CLINICAL AND LABORATORY STANDARDS INSTITUTE®

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C64

Quantitative Measurement of Proteins and Peptides by Mass Spectrometry

This guideline describes the design, development, and validation of quantitative assays for proteins and peptides by mass spectrometry.

A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.

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Quantitative Measurement of Proteins and Peptides by Mass Spectrometry

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Abstract

Clinical and Laboratory Standards Institute guideline C64—*Quantitative Measurement of Proteins and Peptides by Mass Spectrometry* provides a framework for developing clinical protein and peptide assays from conception to validation. This guideline is intended for those who have experience with traditional small-molecule liquid chromatography–mass spectrometry (LC-MS) but not with protein and peptide analysis. Although closely related to traditional small-molecule analysis by LC-MS, protein and peptide analysis involves unique challenges and necessitates complex workflows, which are covered in detail. To enhance translation of assays to clinical use, this guideline focuses on method development aligned with clinically appropriate analytical validation.

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Foreword

This guideline is intended to accompany CLSI documents C62[1] and EP19.[2] Many of the recommendations found in CLSI documents C62[1] and EP19[2] also apply to liquid chromatography–mass spectrometry (MS) protein measurements, and commonalities are highlighted. However, this guideline primarily concentrates on aspects that are unique to quantitative measurement of proteins and peptides by MS.

NOTE: The content of this guideline is supported by the CLSI consensus process and does not necessarily reflect the views of any single individual or organization.

KEY WORDS

Assay development
Bioanalysis
Liquid chromatography
Mass spectrometry
Peptide
Protein
Surrogate peptide
Validation

Chapter 1

Introduction

This chapter includes:

- Guideline's scope and applicable exclusions
- Standard precautions information
- Terminology information, including:
  - Terms and definitions used in the guideline
  - Abbreviations and acronyms used in the guideline

Quantitative Measurement of Proteins and Peptides by Mass Spectrometry

1 Introduction

1.1 Scope

This guideline provides broad recommendations for appropriately developing and validating quantitative protein and peptide assays for clinical applications using electrospray liquid chromatography–mass spectrometry (LC-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) techniques. C64 is practically focused and includes workflow overviews and experimental strategies for developing and validating quantitative assays for soluble proteins and peptides in biofluids (eg, serum, saliva, urine). It covers complex analyses, including measurement of proteins with post-translational modifications (PTMs). Although there are a diverse array of ionization modes and associated mass analyzers (eg, matrix-assisted laser desorption/ionization time-of-flight [MALDI-TOF] mass spectrometry [MS]), this guideline focuses on liquid chromatography (LC) and electrospray ionization (ESI) coupled with tandem mass spectrometry (MS/MS) because of the wide availability and proven utility of this method.

A protein or peptide associated with a medical diagnosis or clinical outcome may exist *in vivo* as a single specified molecular composition or as a complex collection of related proteoforms that differ in molecular composition. Given the heterogenous nature of proteins and the desirability of facilitating results standardization among different assays, the need to appropriately define the measurand is a key difference between small-molecule analysis and protein analysis. In order to design a suitable workflow for measurand assessment, the assay developer needs to consider analyte properties, enrichment and fractionation strategy, and instrument performance characteristics. Subsequently, calibrators and internal standards (IS) are selected based on the chosen workflow and a precisely defined measurand. At the beginning of method development, performance criteria guide the conception and refinement of the path of workflow. Following this iterative process, the developer eventually prepares a candidate method sufficiently robust to pass validation. Finally, rigorous validation studies are performed to demonstrate suitability for routine clinical use.

The intended users of this guideline are medical, research, and public health laboratories; *in vitro* diagnostic instrument manufacturers; and regulatory and accreditation organizations.

Tissues and other nonbiofluid specimens are not within this guideline's scope. Enzyme activity assays are also considered out of scope, as are detailed discussions of software tools and data processing algorithms used for *in silico* analysis of protein and peptide sequences.

1.2 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all known infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of bloodborne pathogens. Published guidelines are available that discuss the daily operations of diagnostic medicine in humans and animals while encouraging a culture of safety in the laboratory.[3] For specific precautions for preventing the laboratory transmission of all known infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all known infectious diseases, refer to CLSI document M29.[4]

Table 2. Exemplar Definitions of Measurands

Analyte	Matrix	Detected Form	Implied Workflow
Concentration of human apoA-I	Human serum	Peptide ATEHLSTLSEK	Single surrogate peptide through a proteolysis-aided workflow
Concentration of human apoA-I	Human plasma	Peptides ATEHLSTLSEK and VQPYLDDFQK	Multiple surrogate peptides through a proteolysis-aided workflow
Concentration of human apoA-I	Human serum	Single intact, processed proteoform (PRO_0000001939)	Direct analysis of 1 specific form of protein apoA-I through an intact workflow
Concentration of oxidized human apoA-I	Human CSF	Peptide WQEEM(ox)ELYR	Single surrogate peptide from modified protein through a proteolysis-aided workflow
Concentration of peptide DRVYIHPFHL (angiotensin I)	Human plasma	Peptide DRVYIHPFHL	*In vivo* peptide through an intact workflow
Ratiometric abundance of glycated-to-total hemoglobin	Human whole blood	V*HLTPE / VHLTPE + V*HLTPE, where * indicates glycation site	Related surrogate peptides from a modified protein through a proteolysis-aided workflow, reported as a ratio

Abbreviations: apoA-I, apolipoprotein A-I; CSF, cerebrospinal fluid.

3.1 Insulin-like Growth Factor 1

Measurand: Concentration of insulin-like growth factor 1 (IGF-1) in human serum as single intact proteoform PRO_0000015664.

IGF-1 has clinical utility in examining growth hormone deficiency or excess. Although immunoassays for IGF-1 are available, they lack standardization and can suffer from interferences due to endogenous binding proteins. IGF-1 is a small protein (7.5 kDa), making it amenable to both intact and proteolysis-aided workflows.[15] Various sample preparation techniques have been published, including one for quantitating IGF-1 and insulin-like growth factor 2 (IGF-2), as well as some of their binding proteins in plasma, by LC-MS or LC-MS/MS.[16] Use of high-resolution LC-MS for intact IGF-1 has enabled identification of an IGF-1 sequence variant when discordant results (compared with an immunoassay) were observed.[17]

3.2 Thyroglobulin

Measurand: Concentration of thyroglobulin in human serum as peptide FSPDDSAGASALLR.

Thyroglobulin is a 660-kDa dimeric protein produced by the thyroid gland, used to evaluate treatment for thyroid cancer and monitor recurrence. Immunoassays can suffer from interferences due to endogenous antithyroglobulin antibodies found in about 20% of patient specimens.[18] Proteolysis-aided LC-MS/MS workflows that degrade the interfering autoantibodies while producing surrogate peptides have been described. Various LC-MS/MS methods and enrichment strategies have been devised, but all include a peptide immunoenrichment step of one or more surrogate peptides: FSPDDSAGASALLR or VIFDANAPVAVR.[19]

5.2 Common Internal Standard Structural Motifs

IS range in complexity from peptides to full-length proteins, with multiple strategies available for incorporating the stable-isotope label (see Figure 6). Chemical and metabolic labeling procedures use readily available isotope-labeled amino acids. Incorporation of ^{13}C and ^{15}N into all atoms is common, although amino acids with positional labels are also available. Either general or selective labeling is possible, so an IS can be specifically designed for the needs of a particular assay.

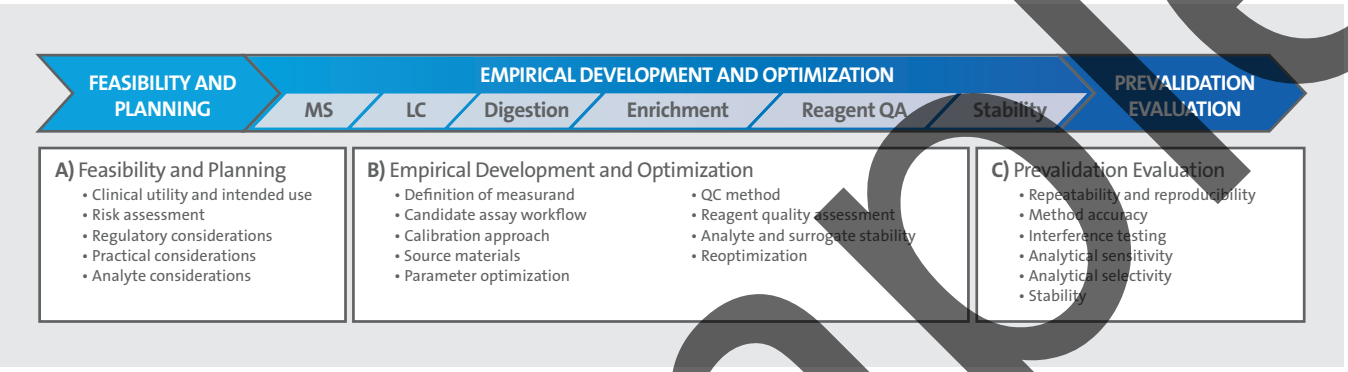
Although many variations of isotope-labeled amino acids are available, there are seven commonly used amino acids in which all carbon and nitrogen atoms have been replaced by ^{13}C and ^{15}N, respectively. They are relatively inexpensive and widely available, in forms ready for use in synthetic or metabolic labeling processes (see Table 4). Subchapter 5.5.2 covers technical specifications regarding optimal isotopic enrichment and required mass differences.

Table 4. Commonly Available Isotope-Labeled Amino Acids, Complete ^{13}C, ^{15}N Replacement

Amino Acid	Single Letter Code	Delta Mass
Alanine	A	+4
Arginine	R	+10
Leucine	L	+7
Lysine	K	+8
Phenylalanine	F	+10
Proline	P	+6
Valine	V	+6

7 Assay Development

This chapter provides guidance on the design, optimization, and preliminary evaluation of analytical methods for protein and peptide analysis in clinical diagnostic applications. It is important to plan properly in the initial stages of the work, so that opportunities and limitations are accounted for early in the project. The review process should cover clinical needs, as well as the feasibility and technical aspects of the method, and may include project economics. Figure 9 summarizes the steps involved in the method development process, from planning to provisional performance evaluation. Although Figure 9 depicts a linear development process, in practice the process is often iterative and interrelated, with significant overlap between phases. Based on observations made during performance evaluations, the assay developer may need to reoptimize method parameters, select new approaches, or even reconsider the assay's feasibility in meeting minimum performance targets.



Abbreviations: LC, liquid chromatography; MS, mass spectrometry; QA, quality assurance; QC, quality control.

Figure 9. Summary of the Method Development Process

7.1 Feasibility Determination and Planning

During the planning phase of the project, the assay developer considers the clinical utility and intended use of the assay, as well as indications for the test, to assess the potential effect of an inaccurate test result and determine whether an inaccurate test result poses a safety risk to patients. Technical aspects to consider during the planning stages are instrumentation requirements for development and routine use of the assay, expected timeline for method development and validation, assay throughput, required turnaround time, sample preparation time, instrumental analysis time, and appropriate use of automation. Additionally, it is important to assess the laboratory's ability to implement the assay and maintain its adequate performance in routine use. Often, assay design characteristics are pragmatically tailored to existing laboratory instrumentation and infrastructure, which supports assay development, validation, and implementation. During the planning stages, the developer should assess finances and expected return on investment for the assay and gather all information required for regulatory compliance.

7.2 Definition of the Measurand

The strategy used for analysis depends on the measurand. As such, it is critical to gather all available structural information for the analyte in the relevant specimen type, including the clinically relevant proteoforms and binding partners of the analyte, in order to create a provisional definition of the measurand(s) and identify the appropriate workflow for the measurement procedure. Subsequently, the assay developer should determine the target performance characteristics of the assay based on physiological concentrations of the measurand, within- and between-individual variation of the concentrations, proteoforms, and clinical need(s) (see Subchapter 8.1).

Related CLSI Reference Materials[a]

C37 **Preparation and Validation of Commutable Frozen Human Serum Pools as Secondary Reference Materials for Cholesterol Measurement Procedures. 1st ed., 1999.** This guideline details procedures for the manufacture and evaluation of human serum pools for cholesterol measurement.

C50 **Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance. 1st ed., 2007.** This guideline provides a general understanding of mass spectrometry and the principles that dictate its application in the clinical laboratory. It includes guidance, references, and quality assurance markers that will assist with the implementation and correct operation of a mass spectrometry (MS) system for its many applications. Information on maintaining optimum performance, approaches to ensuring accurate and precise mass measurement, verification of methods, quality control of assays within and between instruments, instrument troubleshooting, sample preparation, interpretation of results, and limitations of the technology is included.

C52 **Toxicology and Drug Testing in the Medical Laboratory. 3rd ed., 2017.** This guideline provides an overview of drug testing by medical laboratories, including testing for drugs of abuse. It discusses the preexamination, examination, and postexamination considerations for specimen collection, methods of analysis, and the reporting and interpretation of results.

C62 **Liquid Chromatography-Mass Spectrometry Methods. 1st ed., 2014.** This document provides guidance to the clinical laboratorian for the reduction of interlaboratory variance and the evaluation of interferences, assay performance, and other pertinent characteristics of clinical assays. This guideline emphasizes particular areas related to assay development and presents a standardized approach for method verification that is specific to mass spectrometry technology.

EP05 **Evaluation of Precision of Quantitative Measurement Procedures. 3rd ed., 2014.** This document provides guidance for evaluating the precision performance of quantitative measurement procedures. It is intended for manufacturers of quantitative measurement procedures and for laboratories that develop or modify such procedures.

EP06 **Evaluation of Linearity of Quantitative Measurement Procedures. 2nd ed., 2020.** This guideline provides information for characterizing the linearity interval of a measurement procedure, validating a linearity interval claim (to be performed by the manufacturer), and verifying an established linearity interval claim (to be performed by the end user).

EP07 **Interference Testing in Clinical Chemistry. 3rd ed., 2018.** This guideline provides background information, guidance, and experimental procedures for investigating, identifying, and characterizing the effects of interferents on clinical chemistry test results.

[a] CLSI documents are continually reviewed and revised through the CLSI consensus process; therefore, readers should refer to the most current editions.

Related CLSI Reference Materials (Continued)

EP09 **Measurement Procedure Comparison and Bias Estimation Using Patient Samples. 3rd ed., 2018.** This guideline covers the design of measurement procedure comparison experiments using patient samples and subsequent data analysis techniques used to determine the bias between two *in vitro* diagnostic measurement procedures.

EP10 **Preliminary Evaluation of Quantitative Clinical Laboratory Measurement Procedures. 3rd ed., 2014.** This guideline provides experimental design and data analysis for preliminary evaluation of the performance of a measurement procedure or device.

EP14 **Evaluation of Commutability of Processed Samples. 3rd ed., 2014.** This document provides guidance for evaluating the commutability of processed samples by determining if they behave differently than unprocessed patient samples when two quantitative measurement procedures are compared.

EP17 **Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures. 2nd ed., 2012.** This document provides guidance for evaluation and documentation of the detection capability of clinical laboratory measurement procedures (ie, limits of blank, detection, and quantitation), for verification of manufacturers' detection capability claims, and for the proper use and interpretation of different detection capability estimates.

EP19 **A Framework for Using CLSI Documents to Evaluate Clinical Laboratory Measurement Procedures. 2nd ed., 2015.** This report uses the "measurement procedure lifecycle" framework to aid users of CLSI evaluation protocols documents during establishment and implementation of measurement procedures developed by both commercial manufacturers and clinical laboratories, ie, for laboratory-developed tests.

EP21 **Evaluation of Total Analytical Error for Quantitative Medical Laboratory Measurement Procedures. 2nd ed., 2016.** This guideline provides manufacturers and end users with an understanding of concepts related to total analytical error (TAE) for quantitative measurement procedures. An experimental protocol and data analysis method are provided to estimate TAE based upon a comparison of methods experiment with patient specimens, and to assess it relative to a pre-established goal for clinical acceptability.

EP24 **Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves. 2nd ed., 2011.** This document provides a protocol for evaluating the accuracy of a test to discriminate between two subclasses of subjects when there is some clinically relevant reason to separate them. In addition to the use of receiver operating characteristic curves and the comparison of two curves, the document emphasizes the importance of defining the question, selecting the sample group, and determining the "true" clinical state.

EP25 **Evaluation of Stability of *In Vitro* Diagnostic Reagents. 1st ed., 2009.** This document provides guidance for establishing shelf-life and in-use stability claims for *in vitro* diagnostic reagents such as reagent kits, calibrators, and control products.

Related CLSI Reference Materials (Continued)

EP26 **User Evaluation of Between-Reagent Lot Variation. 1st ed., 2013.** This document provides guidance for laboratories on the evaluation of a new reagent lot, including a protocol using patient samples to detect significant changes from the current lot.

EP28 **Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory. 3rd ed., 2010.** This document contains guidelines for determining reference values and reference intervals for quantitative clinical laboratory tests.

EP29 **Expression of Measurement Uncertainty in Laboratory Medicine. 1st ed., 2012.** This guideline describes a practical approach to assist clinical laboratories in developing and calculating useful estimates of measurement uncertainty, and illustrates their application in maintaining and improving the quality of measured values used in patient care.

EP32 **Metrological Traceability and Its Implementation. 1st ed., 2006.** This document provides guidance to manufacturers for establishing and reporting metrological traceability.

EP34 **Establishing and Verifying an Extended Measuring Interval Through Specimen Dilution and Spiking. 1st ed., 2018.** It is often medically necessary to provide results for specimens with concentrations above the analytical measuring interval of an *in vitro* diagnostic measurement procedure. This guideline helps manufacturers and laboratory scientists with establishing, validating, or verifying a dilution scheme that will provide an extended measuring interval for such specimens.

EP35 **Assessment of Equivalence or Suitability of Specimen Types for Medical Laboratory Measurement Procedures. 1st ed., 2019.** This guideline provides recommendations for assessing clinically equivalent performance for additional similar-matrix specimen types and suitable performance for dissimilar-matrix specimen types, such that the laboratory does not necessarily need to repeat the full measurement procedure validation for each specimen type. The recommendations in this guideline apply to both quantitative measurement procedures and qualitative examinations.

EP37 **Supplemental Tables for Interference Testing in Clinical Chemistry. 1st ed., 2018.** This document includes recommended testing concentrations for analytes and endogenous substances that may interfere in clinical chemistry measurement procedures and is intended for use with the evaluation procedures in the Clinical and Laboratory Standards Institute guideline EP07.

M29 **Protection of Laboratory Workers From Occupationally Acquired Infections. 4th ed., 2014.** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

QMS24 **Using Proficiency Testing and Alternative Assessment to Improve Medical Laboratory Quality. 3rd ed., 2016.** This guideline describes an approach for a complete proficiency testing (PT) process and provides assistance to laboratories in using PT as a quality improvement tool.

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