutions is greater than in more concentrated solutions because of the limited binding capacity of the wetted solid surface area *(57)*. To demonstrate the loss of peptides in solution and the effect of storage concentration, 2 peptide mixtures (200 and 1000 fmol/μL) were prepared in nondeactivated glass vials and analyzed by injecting 1 μL of each sample each hour 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 0 peptides, respectively, showed noticeable signal decay over time under the 2 conditions. This effect can be seen by plotting total peak areas of 2 representative peptide sequences, YLGYLEQLLR [Sequence Specific Retention Calculator (SSRC) relative hydrophobicity 41.55] and IYEGSI-

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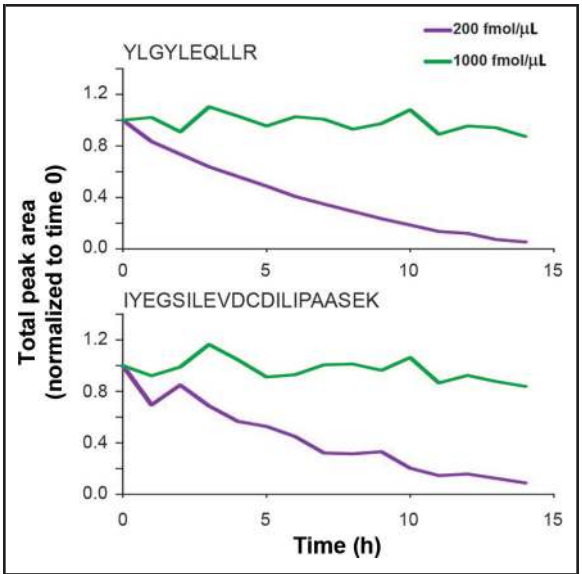

Fig. 3. Evaluation of peptide concentration on stability of signal over time.

The peak areas (normalized to time 0) from 2 representative peptides were plotted vs autosampler storage time (h) 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. For details, see online Supplemental Materials and Methods, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue1.

LEVDCDILIPAASEK (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.

Nonspecific adsorption contributes to carryover, which increases variability and bias due to residual signal in sample runs *(58)*. Carryover in sample preparation can originate from reusing pipette tips to transfer peptide solutions between vials or in dispensing aliquots. Carryover in sample preparation or analysis can negatively affect results through ion suppression of low-abundance peptides (when coelution 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 nonspecific adsorption by transferring a solution of the analyte sequentially from 1 vial to another and analyzing a small aliquot after each transfer step to assess for losses *(59)*. Despite the diverse physicochemical properties of peptides, various strategies can be generically applied to reduce adsorption and cross-contamination phenomena *(56, 60)* leading to carryover. When preparing dilution series, one should never reuse pipette tips, to avoid cross-contamination. Pipette tips should be prerinsed several times with the peptide solution before aspirating the final volume. To minimize nonspecific 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 after 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 with a series of different elution buffers and 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 cleaning the HPLC flow-path and columns.

Different types of vials can introduce significant variability in LC-MS analyses *(61)*. 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 most commonly used to manufacture vials, inserts, and plates. Although a single type of vial might not be optimal in terms of minimizing the nonspecific interaction of all the peptides in an analytical mixture, 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 *(62)*. 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 3 types of autosampler sample vials: nondeactivated 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 hour 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, with cutoffs of <5%, 5%–50%, or >50% peptide loss on the basis of signal intensity over the 15 h. We found that all 3 vial types enabled the recovery of 43 peptides, which accounted for

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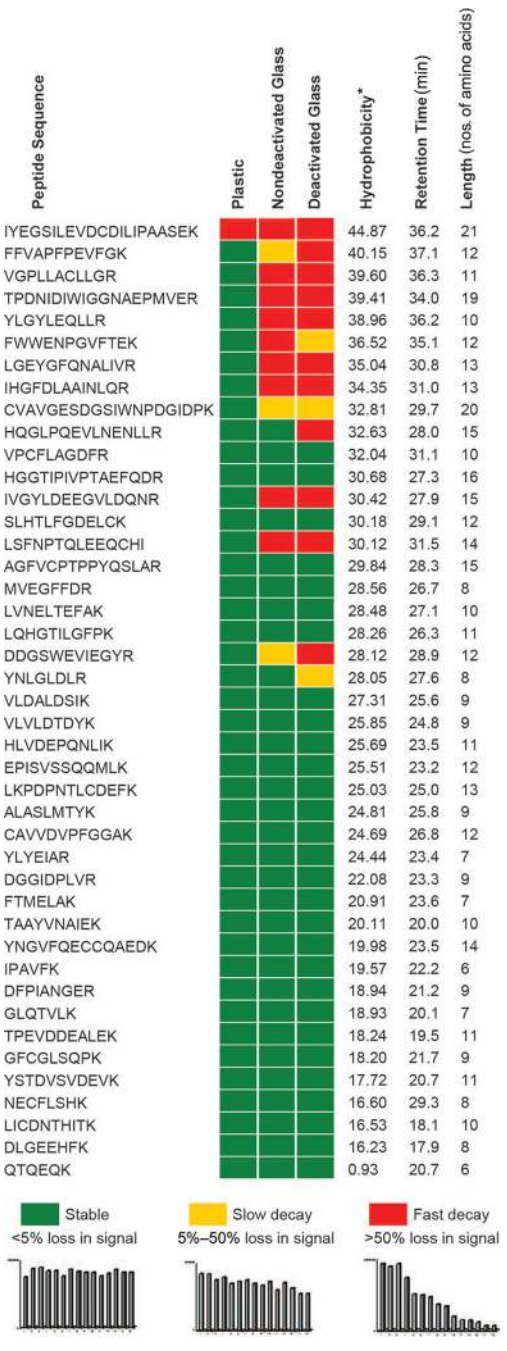

Fig. 4. Effect of autosampler vial material on stability of peptides in a protein digest: plastic, nondeactivated glass, and deactivated glass.

The plastic vial outperforms the 2 glass vials, since it is associated with only 1 "unstable" peptide. The peptide sequences are sorted by relative hydrophobicity. See online Supplemental Materials and Methods for details. * Sequence Specific Retention Calculator relative hydrophobicity.

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 2 glass vials, as only 1 "unstable" peptide with significantly lower recovery was detected, whereas 13 and 14 unstable peptides were detected in nondeactivated 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 1 sample was subjected to 10 freeze–thaw cycles. The remaining 10 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 before the analysis, from each sample over 10 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, 3 plots were made to show peptides with low (peptides with poor ionization), medium, and high signal abundance, respectively. Regardless of how the samples were handled, comparable peak areas of the peptide were observed (Fig. 5A), and there was no significant difference (paired *t*-test, $P < 0.05$) among the mean peak areas for the freeze–thaw experimental conditions.

We next assessed reproducibility under the 3 conditions by comparing imprecision of the replicate analyses (Fig. 5B) and found a number of important observations: (*a*) the variability of peptide peak area (CV) is sequence dependent and closely related to the peptides' hydrophobicity; (*b*) no obvious correlation was observed between peptide peak area and variability (within the limits tested); (*c*) for most peptides, variability of peptide peak area (CV) was lower for freezer storage (approximately 1.5- to 3.5-fold) than for other storage conditions; and (*d*) the variability of peptide peak area (CV) was highest after 10 freeze–thaw cycles. These results suggest that frequent freeze–thaw cycles should be minimized, and that best results are obtained from analysis of samples that are stored frozen and thawed immediately before 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

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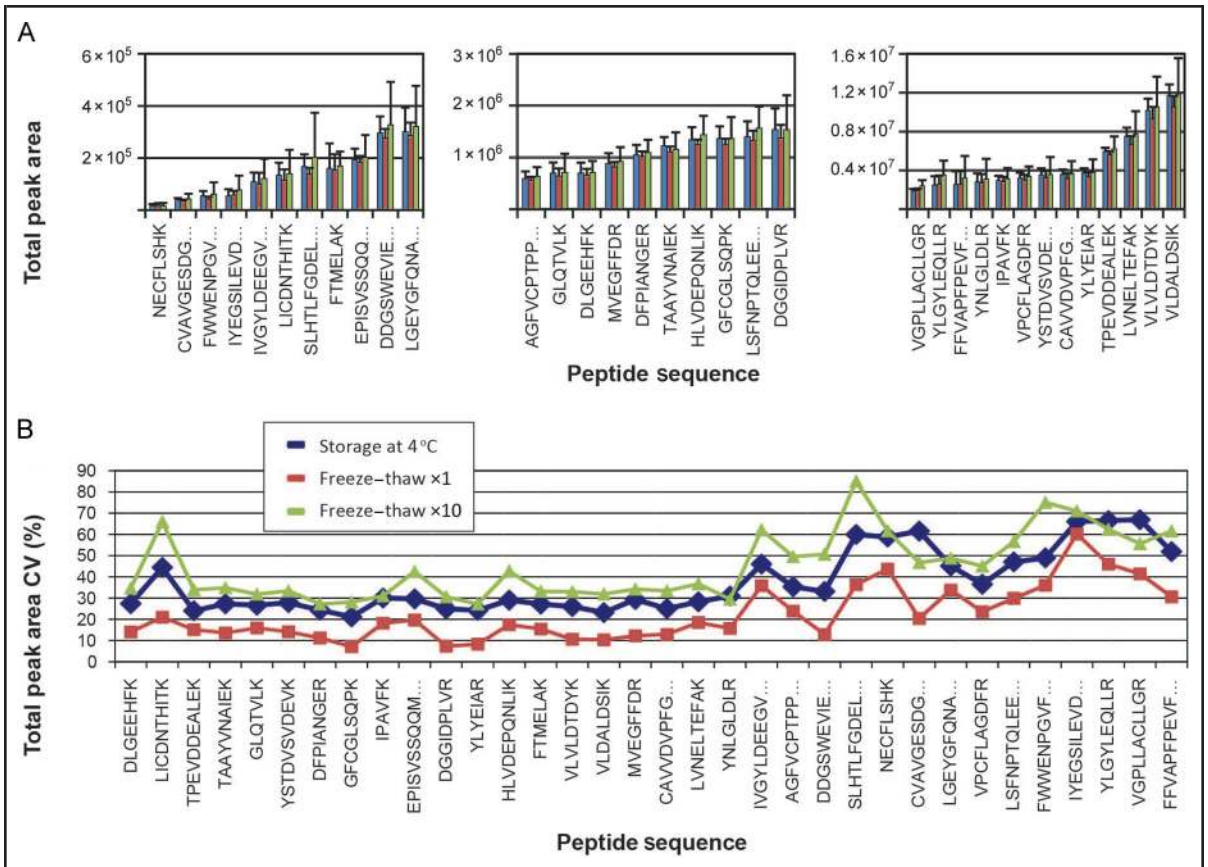

Fig. 5. Evaluation of freeze–thaw effect.

Mean values of total peak area from triplicate injections are plotted for all detected peptides. Error bars show SD. (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 3 conditions: refrigerator storage (control), freezer storage with 1 freeze–thaw cycle, and 10 freeze–thaw cycles. (B), CV is plotted as a function of peptide sequence. Peptides are plotted on the *x* axis in order of increasing retention time. See online Supplemental Materials and Methods for details.

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 MS signal. Peptides should be conditioned to the autosampler tray temperature before injection, as temperature-related differences in peak area have been observed when peptides were not equilibrated *(63)*. 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 *(10)*. Briefly, the guidelines recommend the analysis of peptide peak area variability for 6 temperature- and time-related conditions (6 h at 4 °C, 24 h at 4 °C, 4 weeks at −70 °C, 1 freeze–thaw, and 2 freeze–thaws) compared to the time 0 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 (Table 7).

Ensuring Specificity and Reliability of Quantitative Data on the Basis of 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 stable isotope–labeled internal standard peptides facilitates both of these goals, as described below.

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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 coeluting 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 with Skyline *(14)* or analyzed by automated data processing (e.g., peak picking, quantification and false discovery rate calculation) with software tools such as mProphet *(64)*. Observing the same fragmentation patterns [i.e., the same transition and the same relative peak intensity ratios across multiple transitions *(65)*] 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 transitions by manual inspection or with the software tool AuDIT *(66)*. 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, 10)*, and a summary document can be downloaded from the CPTAC assay portal (https://assays.cancer.gov) *(4)*. Open-source software tools *(14, 67)* are available to facilitate analyses and data sharing. Validation of quantitative assays of proteins in complex mixtures requires additional experiments *(10)*, 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 2 most abundant fragment ions be 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 posttranslational modification, then there must be ≥1 fragment containing the modified residue *(68)*. In parallel reaction monitoring 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 by use of isotopically labeled peptide internal standards is presented below.

1. Check the extracted peaks for both heavy standard and endogenous light peptides with software tools such as Skyline *(14)*, making sure that both peaks coelute.
2. Confirm that the transition pattern (rank and relative intensity, typically requiring ≥3 transitions) is consistent between the isotope-labeled internal standard and endogenous peptide. The confidence of detection of endogenous peptide is ensured 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 by visual inspection or with software tools such as AuDIT *(66)*.
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, LLOQ, 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. On the basis of 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 LLOQ of the assay and within the linear range of the assay.

As mentioned above, there are several reasons this approach may not accurately reflect the amount of endogenous peptide or protein present in the undigested sample: (*a*) liberated peptides are lost or nonspecifically degraded during digestion, especially with high concentrations of trypsin and long digestion times *(69)*; (*b*) proteins in complex mixtures are often not digested to completion when trypsin is added in lower concentrations; (*c*) proteins are most often heterogeneous mixtures

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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

of related macromolecules that differ in primary sequence and posttranslational modifications, which can affect digestion efficiency; (*d*) proteins in macromolecular complexes that are not completely denatured will digest less efficiently; and (*e*) there is substantial interday variability in digestion. Even if inaccurate, the interday quantification of peptides in LC-MS/MS experiments can be relatively precise when the peak area ratio is calibrated with 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, 70)*, 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 report has focused mainly on the approaches and techniques needed to effectively use 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 because of the heterogeneity of protein isoforms in biology. However, if the measurand is defined as the concentration of protein isoforms that contain the peptides analyzed in the experiment, then with proper calibration and QC, 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, and prognosis are based on the numeric results of laboratory tests. Relative accuracy in these assays is established with reference materials whenever possible *(71, 72)*. 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 MS 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 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 MS assays *(73)*.

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 AAA. For proteins in a relevant matrix, there are no reference measurement procedures that use MS/MS to assign protein concentration. The most commonly used methodology is immunoassays with polyclonal antibodies, which have well-known issues in analysis of human biological fluids *(74)*. In these cases, the measurand is difficult to define with any specificity.

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Guidelines have been developed by the International Organization for Standardization (ISO 15194) that ensure the quality of manufactured reference materials. WHO has developed complimentary guidelines for the development and value assignment of their international standards. Table 8 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 AAA will be used to certify the concentration of purified protein reference materials and that trypsin-digestion isotope-dilution MS with well-characterized isotope-labeled protein or peptide internal standards (as described in this document) will become the reference method used to establish the concentration of proteins in matrix-matched reference materials. Once the field establishes assays with enzymatic-digestion isotope-dilution MS 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.

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