available to serve as a calibrant as well. However, this approach assumes that surrogate peptide liberation and recovery are unaffected by the given modification, which may not be true.

6.9.2 Fractional Quantitation of Post-Translational Modifications

When the fraction of a target protein with a specific PTM is the desired measurand (eg, HbA1c or glycated albumin), two options for calibration are possible. The assay developer may independently calibrate and quantitate each proteoform (modified and unmodified), in which case there are truly two measurands subsequently used in a mathematical calculation. Obtaining calibrators with defined quantities for each proteoform may be challenging. Thus, it may be simpler to calibrate the fractional quantity rather than two absolute quantities. In the latter approach, there is only a single measurand. Calibrators with defined fractions of modified and unmodified proteoforms are measured. Then the response factor is obtained by plotting the measured relative ratio of the two proteoforms (modified-to-unmodified) on the y-axis vs the known fractional quantity on the x-axis.[21] Optimally, calibrators should be value assigned by an appropriate RMP (see Subchapter 6.8.1).

6.10 Practical Use for Routine Production

CLSI document C62[1] thoroughly describes practical implementation of calibrators, including their number and placement in the AMI, calibration frequency, and methods for generating and fitting calibration curves. The principles described in CLSI document C62[1] for MS measurement procedures apply to calibration of protein and peptide MS measurement procedures.

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Chapter 7

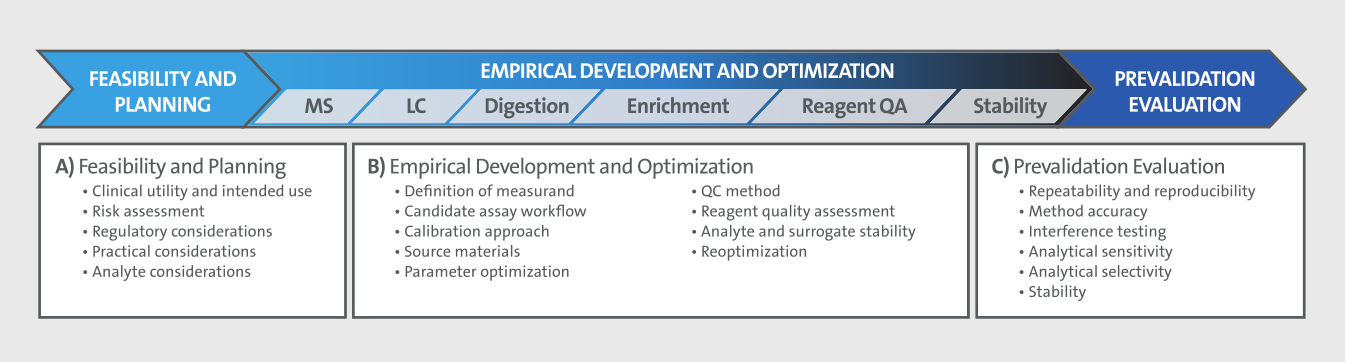
Assay Development

This chapter includes:

- Iterative method development strategies, from planning to empirical optimization
- Guidance on experimental designs (with recommended outcomes) used in assay development
- Recommended prevalidation experiments for tracking progress and reducing risk associated with formal assay validation

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).

7.2.1 Structural Characterization

Defining the measurand generally begins with describing the primary, secondary, tertiary, and quaternary structure of the clinically relevant and irrelevant proteoforms in relation to the measurand (see Chapter 3). This description typically includes compilation of amino acid sequences, sites of PTMs, sequence variants (benign or pathogenic), and known interaction partners. These data can be obtained from both *de novo* experimental characterization and theoretical investigations (eg, sequon identification) or gleaned from the literature. Many databases and bioinformatic resources are available to assist in characterization.[100-103] Although these resources are valuable, users should fully understand their limitations regarding source, quality, completeness, and level of curation used in their assembly.

The literature may thoroughly describe the structure of extensively studied analytes. For less well-studied analytes, the description may rely on inferential data (eg, homology with another protein). Experimental characterization is often preferred but not always possible. The structural information gathered helps the assay developer determine a measurand's suitability for the desired workflow and select surrogate peptides (for digestion workflows) and sample enrichment strategies.

Previously published protein or peptide MS methods, for which the measurand and/or the surrogate peptide have been characterized, can facilitate or eliminate the need for detailed predevelopment investigation. However, some degree of *de novo* analyte characterization is recommended to find any new information and avoid the consequences of incorrect assumptions. For example, a method designed to detect a single proteoform common in one ethnic group may be insensitive to a sequence variant that is common in another.[104]

7.2.2 *In Silico* Digestion

For new methods that use a proteolysis-aided workflow, it is necessary to identify the ideal surrogate peptide(s) to best define the measurand. The first step in this process occurs during the planning stage, using *in silico* digestion of the analyte. The protein or peptide is first represented in text format by the single-letter amino acid code, ordered from N- to C-terminus.[105] Next, a software tool is used to transform the sequence into peptides, using the expected cleavage specificity of the proteolytic enzyme(s) or chemical(s) intended to be used. *In silico* digestion can be performed manually or by one of many freely available software programs,[106,107] which typically use known rules for cleavage specificity or may apply empirical rules based on large-scale analysis of many datasets.[108] Table 5 lists the cleavage specificity of several common proteases.

Table 5. Commonly Used Proteases and Their Cleavage Specificities

Protease	Preferential Cleavage Specificity[a]
Asp-N endopeptidase	N-terminal to Asp
Chymotrypsin	(High specificity) C-terminal to Trp, Tyr, and Phe (Low specificity) C-terminal to Leu, Met, and His
Lys-C lysyl endopeptidase	C-terminal to Lys
Proteinase K	C-terminal to hydrophobic amino acids
Trypsin	C-terminal to Lys or Arg

Abbreviations: Arg, arginine; Asp, aspartic acid; His, histamine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine.
[a] There are exceptions to the stated preferential cleavage specificity, such as certain amino acids (and modifications), as well as certain digestion conditions.[108]

Experimentally, cleavage patterns may vary significantly from predicted digestion products based on several factors, including but not limited to *in vivo* or *in vitro* modifications (eg, extensive phosphorylation blocking cleavage sites, carbamylation by urea) and ragged N- and C-termini generated by endogenous or exogenous proteases. Moreover, the specific digestion conditions (eg, pH, temperature) can affect the rate and specificity of cleavage.[108,109] Therefore, all predicted or previously reported digestion products must be experimentally verified.

7.2.3 Sequence Alignment

For both intact and digestion methods, sequence alignment tools are needed to compare a queried amino acid sequence (eg, intact protein or proteolytic surrogate peptide) against a proteome database.[110] Results are used to evaluate the sequence's specificity for the desired analyte within the relevant proteome(s). Ideally, the sequence should be unique to the analyte in the sample being analyzed, ie, within the human proteome as well as any other proteome introduced in the method (eg, calibrator surrogate matrix).

No proteome database is expected to be comprehensive. They often lack annotation of polymorphic variants and alternative splice variants, as well as proteolytic processing that may occur *in vivo*. Thus, sequence alignments cannot provide definitive evidence for the specificity of a protein or surrogate peptide sequence. However, they can indicate a potential lack of specificity. Even when database searches indicate that a sequence is specific for the desired measurand, detection specificity by MS or MS/MS must be confirmed experimentally. Owing to discrimination by known abundance and/or localization differences (eg, abundant circulating serum protein vs obligate intracellular nuclear transcription factor), it may be justifiable to use nonunique sequences for assay development.

7.2.4 Candidate Surrogate Peptide Selection

For digestion methods, Table 6 defines ideal criteria for selecting surrogate peptides for MS analysis. Compromises may be needed to fulfill the intended purpose of the assay. For example, a PTM may lie in a sequence longer than is typically desirable for a surrogate peptide, or a small protein may yield only two hydrophilic peptides. Selection guidelines can help the assay developer focus empirical development efforts on peptides that meet the greatest number of criteria. Likewise, they can help identify potential analytical challenges when selection of nonideal peptides is necessary.

Table 6. Guidelines for Selecting Proteolytic Surrogate Peptides for MS Analysis[67] (Adapted and reprinted with permission from Oxford University Press on behalf of the American Association of Clinical Chemistry, from Hoofnagle AN, Whiteaker JR, Carr SA, et al. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry-based assays. *Clin Chem*. 2016;62(1):48-69. doi:10.1373/clinchem.2015.250563)

Selection Criterion	Desired Characteristic
Uniqueness (analyte specificity)	Peptides should be unique in sequence to the gene product or proteoform of interest so that the assay is specific for the intended measurand.
Peptide length	Typically, 7–20 amino acids are favorable for analysis by LC-MS/MS and are more likely to be uniquely associated with a single protein.
Observability by MS	Peptides empirically identified in previous MS experiments may be favored, using the instrument on which the method is expected to be developed and validated.
Hydropathy	Extremely hydrophobic or hydrophilic peptides may be problematic, owing to solubility issues and retention time instability by conventional RPLC, respectively.
Labile residues	If possible, the following residues should be avoided, because they may be susceptible to *ex vivo* modifications. Listed in decreasing priority: • Cys (alkylation required, possible oxidation, cyclization if N-terminal) • Met (possible oxidation) • N-terminal Gln (pyroglutamic acid formation) • Asn or Gln when followed by Gly (deamidation) • Asp followed by Gly (dehydration) or Pro (peptide chain cleavage) • Trp (oxidation) • His (additional charge states)
Digestion parameters	Peptides with missed cleavages should be avoided, as should those susceptible to missed cleavage based on inhibitory motifs, such as ragged ends (eg, KK and RR for trypsin), which may display low and variable digestion yields.
Modification sites	Unless the goal is to quantitate a specific isoform, peptides near or containing known sites of polymorphism or PTM, including sites near known PTM motifs (eg, phosphorylation, N-glycosylation [NXS/T[a]]), should be avoided if possible, because they may affect assay results by altering the recoverability or detection of the surrogate peptide.

Abbreviations: Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Gly, glycine; His, histidine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; Met, methionine; MS, mass spectrometry; Pro, proline; PTM, post-translational modification; RPLC, reversed-phase liquid chromatography; Trp, tryptophan.

[a] N = asparagine; X = any amino acid; S = serine; T = threonine.

7.3 Empirical Optimization

The definition of the measurand established during project planning largely dictates the provisional assay workflow, providing direction for method development. For analyses of intact proteins, the measurand and basic assay workflow are often well defined and can be used to optimize method parameters to achieve the predefined analytical requirements for the assay. For digestion methods, the optimal surrogate peptide for the measurand may also be well defined by a predicate method procedure or by a specific modification site intended to be measured. When the assay developer designs a novel method that uses digestion or that has a measurand that is less well defined by the predicate method (eg, ligand-binding assays or immunoassays), the focus of assay development is different. In these cases, development efforts necessarily focus on identifying, based on experimental evidence, surrogate peptide(s) that adequately define the measurand from among the list of *in silico* candidates (see Subchapter 7.2). Figure 10 depicts the most expedient refinement process, wherein easily identifiable analytical characteristics are used to empirically eliminate from consideration and/or rank large numbers of *in silico* candidates early in development.

As development progresses, the developer should perform cursory tests of the method's selectivity, accuracy, stability, reproducibility, and practicality (from sample preparation to chromatography and MS analysis) to evaluate the measurand and/or refine the possible surrogate peptides that may be used in the assay. Table 7 lists recommended experimental considerations for refining the measurand definition.

Table 7. Summary of Empirical Refinement Opportunities

Refinement Opportunity	Considerations
Specimen type	• Suitability of desired specimen collection method and tube type (eg, serum vs EDTA plasma) • Uniqueness and stability of measurand in relevant specimen and calibrator matrix
Enrichment (when applicable)	Ability to reproducibly enrich for target analyte or surrogate peptide with necessary selectivity, using available reagents (eg, over entire physiological concentration range)
Digestion (when applicable)	• Digestion effectiveness, speed, and reproducibility used to differentiate between surrogate peptides • Method reproducibility possibly affected by speed and stability (eg, kinetics) of peptide formation, making some surrogate peptides preferable
Stability	• Stability throughout assay protocol (eg, during all stages of sample preparation) • Surrogate peptides differentiated based on stability during postextraction procedures and/or digestion (eg, in autosampler)
Chromatography	• Ability to retain on chromatographic media and sufficiently separate from interfering substances in sample • Surrogate peptides differentiated by chromatographic performance (eg, peak shape) and selectivity, in combination with MS detection
MS	• (Method-dependent) ability to ionize and effectively fragment • Ability to selectively detect target free of interferences • Ability to detect target in necessary physiological range • Surrogate peptides differentiated based on sensitivity and selectivity of MS detection
Selectivity	• Molecular specificity of clinically relevant measurand, compared with a predicate assay • A surrogate peptide's specificity, inferred from *in silico* analyses, corroborated by correlated measurements with multiple other surrogate peptides or with a predicate method[111]

Abbreviations: EDTA, ethylenediaminetetraacetic acid; MS, mass spectrometry.

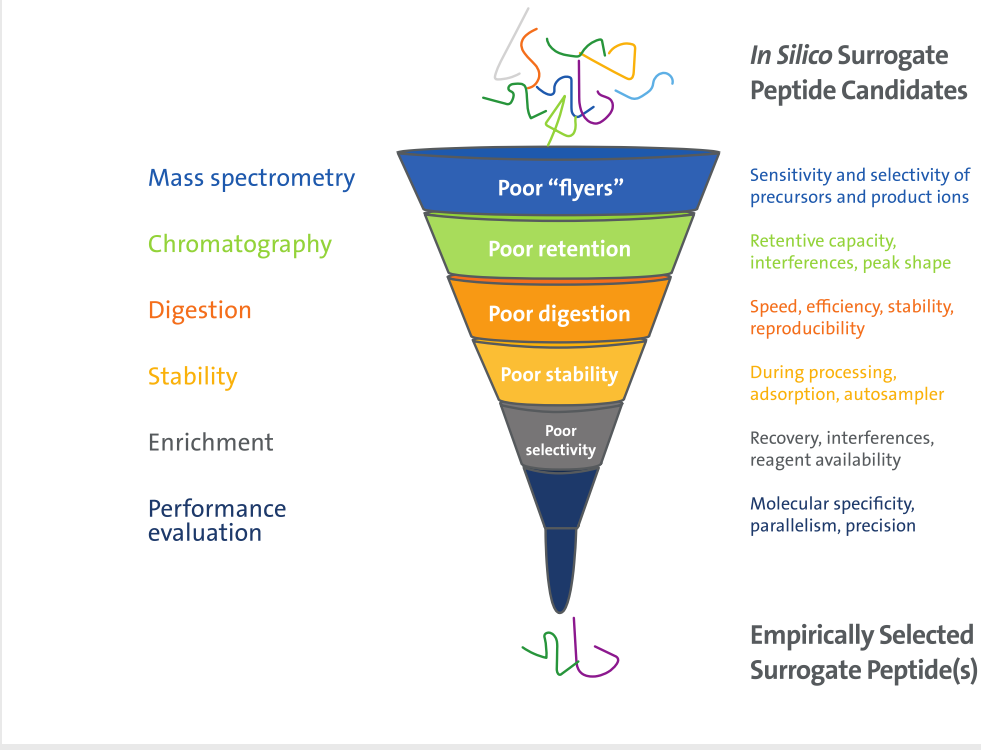


Figure 10. Summary of Empirical Refinement Opportunities (Printed with permission of Hendrik Neubert.)

7.3.1 Standardization

Early in the project lifecycle, it is important to determine whether the assay will fall under a standardization or harmonization program. The assay developer should identify approaches to calibration and QC and determine the availability of calibrants, RMs, and commercial QC materials, which are required for assay development, validation, and subsequent routine use. Enrollment in or development of a suitable proficiency testing program can be useful from development onward.

During analytical method validation for proteins and peptides, it is important to compare the method with other available measurement procedures for the same measurand to assess accuracy and trueness. Thus, early in development, it is often beneficial to determine the availability of a comparator method (ie, reference procedure or other validated method) for the same measurand. For novel methods with no comparative measurement procedures, the developer needs to evaluate the clinical performance of the new assay with clinically characterized patient samples and establish a mechanism to provide traceability over the assay's lifetime (see Chapter 6).

7.3.2 Liquid Chromatography–Tandem Mass Spectrometry

The principles described in CLSI document C62[1] also apply to development of LC-MS/MS detection methods for proteins and peptides. A purified, authenticated standard of the analyte is prepared in neat form and analyzed by MS to characterize the precursor ion mass and charge state, as well as characteristic product ions for MS/MS. The assay developer first optimizes various ionization, fragmentation, and ion optic parameters without

online chromatographic separation, then reoptimizes or verifies them after determining the chromatographic conditions. For assays targeting surrogate peptides following digestion, it is often convenient to use crude synthetic peptides or the digested protein standard for this purpose in early development. However, these materials are inherently impure with respect to the detected molecule (ie, surrogate peptide) and may be confounded by unexpected isobaric interferences. For example, digestion of a larger protein standard may produce hundreds of unique surrogate peptides that could interfere with one another, even if they are separated upfront. As such, the characteristic MS and MS/MS properties used to define the detection of the analyte (ie, intact protein or surrogate peptide) should ultimately be established with a purified, authenticated standard of the analyte (ie, purified intact protein or purified synthetic peptide). The developer may use purified, SIL peptides in place of an unlabeled synthetic peptide, given that they are expected to ionize and fragment in the same manner. For analyses of intact proteins, the native protein analyte should be used for MS method development.

7.3.2.1 Ionization Effects

In addition to LC mobile-phase composition, several substances (eg, salts, lipids, other peptides) affect the analyte's ionization efficiency.[112] Although use of SIL IS corrects for the effects of ionization suppression (and enhancement) in the linear dynamic range of the mass analyzer, it is ideal to avoid these sources of interferences because they may affect the assay's sensitivity.

The assay developer can assess ionization effects for LC-MS by postcolumn T-infusion of the detected analyte (or IS) during injection of extracted patient samples, extracted calibrator matrix, and matrix-free control (eg, mobile phase). The infusion profile observed when the matrix-free control is injected indicates the optimal ionization in the absence of a matrix effect. Comparing the matrix-free control infusion profile with the infusion profile observed when prepared samples are injected can help identify regions of ionization effects.[113,114]

Alternatively, as the final sample preparation step before MS or LC-MS analysis, the developer may evaluate ionization effects by spiking IS into extracted samples and extracted calibrators, along with a matrix-free control (eg, solvent) spiked with the same amount of IS. The IS response in the matrix-free control indicates the optimal ionization in the absence of a matrix effect. Comparing the IS response in the matrix-free control with the IS response in the spiked extracted samples can help identify matrix effects. For exogenous analytes, the developer may use the unlabeled analyte for spiking[115] or spike at a concentration at least 20-fold greater than the endogenous analyte concentration (ie, such that the endogenous analyte signal is negligible). Notably, this approach is amenable to matrix-assisted laser desorption/ionization assays or LC-MS assays, for which postcolumn infusion is not feasible (because the ESI emitter is integrated with the LC column). However, this experiment may be confounded by adsorptive loss of the spiked IS in the matrix-free control.

Altering the separation or sample preparation conditions may reduce ionization effects. However, the chemical extraction techniques that are generally successful with small molecules often do not improve results with digested peptides, because the interferences are usually caused by high-abundance peptides with similar chemical properties. If the workflow allows, the best way to resolve a matrix effect is to first separate the protein analyte from the more abundant protein that is causing the ionization effect or that is the substrate for the interfering peptide (derived from the protein by digestion). Subchapter 7.3.5 discusses techniques for separating interfering proteins. In some instances, adjusting the chromatographic separation to shift the interference away from the analyte may resolve the problem.

7.3.2.2 Mass Spectrometry Detection Selectivity

The assay developer can evaluate the selectivity of MS analysis through blank matrix control studies and through ion ratio monitoring, wherein multiple ions for the detected molecule are monitored and their intensities compared (see Subchapter 8.9).[116] In MS/MS, the relative intensity of product ions under fixed experimental conditions (ie, the instrument parameters) is a fundamental property of the molecular ion. It is also the basis of

the spectral fingerprinting used in traditional toxicology screening.[117] During MS/MS, most proteins and peptides produce a series of characteristic product ions that may be useful for quantitative analysis as well as ion ratio monitoring, as long as the fragmentation mode is appropriately matched to the analyte's size (see Subchapter 4.1). In the absence of unique secondary product ions generated by MS/MS, isotope ratios[53] may be monitored by comparing the relative abundance of isotopic peaks. Their relative abundance depends predominantly on the number of carbon atoms within the molecule or the number of other atoms with abundant natural isotopes (eg, sulfur, chlorine).

Multiple product ions or SRM transitions are monitored during LC-MS/MS analysis for neat standards and extracted and/or digested patient samples. The observed peak areas associated with each product ion or SRM transition in a given analysis are used to calculate a transition ratio, which is conventionally calculated as the ratio of the qualifying ion peak area divided by the quantifying ion peak areas.[116] Optimally, the transition ratio observed in a matrix-free analysis can be used to define the expected transition ratio in the absence of interference. Thus, if the transition ratio(s) measured in extracted samples differs significantly from that measured with standards, an isobaric interference is possible.

Matrix effects can alter the fragmentation pathway for precursors of the same molecular ion by altering the placement of charge.[118] As such, transition ratios measured in extracted samples may not be identical to those observed in the absence of matrix and associated isobaric interferences. In these cases, the transition ratios measured in calibrators may be used to establish the expected transition ratio. Additionally, transition ratios of the unlabeled surrogate peptide can be compared against the labeled IS peptide using matched SRM transitions.[119] Expected transition ratios may shift over time as instrument performance drifts, a possibility that should be considered when target criteria are established.[120]

During early assay development, it is beneficial to monitor several SRM transitions for each analyte and examine transition ratio stability to identify the most selective transitions. Subsequently, the developer should test ratios during validation to confirm the selectivity of the transition in patient samples. This step should be performed for each analyte, as well as each IS.

In the absence of viable qualifying transitions, the quantifying transition may be used, with an alternate collision energy, as the qualifying transition.[116] Alternatively, the precursor ion *m/z* that is monitored for the quantifying transition may be increased by one *m/z,* such that different isotopes of the same precursor are monitored as the qualifying transition.[28] However, using the same pairs of precursor and product ions, with a different collision energy or isotope, as both quantifying and qualifying transitions makes ratio monitoring less sensitive to detection of isobaric interferences.

7.3.2.3 Liquid Chromatography Development

The principles for chromatographic separation of small molecules outlined in CLSI document C62[1] apply to proteins and peptides as well. Most high-fidelity separations of proteins and peptides are accomplished by reverse-phase separation using ion-pairing agents. However, this guideline neither makes assumptions nor imposes requirements regarding the chromatographic mode used in a clinical assay targeting proteins or peptides, as long as the separation is robust and capable of routinely providing the chromatographic selectivity necessary to meet the intended clinical need. The performance of the LC method depends on the proper optimization of both the LC separation and the upstream sample preparation steps, as well as the selectivity afforded by the downstream MS detection. Therefore, the LC parameters are often optimized multiple times throughout assay development, along with other method parameters. Often, the final step of assay development is reduction of the LC run time in order to enable higher-throughput analyses, without compromising selectivity, sensitivity, reproducibility, or ruggedness.