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Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology

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410 North 21st Street
Colorado Springs, CO 80904

This document may be downloaded from: www.aafs.org/academy-standards-board

Foreword

This standard provides the minimum requirements for selecting, developing, validating, and verifying test methods in forensic toxicology laboratories.

Different options are often considered when a forensic toxicology laboratory needs a new test method to enhance or broaden its testing capabilities. It can use a standard test method as published or with modification, or it can use a non-standard test method, including one developed in-house. These options enable laboratories to maintain flexibility and adaptability in their testing approaches, allowing them to meet their diverse analytical needs.

Method development is the process of designing and optimizing procedures for conducting qualitative or quantitative analyses in forensic toxicology. It involves identifying the most effective technique, instrument, parameters, and conditions to achieve the needed sensitivity, bias, precision, or efficiency of the method.

Method validation is the process of performing experiments to obtain objective evidence establishing that the developed method is fit for purpose and to identify the method's limitations under normal operating conditions.

Method verification is a type of assessment limited to a laboratory's use of an unmodified standard test method. Method verification experiments enable a laboratory to demonstrate its ability to use the standard test method and ensure it performs as intended by meeting or exceeding the published parameters.

Revalidation is necessary when modifications are made to a previously validated method. Possible modifications include adding compounds to a method's scope, adjusting the calibration range or model, or upgrading instrumentation. Full revalidation is necessary unless an abbreviated revalidation is justified.

This 2nd Edition includes substantive changes from the 1st Edition. A section was added to address method selection. The method development section was enhanced to provide detailed recommendations. The method validation section added a requirement for determining rates of false positives and negatives for qualitative methods. A section was added to address the verification of standard test methods. Detailed development, validation, and verification instructions were moved into separate annexes. The examples contained within the annexes of the 1st Edition are now included in a separate guidance document, ASB Guideline 236, *Guideline for Conducting Test Method Development, Validation, and Verification in Forensic Toxicology*.

The American Academy of Forensic Sciences established the Academy Standards Board (ASB) in 2015 with a vision of safeguarding Justice, Integrity and Fairness through Consensus Based American National Standards. To that end, the ASB develops consensus based forensic standards within a framework accredited by the American National Standards Institute (ANSI), and provides training to support those standards. ASB values integrity, scientific rigor, openness, due process, collaboration, excellence, diversity and inclusion. ASB is dedicated to developing and making freely accessible the highest quality documentary forensic science consensus Standards, Guidelines, Best Practices, and Technical Reports in various forensic science disciplines as a service to forensic practitioners and the legal system.

The Toxicology Consensus Body of the AAFS Standards Board revised, prepared, and finalized this document as a standard.

Questions, comments, and suggestions for improving this document can be sent to the AAFS-ASB Secretariat at asb@aafs.org or 401 N 21st Street, Colorado Springs, CO 80904.

All hyperlinks and web addresses shown in this document are current as of the publication date of this standard.

ASB procedures are publicly available, free of cost, at www.aafs.org/academy-standards-board.

Keywords: *method selection, method development, method validation, method verification, forensic toxicology*

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Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology

1 Scope

This document delineates minimum requirements for selecting, developing, validating, and verifying test methods used in forensic toxicology that target specific analytes or analyte classes. It is specifically intended for the subdisciplines of postmortem forensic toxicology, human performance toxicology, non-regulated workplace drug testing, and court-ordered toxicology. This document does not address calibration or testing in breath alcohol programs.

2 Normative References

The following references are indispensable for applying this standard. For dated references, only the edition cited applies. For undated references, the document's latest edition (including any amendments) applies.

ANSI/ASB Standard 017, *Standard for Metrological Traceability in Forensic Toxicology* [a]

ANSI/ASB Standard 098, *Standard for Mass Spectral Analysis in Forensic Toxicology* [a]

ANSI/ASB Standard 113, *Standard for Identification Criteria in Forensic Toxicology* [a]

ANSI/ASB Standard 119, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Medicolegal Death Investigations* [a]

ANSI/ASB Standard 120, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Impaired Driving Investigations* [a]

ANSI/ASB Standard 121, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Urine in Drug-Facilitated Crime Investigations* [a]

ASB Technical Report 208, *Forensic Toxicology: Terms and Definitions* [a]

3 Terms and Definitions

For purposes of this document, the following terms and definitions apply. Additional applicable terms are defined in ASB Technical Report 208*, Forensic Toxicology: Terms and Definitions*.

3.1
calibration range
The range of concentrations between a quantitative method's lowest and highest calibrators.

3.2
fortified matrix sample
A blank matrix sample spiked with target analyte and/or internal standard using reference materials.

[a] Available from https://www.aafs.org/academy-standards-board.

3.3
ionization suppression/enhancement
The direct or indirect alteration or interference in the instrument response due to the presence of co-eluting compounds and background components.

3.4
upper and lower limits of reliability
The limits within which the rate of false negatives and false positives are such that the obtained results are deemed to be reliable.

4 Requirements for Method Selection

4.1 Forensic toxicology laboratories often need to establish test methods to improve, expand, or adjust their scope of testing. When a new test method is required, different options may be considered:

— standard test methods that are used without modification;

— standard test methods used outside the intended scope or otherwise modified; or

— non-standard test methods, to include laboratory-developed test methods.

4.1.1 Test methods specified in law or regulations shall be followed per those requirements.

4.1.2 Standard test methods should be used, if available.

4.2 The type of test method selected directly impacts how a laboratory demonstrates its ability to use the test method and achieve the defined performance specifications.

4.2.1 At a minimum, method verification experiments shall be performed when unmodified standard test methods are selected (see Section 7).

NOTE Method verification allows the laboratory to demonstrate its ability to use the standard test method within its intended scope and achieve the method's defined performance specifications.

4.2.2 When modified standard, non-standard, or laboratory-developed test methods are selected, method development (Section 5) and method validation (Section 6) experiments shall be performed.

5 Requirements and Recommendations for Method Development

5.1 General

Method development enables a laboratory to establish a development plan, define how metrological traceability will be established, optimize the steps for sample preparation and instrumental analysis parameters, and determine how observations, data, or calculations will be interpreted.

5.2 Method Development

Method development shall occur before method validation.

NOTE 1 It is recognized that the results of validation experiments may trigger further method development.

NOTE 2 Data acquired during method development may be used toward validation requirements, provided they are documented and the method has not changed after the data are generated.

5.3 Requirements for Establishing a Method Development Plan

5.3.1 A plan shall be established and approved before initiating method development.

5.3.2 The method development plan shall address the questions to be answered by the test method to include, as applicable:

a) analyte(s);

b) matrix or matrices;

c) concentration range(s);

d) decision point concentration(s);

e) internal standard(s);

f) sample preparation technique(s) ;

g) instrumentation;

h) automation;

i) customer needs for the method; and

j) individual(s) assigned to conduct the method development experiments.

5.3.3 The following documents shall be adhered to, as appropriate, for establishing the test method's analytical scope and sensitivity in the method development plan:

— ANSI/ASB Standard 119, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Medicolegal Death Investigations;*

— ANSI/ASB Standard 120, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Blood in Impaired Driving Investigations;*

— ANSI/ASB Standard 121, *Standard for the Analytical Scope and Sensitivity of Forensic Toxicological Testing of Urine in Drug-Facilitated Crime Investigations.*

5.3.4 Modifications to the original method development plan shall be approved and authorized.

5.4 Requirements for Conducting Method Development Experiments

5.4.1 General

For the purposes of this document, method development is considered in three phases:

a) development and optimization of instrumental parameters;

b) defining observations, data processing, and calculations; and

c) development and optimization of sample preparation steps.

NOTE Method development experiments depend on numerous variables, so a defined minimum number of analyses for each experiment is unnecessary. Ultimately, the laboratory will be required (in Section 6.4) to conduct validation experiments with a specified minimum number of samples. Failure to fully assess a phase listed above during method development may lead to failures during method validation.

5.4.2 Development and Optimization of Instrumental Parameters

5.4.2.1 Instrumental parameters shall be established through analysis of reference materials of the analyte(s) of interest to achieve the required instrumental performance.

5.4.2.1.1 For chromatography-based test methods, the following parameters shall be established during method development:

a) column (e.g., guard column, composition, dimensions, temperature program);

b) mobile phase (e.g., composition, flow rate, gradient) or carrier gas (e.g., type, flow rate);

c) injection type and volume.

NOTE Additional chromatography conditions may need to be optimized based on the type and manufacturer of the instrument.

5.4.2.1.2 For mass-spectral test methods, the following parameters shall be established during method development:

a) source type and conditions;

b) data collection mode [e.g., scan, selected ion monitoring (SIM), multiple reaction monitoring (MRM)];

c) mass range or diagnostic ions;

d) voltages;

e) temperatures;

f) gas flows;

g) cycle/dwell times.

NOTE Additional mass spectral conditions may need to be optimized depending on type and manufacturer of the instrument.

5.4.2.1.3 For all other instrument-based test methods, applicable parameters (e.g., wavelength range) shall be established.

5.4.3 Defining Observations, Data Processing, and Calculations

5.4.3.1 The acceptance criteria for observations, data processing, and calculations shall be defined.

5.4.3.2 The following shall be defined:

a) data processing parameters (e.g., integration parameters, chromatographic smoothing, preliminary calibration model);

b) requirements for identification and quantitation:

   1) chromatographic requirements (e.g., peak shape, retention time tolerance, signal-to-noise);

   2) mass spectral requirements (e.g., ion ratios, library match scores) according to ANSI/ASB Standard 098, *Standard for Mass Spectral Analysis in Forensic Toxicology;*

   3) visual observations (e.g., color change);

   4) bias and precision acceptance criteria;

   5) calculations to be performed;

c) identification points (e.g., the number of identification points the test method will contribute to the overall identification) according to ANSI/ASB Standard 113, *Standard for Identification Criteria in Forensic Toxicology*.

5.4.4 Development and Optimization of Sample Preparation Steps

5.4.4.1 The sample preparation technique shall be established using reference materials of the analyte(s) and internal standard(s) in matrices of interest.

NOTE This step demonstrates that the sample preparation steps allow for adequate clean-up and extraction of the analyte(s) from the matrices of interest.

5.4.4.2 The following shall be established for the sample preparation steps that will be used in the test method:

a) equipment;[b]

b) homogenization;

[b] ISO/IEC 17025, 6.4.1 "… equipment (including, but not limited to, measuring instruments, software, measurement standards, reference materials, reference data, reagents, consumables or auxiliary apparatus) …"

c) sample hydrolysis;

d) sample amount or volume and routine need for dilutions;

e) extraction technique (e.g., solvent extraction, solid-phase extraction) and related materials (e.g., solvents, volumes, buffers);

f) sample derivatization;

g) reconstitution solvent and volume;

h) ionization suppression/enhancement (see Annex F);

i) recovery (see Annex L);

j) processed sample stability (see Annex J);

k) upper and lower reliability limits for assays that rely on decision point concentrations to determine if the sample will be "positive" or "none detected" (Annex M);

l) calibration range; [c]

m) automation parameters (e.g., sample extraction robotics).

5.5 Requirements for Establishing a Statement of Metrological Traceability

5.5.1 The method development documentation shall include a statement identifying how metrological traceability will be established for the test method, when applicable.

5.5.2 Metrological traceability shall comply with the requirements stated in ANSI/ASB Standard 017, *Standard for Metrological Traceability in Forensic Toxicology.*

6 Requirements for Method Validation

6.1 General

Method validation experiments shall be performed when modified standard, non-standard, or laboratory-developed test methods are selected.

6.2 Historical Methods

6.2.1 For historical methods in use, laboratories shall meet all requirements for method validation in this document.

6.2.2 Historical calibration and control data, as well as previously analyzed casework sample results, may be used in method validation experiments.

[c] The calibration range typically includes those concentrations expected in daily casework.

6.2.3 Historical data shall have used the same instrumental parameters and sample preparation steps as the method being validated.

6.2.4 In the absence of sufficient historical data, appropriate validation experiments shall be conducted to ensure compliance with this document.

6.3 Requirements for Establishing a Method Validation Plan

6.3.1 A plan shall be established and approved before initiating validation experiments.

NOTE 1 The experiments included in method validation will depend upon the circumstances in which the method is to be used (i.e., the scope of the method).

NOTE 2 Data acquired during method development may be used toward method validation experiment requirements, provided they were documented and the method used to gather the data was the same as the method being validated.

6.3.2 The method validation plan shall include the sample preparation steps and instrumental parameters(s) to be used, the validation experiments to be conducted, and the acceptance criteria for each assessment that will allow the method to be considered fit-for-use.

6.3.3 *Required Validation Experiments Based on the Scope of the Method*—The scope of forensic toxicology methods is classified into two categories: qualitative and quantitative.

6.3.3.1 The following performance characteristics shall be assessed based on the method's scope.

NOTE The normative Annexes in this document provide detailed requirements for conducting method validation experiments.

a) Qualitative Methods:

   1) carryover (see Annex C);

   2) interference studies (see Annex E);

   3) ionization suppression/enhancement for applicable techniques, such as LC/MS (see Annex F);

   4) limit of detection (see Annex G);

   5) processed sample stability (see Annex J); and

   6) rates of false positives and false negatives (see Annex K).

b) Quantitative Methods:

   1) bias (see Annex A);

   2) calibration model (see Annex B);

   3) carryover (see Annex C);

4) dilution integrity (if applicable) (see Annex D);

5) interference studies (see Annex E);

6) ionization suppression/enhancement for applicable techniques, such as LC/MS (see Annex F);

7) limit of detection (see Annex G);

8) lower limit of quantitation (see Annex H);

9) precision (see Annex I); and

10) processed sample stability (see Annex J).

c) If a laboratory will use a quantitative method in a qualitative manner, false positive and negative rates shall be established.

6.4 Requirements for Conducting Method Validation Experiments

6.4.1 All method validation experiments shall utilize the same instrumental parameters and sample preparation steps as the final developed method.

NOTE Daily instrument performance requirements used for casework shall be met for conducting method validation experiments.

6.4.2 All method validation experiments shall be conducted using fortified samples for each matrix type for which the method is intended unless otherwise noted within this document.

NOTE 1 It may be appropriate to include previously analyzed samples for some method validation experiments (e.g., interference studies).

NOTE 2 Blood products (i.e., whole blood, serum, plasma) are different matrix types.

Example:

A method for blood and urine specimens would include complete validation experiments using fortified blank blood samples and complete validation experiments using fortified blank urine samples.

6.4.2.1 For methods that include the analysis of postmortem and antemortem specimens, validation experiments should be conducted using both types of specimens.

Example:

A method for postmortem and antemortem blood is best conducted with validation experiments using fortified blank postmortem blood samples and separate validation experiments using fortified blank antemortem blood samples.

6.4.3 Blank matrix used during method validation experiments shall represent the quality of samples typically encountered in casework.

6.4.4 Reference materials used to prepare calibrators and fortified matrix samples shall be obtained in the following preferential order to achieve the greatest level of independence:

a) from different manufacturers;

b) from the same manufacturer but from different lot numbers; or

c) from the same manufacturer's lot number but prepared by different analysts.

6.4.5 Validation experiments should be conducted over multiple days by different analysts on all instruments to be utilized for the assay.

7 Requirements for Method Verification

7.1 Verification of a Standard Test Method

7.1.1 Method verification experiments shall be performed when a standard test method is selected.

NOTE A laboratory may prefer to exceed this requirement and conduct its own validation of the standard test method.

7.1.2 The method performance characteristics to be evaluated for verifying a standard test method will depend on whether the method is qualitative or quantitative.

7.1.3 All verification experiments shall utilize the same instrumental parameters and sample preparation steps listed in the standard test method.

7.1.4 For a historical standard test method in use, laboratories shall meet all requirements for method verification in this document.

7.1.4.1 Historical calibration and control data, as well as previously analyzed casework sample results, may be used in method verification experiments.

7.1.4.2 Historical data shall have used the same instrumental parameters and sample preparation steps as the standard test method being verified.

7.1.4.3 In the absence of sufficient historical data, appropriate verification experiments shall be conducted to ensure compliance with this document.

7.2 Requirements for Establishing a Method Verification Plan

7.2.1 A plan shall be established and approved before initiating method verification.

7.2.2 The method verification plan shall include the sample preparation steps and instrumental parameters to be used, the verification experiments to be conducted, and the acceptance criteria for each assessment that will allow the method to be considered fit for use.

7.3 Required Verification Experiments Based on the Scope of the Method

7.3.1 The scope of forensic toxicology methods is classified into two categories: qualitative and quantitative.

7.3.2 The following performance characteristics shall be assessed based on the method's scope

NOTE The normative Annexes in this document provide detailed requirements for conducting method verification experiments.

a) Qualitative Methods:

1) carryover (see Annex C);

2) interference studies (see Annex E);

3) limit of detection (see Annex G); and

4) rates of false positives and false negatives (see Annex K).

b) Quantitative Methods:

1) bias (see Annex A);

2) calibration model (see Annex B);

3) carryover (see Annex C);

4) interference studies (see Annex E);

5) limit of detection (see Annex G);

6) lower limit of quantitation (see Annex H); and

7) precision (see Annex I).

7.3.3 If a laboratory uses a method in both a qualitative and quantitative manner, all required experiments in 7.3.2.a) and 7.3.2.b) shall be completed.

7.4 Requirements for Conducting Method Verification Experiments

7.4.1 All method verification experiments shall be conducted using fortified samples for each matrix type for which the method is intended unless otherwise noted within this document.

NOTE 1 It may be appropriate to include previously analyzed samples for some method validation experiments (e.g., interference studies).

NOTE 2 Blood products (i.e., whole blood, serum, plasma) are different matrix types.

Example:

A method to analyze blood and urine specimens would include all required verification experiments using fortified blank blood samples and all required verification experiments using fortified blank urine samples.

7.4.2 Blank matrix used during method verification shall represent the quality of samples typically encountered in casework.

7.4.3 Reference materials used to prepare calibrators and fortified matrix samples shall be obtained in the following preferential order to achieve the greatest level of independence:

a) from different manufacturers;

b) from the same manufacturer but from different lot numbers; or

c) from the same manufacturer's lot number but prepared by different analysts.

8 Revalidation of Changes to Previously Validated Methods

8.1 After validation has occurred, methods may be revised. The extent and frequency of revalidation of previously validated methods will depend upon the nature of the intended changes or laboratory policy.

8.2 A laboratory shall evaluate the impact on performance characteristics listed in 6.3.3.1 when changes are made to a previously validated method.

Examples of typical changes include:

— compounds added to a method's scope;

— changes to the calibration range or calibration model;

— sample preparation modifications (e.g., change in extraction technique, different internal standard, change in reconstitution volume);

— instrumentation or instrumental parameter changes (e.g., newer model, different vendor, different chromatographic parameters, additional mass spectral acquisition parameters); or

— data processing updates (e.g., switch in qualification and quantitation ions, software updates).

8.3 A full revalidation shall be conducted unless the elimination or reduction of some validation experiments is justified by logically assessing the change's impact on specific method performance characteristics.

9 Documentation Requirements for Method Development, Validation, and Verification

9.1 The records generated during method development (e.g., method development plan, activities conducted, data, results) should be retained for at least 10 years after the method is retired.

9.1.1 Method development records should summarize the experiments conducted and their results.

9.2 The records generated during method validation and verification (e.g., validation/verification plan, activities conducted, data, results) shall be retained for at least 10 years after the method is retired.

9.2.1 Method validation and verification records shall include a summary of the experiments conducted and their results.

9.2.2 The summary shall minimally include the following:

a) scope (e.g., qualitative vs quantitative; specific matrices included; analytes);

b) validation/verification plan that describes the experiments conducted and justification for any required experiments that were not conducted;

c) validation/verification results;

d) statement as to the method's fitness for intended use and any identified limitations of use; and

e) documentation of management's review and approval.

9.2.3 The method validation and verification records shall also contain specific details regarding the experiments conducted, including:

a) individuals involved in the method validation/verification;

b) sample preparation steps used;

c) specific instrumentation and parameters;

d) raw instrumental data;

e) calculations; and

f) dates of validation/verification experiments.

Annex A
(normative)

Requirements for Assessing Bias

A.1 General Requirements for Assessing Bias

NOTE Annex B (B.1.6.2), Annex D (D.1), and Annex F (F.2.3) contain requirements that may impact the design of bias experiments.

A.1.1 The same data generated from bias experiments may also be used for precision experiments.

A.1.2 Bias shall be evaluated with at least three fortified matrix concentration pools for each matrix type at low, medium, and high concentrations.

A.1.2.1 If significant ionization suppression/enhancement has been demonstrated, at least three unique sources of blank matrices for each matrix type shall be used (see Annex F.2.3).

A.1.2.2 Low concentrations shall be no more than approximately 3 times the lowest calibrator.

A.1.2.3 High concentrations shall be no less than approximately 80% of the highest calibrator.

A.1.2.4 Medium concentrations shall be near the midpoint of the low and high concentrations.

A.1.3 A single bias value shall be calculated for each concentration using the following formula:

$$\text{Bias (\%) at Concentration}_x = \left[\frac{\text{Grand Mean of Calculated Concentration}_x - \text{Nominal Concentration}_x}{\text{Nominal Concentration}_x}\right] \times 100$$

A.2 Specific Requirements for Method Validation

A.2.1 Bias experiments shall be carried out for all quantitative methods for each matrix type for which the method is intended to be used.

A.2.2 Bias shall be evaluated using a minimum of triplicate analysis per concentration pool in each of five or more independently calibrated analytical runs.

NOTE In some instances, analyte instability may preclude the ability to use concentration pools of fortified samples (e.g., cocaine in unpreserved whole blood). A laboratory may fortify different samples with each independent run in these instances.

A.2.3 The maximum acceptable bias at each concentration shall be ±10% for ethanol and ±20% for all other analytes.

A.3 Specific Requirements for Method Verification

A.3.1 Bias experiments shall be carried out for all quantitative standard test methods for each matrix type designated within the method.

A.3.2 Bias shall be evaluated using a minimum of quintuple analysis per concentration pool in each of three or more independently calibrated analytical runs.

NOTE In some instances, analyte instability may preclude the ability to use concentration pools of fortified samples (e.g., cocaine in unpreserved whole blood). A laboratory may fortify different samples with each independent run in these instances.

A.3.3 The maximum acceptable bias at each concentration shall be no more than that listed within the standard test method.

Annex B
(normative)

Requirements for Assessing Calibration Model

B.1 General Requirements for Assessing Calibration Model

B.1.1 The appropriate calibration model shall be determined or verified for all quantitative methods.

NOTE The selection of an appropriate model (i.e., weighted or unweighted; linear or quadratic) is necessary for accurate and reliable quantitative results.

B.1.2 The origin shall not be included as a calibration point.

B.1.3 A minimum of six different non-zero concentrations shall be used to evaluate the calibration model.

B.1.4 The simplest calibration model that best fits the concentration-response relationship shall be used.

B.1.4.1 An unweighted regression shall be used when there is constant variance over the entire concentration range (homoscedasticity).

B.1.4.2 When there is a significant difference between variances at the lowest and highest concentrations (heteroscedasticity), a weighted regression shall be applied (e.g., $1/x$, $1/x^2$).

NOTE Data are generally heteroscedastic when the concentration range exceeds one order of magnitude.

B.1.4.2.1 Presence of heteroscedasticity should be evaluated via statistical means, such as hypothesis tests (F-test, Levene's test) or plots (variance plots, residual plots).

B.1.4.2.2 In the presence of heteroscedasticity, an adequate weighting factor should be selected via statistical means, such as the total weighted normalized variance, variance plots, or weighted residual plots.

B.1.4.3 A linear least squares regression shall be used unless it can be demonstrated that a non-linear (e.g., quadratic) regression better fits the data.

B.1.4.3.1 The presence of non-linearity should be evaluated via statistical means (e.g., partial F-test, significance of the second-order term of a quadratic model, residual plot).

B.1.5 The selected calibration model shall be evaluated for goodness of fit.

B.1.5.1 A calibration model shall not be evaluated simply via its correlation coefficient (r) or coefficient of determination (r^2 or R^2).

B.1.5.2 Goodness of fit shall be evaluated via statistical means, such as hypothesis tests (e.g., normality testing of the residuals (Kolmogorov-Smirnov, Cramer-von Mises)) or residuals plot.

B.1.6 After the calibration model has been established, the number of calibrators required for daily use of the method may be reduced.

B.1.6.1 The lowest and highest calibrator concentrations used to establish the model shall remain when the number of calibrators is reduced.

B.1.6.1.1 No fewer than four non-zero calibrators shall be used with linear calibration models.

B.1.6.1.2 No fewer than six non-zero calibrators shall be used with non-linear calibration models.

B.1.6.2 Bias and precision studies shall be conducted using the decreased number of calibrators and the same concentrations.

B.1.7 Matrix-matched calibrator samples (for each unique matrix) should be used.

NOTE Annex A (Bias) and Annex I (Precision) require bias and precision studies to be performed using samples prepared in the matrices intended for the method. This still applies when non-matrix-matched calibrator samples are used. For example, blood alcohol methods may use aqueous calibrator samples, provided they demonstrate acceptable bias and precision with whole blood controls. Likewise, blood calibrator samples may be used to quantitate analytes in tissue samples once it has been demonstrated that acceptable bias and precision can be achieved using the blood calibrators with tissue-based controls.

B.2 Specific Recommendations for Method Development

B.2.1 Calibrator samples should be analyzed to determine a calibration range and preliminary calibration model.

B.2.2 A minimum of three replicates per concentration should be used, which may be analyzed in the same or separate runs.

B.3 Specific Requirements for Method Validation and Method Verification

B.3.1 Calibrator samples spanning the calibration range shall be analyzed to establish the calibration model.

B.3.2 For method validation experiments, a minimum of five replicates per concentration shall be used, which may be analyzed in the same or separate runs.

B.3.3 For method verification experiments, a minimum of five replicates per concentration shall be used; however, two replicates from each concentration may be reinjected with calibrator samples.

NOTE All replicates may be analyzed in the same or separate runs.

Annex C
(normative)

Requirements for Assessing Carryover

NOTE Analyte carryover into a subsequent sample may lead to an inaccurate qualitative or quantitative result when using instrumental methods.

C.1 For Method Validation

C.1.1 Carryover shall be evaluated during method validation of qualitative and quantitative methods by analyzing blank matrix samples immediately after high-concentration samples or reference materials for each analyte in the test method.

C.1.2 The laboratory shall define what constitutes unacceptable carryover for the method (e.g., exceeds 10% of the LOD and all detection criteria are met).

NOTE In quantitative assays, a laboratory may limit the carryover study to the highest point of the calibration curve.

C.1.3 The highest concentration without carryover for each analyte shall be determined in at least five separate analytical runs using at least one blank matrix sample for each matrix type per run.

C.1.4 Carryover shall either be eliminated through method modification or addressed through quality assurance practices during method use.

C.1.4.1 If the method is modified, all validation experiments previously conducted shall be repeated with the modified method, unless justified by logically assessing the change's impact on the previously conducted experiments.

C.2 For Method Verification

C.2.1 Carryover shall be evaluated during method verification of qualitative and quantitative methods for each analyte in the standard test method.

C.2.2 Carryover studies shall be conducted for each unique matrix designated within the standard test method.

C.2.3 Injections of fortified samples or reference materials shall be immediately followed by injections of prepared blank matrix samples.

C.2.3.1 This shall be repeated for at least three series of injections in one or more analytical runs.

NOTE 1 The highest fortified concentration at which no analyte carryover is observed (above the method's LOD) in the blank matrix sample establishes the concentration at which the method is free from carryover.

NOTE 2 It is acceptable to limit the carryover study to the highest concentration identified within a standard test method in which carryover was not observed.

C.2.4 If carryover is determined to occur at a concentration lower than that established within the standard test method, it shall be addressed through quality assurance practices during method use.

Annex D
(normative)

Requirements for Assessing Dilution Integrity

NOTE Low specimen volumes may require a lower sample volume to be used for a method. In other instances, excessively high concentrations may be encountered that exceed the established calibration range. In both instances, sample dilution may be necessary.

D.1 If sample dilutions are performed for a quantitative method, the laboratory shall evaluate the impact of these dilutions on the method's bias and within-run precision (see Annex A and Annex I) on at least one concentration pool during method validation experiments.

D.2 Commonly employed dilution ratios (e.g., 1:2; 1:5; 1:10) shall be evaluated to determine if performance criteria for bias and within-run precision are met after dilutions.

Annex E
(normative)

Requirements for Assessing Interferents

E.1 General

Interference from the matrix, the internal standard, and non-method analytes shall be evaluated in all qualitative and quantitative methods, during method validation and method verification.

NOTE The laboratory may determine that an interferent below the LOD is insignificant.

E.2 For Method Validation

E.2.1 General

E.2.1.1 Identified interferents shall either be eliminated through method modification or addressed through laboratory policies or procedures.

E.2.1.2 If the method is modified, all validation experiments previously conducted shall be repeated with the modified method, unless justified by logically assessing the change's impact on the previously conducted experiments.

E.2.2 Evaluating Interference from Matrix

E.2.2.1 Blank matrix samples without addition of internal standard (when used in the method) shall be analyzed to demonstrate the absence of common interferences from the matrix.

E.2.2.2 When possible, a minimum of ten unique sources of blank matrix for each matrix type shall be used.

NOTE While this approach may detect the more common matrix interferents, it is recognized that less common interferents may not be detected.

E.2.3 Evaluating Interference from Internal Standard

E.2.3.1 Interference from the internal standard shall be assessed for all qualitative and quantitative chromatographic methods by analyzing a single blank matrix sample, for each matrix type, fortified with internal standard at the concentration used in the method and monitoring the signal of the analyte(s) of interest.

NOTE Isotopically-labeled compounds may contain the non-labeled compound as an impurity for methods employing stable isotope internal standards. Additionally, the mass spectra of the labeled analogs may contain fragment ions with the same mass-to-charge ratios as the significant ions of the target analyte. In both instances, analyte identification or quantitation could be impacted.

E.2.3.2 For all qualitative and quantitative chromatographic methods, a single blank matrix sample, for each matrix type, fortified with the analyte(s) of interest at a high concentration shall be analyzed without an internal standard to evaluate whether there are relevant interferences by monitoring the signal of the internal standard.

NOTE A high concentration for evaluating analytes interfering with the internal standard may be near the upper limit of the calibration range for quantitative methods or at the highest concentration expected in routine casework for qualitative methods.

E.2.4 Evaluating Interferences from Non-Method Analytes

E.2.4.1 For all methods other than immunoassays, laboratories shall evaluate non-method analytes for their potential to interfere with the method's analyte(s) of interest and internal standard(s).

NOTE Non-method analytes can include drugs, metabolites, and other chemicals other than those included in the scope of the method that are routinely encountered in casework.

E.2.4.2 This evaluation shall be accomplished by analyzing fortified matrix samples, previously analyzed case samples, or neat reference materials of the potential interference(s) at high therapeutic or lethal concentrations, depending on the analyte, the matrix, and the laboratory's mission.

E.3 For Method Verification

E.3.1 General

Identified interferents shall be addressed through laboratory policies or procedures.

E.3.2 Evaluating Interferences from Non-Method Analytes

E.3.2.1 Laboratories shall evaluate undocumented non-method analytes in the standard test method for their potential to interfere with the method's analyte(s) of interest and internal standard(s).

NOTE Undocumented non-method analytes can include drugs, metabolites, and other chemicals other than those already evaluated by the standard test method that are routinely encountered in casework.

E.3.2.2 This evaluation shall be accomplished by analyzing fortified matrix samples, previously analyzed case samples, or neat reference materials of the potential interference(s) at high therapeutic or lethal concentrations, depending on the analyte, the matrix, and the laboratory's mission.

Annex F
(normative)

Recommendations and Requirements for Assessing Ionization Suppression/Enhancement

NOTE Co-eluting substances may suppress or enhance an analyte's ionization in LC-MS applications.

F.1 For Method Development

F.1.1 General

Laboratories shall assess the impact of ionization suppression/enhancement on all of the method's target analytes and internal standards during method development experiments using one of the following approaches:

NOTE Monitoring a single precursor to diagnostic product ion transition for each target analyte and internal standard in LC-MS/MS applications is sufficient for assessing the degree of ionization suppression/enhancement.

F.1.2 Post-Column Infusion to Assess Ionization Suppression/Enhancement

NOTE This approach provides information on where ionization suppression/enhancement occurs within a chromatogram and estimates its significance.

F.1.2.1 Neat solutions at a low analyte concentration shall be prepared.

F.1.2.2 A minimum of three unique blank matrix samples for each matrix type shall be processed following the sample preparation steps.

F.1.2.3 The baseline signal for the analyte shall be monitored using the low-concentration solution infused (using a syringe pump) into the column eluent via a post-column "T"-connector.

F.1.2.4 A solvent blank shall be injected to establish the impact of the solvent on the baseline signal.

F.1.2.5 The prepared blank matrix samples shall be sequentially injected into the LC/MS, and changes in the baseline signal should be monitored, looking for changes that are greater than those observed from the solvent blank.

F.1.2.6 A >25% change in the infused analyte signal at the retention time of the analyte shall be considered as an indication of significant suppression/enhancement.

NOTE Modifying the chromatographic system or the sample preparation steps may be required to minimize the impact.

F.1.3 Post-Extraction Addition to Assess Ionization Suppression/Enhancement

F.1.3.1 This approach may be used during method development. It allows for the calculation of the extent of ionization suppression/enhancement that occurs during analyte elution.

F.1.3.2 Two different sets of samples shall be prepared and analyzed.

F.1.3.2.1 "Set 1" shall consist of a neat standard prepared at a low concentration containing each target analyte and the internal standard.

F.1.3.2.1.1 The neat standard shall be injected a minimum of three times.

F.1.3.2.2 "Set 2" shall consist of a minimum of three unique blank matrix sources for each matrix type.

NOTE Given the variety of sample conditions typically encountered in postmortem toxicology, additional matrix samples may be needed.

F.1.3.3 Each blank matrix source for Set 2 shall be extracted and reconstituted/fortified at the same low concentration used in Set 1.

F.1.3.4 The internal standard shall be added to all Set 2 samples at the method's defined concentration.

F.1.3.5 Dilution effects shall be compensated for to ensure consistency between the concentrations evaluated in the Set 1 and Set 2 samples.

F.1.3.6 Each sample shall be injected one time.

F.1.3.7 The average peak areas of the neat standard (Set 1) shall be compared to the peak area of the individual Set 2 samples, as follows:

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{\text{Area of Individual Set 2 Samples}}{\text{Average Area of Set 1}}\right) \times 100$$

F.1.3.8 The individual ionization suppression/enhancement percentages for each matrix type shall be averaged, and the % CV should be calculated.

NOTE This will result in two average ionization suppression/enhancement percentages and % CVs for each matrix type: one for the low concentration and one for the internal standard.

F.1.3.9 An average instrumental response that drops to less than 75%, or increases to more than 125%, or has a % CV exceeding 20% shall be considered an indication of significant suppression/enhancement.

NOTE Modifying the chromatographic system or the sample preparation technique may be required to minimize the impact.

F.2 For Method Validation

F.2.1 General

To address ionization suppression/enhancement during method validation experiments, laboratories shall do one of the following:

a) assume ionization suppression/enhancement is present and follow the requirements of F.2.3 below; or

b) evaluate the significance of ionization suppression/enhancement via the post-extraction addition assessment approach.

NOTE Monitoring a single precursor to diagnostic product ion transition for each target analyte and internal standard in LC-MS/MS applications is sufficient to assess the degree of ionization suppression/enhancement.

F.2.2 Post-Extraction Addition Approach to Assess Ionization Suppression/Enhancement

F.2.2.1 Two different sets of samples shall be prepared and analyzed.

F.2.2.1.1 "Set 1" shall consist of two neat standards prepared at low and high concentrations containing each target analyte and the internal standard(s) at the method's defined concentration. These neat standards shall be injected a minimum of three times each.

F.2.2.1.2 "Set 2" shall consist of at least ten unique blank matrix sources, per matrix type.

NOTE Given the variety of sample conditions typically encountered in postmortem toxicology, additional matrix samples may be needed.

F.2.2.2 Each blank matrix source shall be extracted in duplicate.

F.2.2.3 One replicate from each extracted blank matrix source shall be reconstituted/fortified at the low concentration used in Set 1 and the other replicate at the high concentration used in Set 1 for each target analyte.

F.2.2.4 The internal standard(s) shall be added at the method's defined concentration to all Set 2 samples.

F.2.2.5 Dilution effects shall be compensated for to ensure consistency between the concentrations evaluated in the Set 1 and Set 2 samples.

F.2.2.6 Each sample shall be injected one time.

F.2.2.7 The average peak areas of neat standards (Set 1) shall be compared to the peak area of the individual Set 2 samples, as follows:

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{\text{Area of Individual Set 2}}{\text{Average Area of Set 1}}\right) \times 100$$

F.2.2.8 The individual ionization suppression/enhancement percentages for each matrix type and at each concentration shall be averaged, and the % CV shall be calculated.

NOTE This will result in three average ionization suppression/enhancement percentages and % CVs for each matrix type: low concentration, high concentration, and internal standard.

F.2.2.9 An average instrumental response that drops to less than 75%, increases to more than 125%, or has a % CV exceeding 20% shall indicate significant suppression/enhancement.

F.2.3 Addressing Significant Ionization Suppression/Enhancement

F.2.3.1 If significant ionization suppression/enhancement is assumed or demonstrated, the influence on other validation experiments shall be determined by at least tripling the number of unique sources of blank matrices used for their evaluation.

F.2.3.1.1 For a qualitative method, the influence of significant ionization suppression/enhancement shall be determined for the method's LOD and rates of false positives and false negatives.

F.2.3.1.2 For a quantitative method, the influence of significant ionization suppression/enhancement shall be determined for the method's LOD, LLOQ, bias, and precision.

Annex G
(normative)

Requirements for Estimating Limit of Detection

G.1 For Method Validation

G.1.1 LOD experiments shall be performed for all method validations.

G.1.2 The LOD shall be evaluated over multiple runs using fortified samples prepared from at least three unique sources of blank matrix for each matrix type.

Example:

At least three unique blood sources are needed if the assay is to be used for blood samples.

G.1.3. The number of unique sources of blank matrix for each affected matrix type shall be increased to at least nine if a method demonstrates significant ionization suppression/enhancement (see Annex F.2.3).

G.1.4 The LOD shall be determined by one of the approaches in G.2 through G.6.

G.2 Estimating LOD for Immunoassays

G.2.1 Using Manufacturer's Specifications

When a laboratory uses an immunoassay following all of the manufacturer's specifications (e.g., same matrix, same target analyte, same cutoff concentration), the laboratory shall:

a) use the manufacturer-stated cutoff concentration for the target analyte's LOD,

b) use the manufacturer-defined equivalent concentrations as an estimated LOD for other analytes, or

c) use manufacturer-provided cross-reactivity data compared to the cutoff concentration of the target analyte (often as percentages) to calculate an estimated LOD for other analytes.

Example:

A manufacturer's benzodiazepine immunoassay uses oxazepam at 50 ng/mL as the target analyte in blood. The cross-reactivity for clonazepam is 80%. To estimate the clonazepam LOD, 50 ng/mL is divided by 80% (0.8) to yield approximately 63 ng/mL.

G.2.2 Using Modified Manufacturer's Specifications

G.2.2.1 When a laboratory modifies a manufacturer's immunoassay method (e.g., different matrix, different target analyte, different cutoff concentration), the laboratory shall experimentally determine the LODs for individual analytes.

G.2.2.2 For the target analyte, the cutoff concentration shall be demonstrated as appropriate, as follows:

G.2.2.2.1 The laboratory shall prepare a "cutoff sample" using a single blank matrix sample fortified with the cutoff concentration of the target analyte and analyze the sample at least three times to establish the within-run range of cutoff responses.

NOTE A single matrix type (e.g., blood) may be used to prepare the cutoff sample to validate other matrices (e.g., vitreous, urine, tissues).

G.2.2.2.2 For each matrix type being validated, at least three unique blank sources shall be analyzed at least three times each in a single run to establish individual blank responses.

G.2.2.2.3 The cutoff and blank samples shall be reanalyzed over a minimum of two additional independent runs.

G.2.2.2.4 The within-run range of cutoff responses from each run shall be evaluated for overlap with the individual blank responses from the same run.

G.2.2.2.5 The responses for the blank and cutoff samples shall not overlap in at least 95% of all samples.

NOTE The above experiments provide objective evidence of the immunoassay's ability to distinguish blank matrix from the proposed cutoff concentration of the target analyte.

G.2.2.3 For analytes with cross-reactivities that are the same or better than the target analyte, the laboratory shall declare that the LOD for the analyte is the same as that of the target analyte.

Example:

A manufacturer's benzodiazepine immunoassay uses oxazepam at 50 ng/mL as the target analyte in blood. The lab changes the cutoff concentration to 20 ng/mL. The cross-reactivity for alprazolam is 150%. The LOD for alprazolam is set at the same modified cutoff concentration of 20 ng/mL.

G.2.2.4 For analytes with cross-reactivities that are poorer than the target analyte (i.e., <100%), the new LOD concentration for these analytes shall be estimated through calculations using the manufacturer's provided data.

Example:

A manufacturer's benzodiazepine immunoassay uses oxazepam at 50 ng/mL as the target analyte in blood. The lab changes the cutoff concentration to 20 ng/mL. The cross-reactivity for clonazepam is 80%. To estimate the clonazepam LOD at the new cutoff concentration, 20 ng/mL is divided by 80% (0.8) to yield approximately 25 ng/mL.

G.2.3 Adding Analytes to Immunoassay Panels

G.2.3.1 When an analyte is added to an immunoassay panel without the manufacturer's cross-reactivity or equivalent concentration data, the laboratory shall experimentally determine the LOD as follows:

G.2.3.1.1 At least three unique blank matrix sources for each matrix type shall be fortified with decreasing concentrations of the new analyte *or* at a decision point concentration.

G.2.3.1.2 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

G.2.3.1.3 The LOD shall be the lowest concentration of analyte that yields a positive result compared to the target analyte at the cutoff concentration in at least 95% of the replicate results.

G.3 Estimating LOD for a Non-Instrumental Method

NOTE This approach is often used when screening for the presence or absence of a specified analyte or class of analytes (e.g., color tests).

G.3.1 At least three unique blank matrix sources for each matrix type shall be fortified with decreasing analyte concentrations.

G.3.2 Each fortified blank matrix sample shall be analyzed by at least two analysts in a minimum of three runs.

G.3.3 The LOD shall be the lowest concentration of analyte that yields a positive result in at least 95% of the replicate results from all analysts.

G.4 Using the Lowest Non-Zero Calibrator as the LOD

G.4.1 At least three unique blank matrix sources for each matrix type shall be fortified with the analyte at the lowest non-zero calibrator concentration.

G.4.2 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

NOTE It is acceptable to use the same calibrator replicates used to establish the calibration model (Annex B) for this approach; however, additional samples/replicates may be needed to meet the minimum of nine data points, including at least three sources per matrix type.

G.4.3 The lowest non-zero calibrator shall be established as the method's LOD if all detection and identification criteria are observed in at least 95% of the replicate results.

G.5 Using the Decision Point Concentration as the LOD

G.5.1 The laboratory may define the LOD as an administratively-defined decision point.

Example:

A laboratory may define a method's LOD for ethanol as 0.02 g/dL for blood based on the laboratory's administratively defined decision point for reporting this analyte, even though a lower LOD is analytically achievable.

G.5.2 At least three unique matrix sources for each matrix type shall be fortified with the analyte at the decision point concentration.

G.5.3 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

G.5.4 The decision point concentration shall be established as the method's LOD if all detection and identification criteria are observed in at least 95% of the replicate results.

G.6 Estimating LOD Using Background Noise

NOTE The following approaches are only useful for instrumental methods demonstrating background noise.

G.6.1 Estimating LOD Using Reference Materials

G.6.1.1 At least three unique matrix sources for each matrix type shall be fortified with the analyte at decreasing concentrations.

G.6.1.2 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

G.6.1.3 The signal-to-noise ratio shall be determined through software or manual calculations.

G.6.1.3.1 If manually calculated, the following equation shall be used:

$$\text{Signal-to-Noise} = \frac{\text{height of analyte}}{\text{height of noise}}$$

G.6.1.4 The LOD shall be established as the lowest concentration that yields:

a) responses greater than or equal to 3.3 times the noise level of the background signal in each of the replicates, and

b) detection and identification criteria observed in at least 95% of the replicate results.

G.6.2 Estimating LOD Using Statistical Analysis of Background Noise

G.6.2.1 Two sets of samples shall be prepared and analyzed in triplicate in at least three runs.

G.6.2.1.1 "Set 1" shall consist of at least three unique blank matrix sources for each matrix type.

G.6.2.1.2 "Set 2" shall consist of the same blank matrix sources fortified with the analyte at decreasing concentrations.

G.6.2.2 The average signal (e.g., integrated signal area at the analyte's retention time) from the Set 1 data of a given matrix type and respective standard deviation shall be calculated.

G.6.2.3 For each matrix type, the average signal from the Set 1 samples plus 3.3 times the respective standard deviation shall define the threshold signal.

G.6.2.4 The LOD shall be defined as the lowest concentration where 95% of the individual Set 2 samples yield a signal greater than the threshold signal for that matrix.

G.6.3 Estimating LOD Using a Linear Calibration Curve

G.6.3.1 This technique shall only be used for quantitative methods that follow a linear calibration model.

G.6.3.2 At least three unique matrix sources for each matrix type shall be used to estimate the LOD using this approach.

G.6.3.3 For each matrix type, a minimum of three independent calibration curves shall be constructed across the calibration range of the test method in different runs.

G.6.3.4 The LOD shall be calculated using the standard deviation of the *y-intercept* and the average slope:

803 $$\text{LOD} = \frac{(3.3 \times \text{standard deviation of the y-intercept})}{\text{average slope}}$$

804 ## G.7 For Method Verification

805 **G.7.1** LOD studies shall be performed for all method verifications.

806 **G.7.2** The LOD specified in a standard test method shall be used as an administratively-defined
807 decision point concentration.

808 **G.7.3** At least three unique matrix sources for each matrix type shall be fortified with the analyte
809 at the decision point concentration.

810 **G.7.4** Each fortified blank matrix sample shall be analyzed in triplicate in one or more runs.

811 **G.7.5** The decision point concentration shall be verified as the method's LOD if all detection and
812 identification criteria are observed in at least 95% of the replicate results.

813

Annex H
(normative)

Requirements for Assessing Lower Limit of Quantitation

H.1 For Method Validation

H.1.1 LLOQ experiments shall be performed for method validation of all quantitative methods.

H.1.2 The LLOQ shall be evaluated over multiple runs using fortified matrix samples prepared from at least three unique sources of blank matrix for each matrix type.

Example:

At least three unique blood sources are needed if the assay is to be used for blood samples.

H.1.3. The number of unique sources of blank matrix for each affected matrix type shall be increased to at least nine if a method demonstrates significant ionization suppression/enhancement (see Annex F.2.3).

H.1.4 The LLOQ shall be determined by one of the approaches in H.2 and H.3.

H.2 Using the Lowest Non-Zero Calibrator as the LLOQ

H.2.1 At least three unique blank matrix sources for each matrix type shall be fortified with the analyte at the lowest calibrator concentration.

H.2.2 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

NOTE It is acceptable to use the same calibrator replicates used to establish the calibration model (Annex B) for this approach; however, additional samples/replicates may be needed to meet the minimum of nine data points including at least three sources per matrix type.

H.2.3 The lowest non-zero calibrator shall be established as the method's LLOQ if bias and precision for these fortified samples remain within the requirements specified for the method.

H.3 Using the Decision Point Concentration as the LLOQ

H.3.1 The laboratory may define the LLOQ as an administratively-defined decision point.

Example:

A laboratory may choose to define a method's LLOQ for GHB as 5 mg/L for antemortem blood based on the laboratory's administratively defined decision point for reporting this analyte, even though a lower LLOQ is analytically achievable.

H.3.2 At least three unique matrix sources for each matrix type shall be fortified with the analyte at the decision point concentration.

H.3.3 Each fortified blank matrix sample shall be analyzed in a minimum of three runs.

H.3.4 The decision point concentration shall be established as the method's LLOQ if bias and precision for these fortified samples remain within the requirements specified for the method.

H.4 For Method Verification

H.4.1 LLOQ studies shall be performed for all quantitative method verifications.

H.4.2 The LLOQ specified in a standard test method shall be used as an administratively-defined decision point.

H.4.3 At least three unique matrix sources for each matrix type shall be fortified with the analyte at the decision point concentration.

H.4.4 Each fortified blank matrix sample shall be analyzed in triplicate in one or more runs.

H.4.5 The decision point concentration shall be verified as the method's LLOQ if bias and precision for these fortified samples remain within the requirements specified for the method.

Annex I

(normative)

Requirements for Assessing Precision

I.1 General Requirements for Assessing Precision

NOTE Annex B (B.1.6.2), Annex D (D.1), and Annex F (F.2.3) contain requirements that may impact the design of precision experiments.

I.1.1 Precision experiments may be carried out concurrently with bias experiments.

I.1.2 Both within-run and between-run precision shall be evaluated.

I.1.3 Precision shall be evaluated with at least three fortified matrix concentration pools for each matrix type at low, medium, and high concentrations.

I.1.3.1 If significant ionization suppression/enhancement has been demonstrated, at least three unique sources of blank matrices for each matrix type shall be used (see Annex F.2.3).

I.1.3.2 Low concentrations shall be no more than approximately 3 times the lowest calibrator.

I.1.3.3 High concentrations shall be no less than approximately 80% of the highest calibrator.

I.1.3.4 Medium concentrations shall be near the midpoint of the low and high concentrations.

I.1.4 Precision shall be expressed as the coefficient of variation (% CV) using the mean and standard deviation (*std dev)* for each concentration using the following formula:

$$\%\ CV = \frac{\text{std dev}}{\text{mean}} \times 100$$

I.2 Within-Run Precision Calculations

Each concentration shall have one within-run precision calculated per run using the following formula:

$$\text{Within-run CV}(\%) = \frac{\text{std dev of a single run of samples}}{\text{mean calculated value of a single run of samples}} \times 100$$

I.3 Between-Run Precision Calculations

Each concentration shall have a between-run precision calculated using the following formula:

$$\text{Between-run CV}(\%) = \frac{\text{std dev of all observations for each concentration}}{\text{grand mean for each concentration}} \times 100$$

I.4 One-Way Analysis of Variance (ANOVA) Approach to Calculate Within-Run and Between-Run Precision

I.4.1 Instead of the formulas listed in sections I.2 or I.3, within-run and between-run precisions may be calculated using the one-way ANOVA approach with the run number as the grouping variable.

NOTE The ANOVA calculations may be performed using a spreadsheet or a statistical software program.

I.4.1.1 Within-run precision using the ANOVA approach shall be calculated for each concentration as:

$$\text{Within-run CV}(\%) = \left[\frac{\sqrt{MS_{wg}}}{\text{grand mean for each concentration}}\right] \times 100$$

where MS_{wg} is the mean square within groups obtained from the ANOVA table.

I.4.1.2 Between-run precision using the ANOVA approach shall be calculated as:

$$\text{Between-run CV}(\%) = \left[\frac{\sqrt{\frac{MS_{bg} + (n-1) \times MS_{wg}}{n}}}{\text{grand mean for each concentration}}\right] \times 100$$

where MS_{bg} is the mean square between groups obtained from the ANOVA table and n is the number of observations in each group (e.g., n=3 if doing triplicate analyses per run).

I.5 Specific Requirements for Method Validation

I.5.1 Precision experiments shall be carried out for all quantitative methods for each matrix type for which the method is intended to be used.

I.5.2 Precision shall be evaluated using a minimum of triplicate analysis per concentration pool in each of five or more independently calibrated analytical runs.

NOTE In some instances, analyte instability may preclude the ability to use concentration pools of fortified samples (e.g., cocaine in unpreserved whole blood). A laboratory may fortify different samples with each independent run in these instances.

I.5.3 The maximum % CV shall be 10% for ethanol and 20% for all other analytes at each concentration.

I.5.4 The largest within-run % CV for each concentration pool and the between-run % CV for each concentration pool shall be used to assess the acceptability of precision.

I.6 Specific Requirements for Method Verification

I.6.1 Precision experiments shall be carried out for all quantitative standard test methods for each matrix type designated within the method.

I.6.2 Precision shall be evaluated using a minimum of quintuple analysis per concentration pool in each of three or more independently calibrated analytical runs.

NOTE In some instances, analyte instability may preclude the ability to use concentration pools of fortified samples (e.g., cocaine in unpreserved whole blood). A laboratory may fortify different samples with each independent run in these instances.

I.6.3 The maximum acceptable % CV at each concentration shall be no more than that listed within the standard test method.

Annex J
(normative)

Requirements for Assessing Processed Sample Stability

NOTE Processed samples are typically analyzed in batches on the same day of preparation; however, circumstances may arise in which they cannot be analyzed within a reasonable amount of time due to atypical events (e.g., instrument failures or loss of power). Analyzing processed samples the following day or even later may be necessary.

J.1 Processed sample stability experiments shall be performed during method development or method validation when a laboratory will allow samples to be analyzed more than 24 hours after processing (e.g., extracted).

NOTE The following approach provides a means of evaluating the loss of analytes in stored, processed samples at low and high concentrations that could impact the ability to detect, identify, and quantify them accurately.

J.2 Processed sample stability assessments shall evaluate the length of time processed samples can be maintained before they undergo unacceptable changes.

J.3 For qualitative methods, the laboratory shall define acceptable limits for processed sample stability experiments.

J.4 For quantitative methods, the method's bias requirements (as defined in the method development or method validation plan) shall serve as the acceptable limits for processed sample stability experiments.

Example:

> *A method's bias requirement is ±15%, and the time zero average signal is 100,000. The laboratory's processed samples are placed into different autosampler vials and are analyzed repeatedly for up to 72 hours. For this example, the processed sample's analyte is considered stable until the average signal falls outside of the 85,000 – 115,000 range (±15% of the time zero average signal).*

J.5 Processed sample stability experiments shall utilize blank matrix samples fortified at low and high concentrations.

J.6 A single source of blank matrix for each matrix type may be used to evaluate processed sample stability.

J.6.1 The samples may be: prepared by the laboratory, purchased from a commercial source, or previously analyzed, pooled samples.

J.6.2 A large enough sample volume should be used to complete the studies.

J.7 Numerous aliquots from each concentration set shall be processed (e.g., extracted) using the method under validation.

J.8 The processed samples for a given concentration pool shall be combined, mixed, and then divided into different (autosampler) vials for instrumental analysis.

J.9 The first vials of each concentration shall be immediately analyzed in triplicate to establish the time zero responses.

J.10 All remaining vials shall be maintained as they would typically be stored during routine analysis (e.g., at refrigerated or room temperature on autosampler).

J.11 The remaining vials shall be analyzed in triplicate at different time intervals, representing the typical time range expected for processed samples to wait before being analyzed.

J.12 The analyte shall be considered stable until the average signal (e.g., peak area or ratios of analyte peak area to internal standard peak area) compared to the time zero average signal falls outside established limits.

NOTE For each concentration pool, a plot of the average response against each time point with linear regression allows for an assessment of trends.

Annex K
(normative)

Requirements for Assessing Rates of False Positives and False Negatives

NOTE False positive and false negative rates provide information about the probability of a mischaracterized result in qualitative methods.

K.1 General

K.1.1 Determination of false positive and false negative rates (expressed as percentages) shall be carried out for all qualitative methods, as well as for all quantitative methods used qualitatively, during method validation and method verification.

K.1.2 The assessment of rates of false results shall be conducted over no less than three days.

K.2 Assessing Rates of False Positives and False Negatives for Immunoassays

K.2.1 A sample shall be prepared in a blank matrix fortified with the cutoff concentration of the target analyte and analyzed at least three times to establish the average response for this concentration.

NOTE A single matrix type (e.g., blood) may be used to prepare the cutoff concentration sample to validate other matrices (e.g., vitreous, urine, tissues).

K.2.2 A minimum of 10 unique blank matrix sources for each matrix type shall be obtained.

K.2.3 Each of the unique blank matrix sources shall be divided into two subsamples.

K.2.3.1 The first subsample for each unique blank matrix source shall be fortified with the immunoassay's target analyte at approximately 50% below its cutoff concentration.

K.2.3.1.1 The first subsamples shall serve as "negatives."

K.2.3.1.2 Each negative subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently prepared and tested.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.2.3.1.2.1 The number of data points below the average response of the cutoff concentration shall be recorded as "true negatives" (TN).

K.2.3.1.2.2 The number of data points above the average response of the cutoff concentration shall be recorded as "false positives" (FP).

K.2.3.2 The second subsample for each unique blank matrix source shall be fortified with the immunoassay's target analyte at approximately 50% above its cutoff concentration.

K.2.3.2.1 The second subsamples shall serve as "positives."

K.2.3.2.2 Each positive subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently prepared and tested.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.2.3.2.2.1 The number of data points above the average response of the cutoff concentration shall be recorded as "true positives" (TP).

K.2.3.2.2.2 The number of data points below the average response of the cutoff concentration shall be recorded as "false negatives" (FN).

K.3 Assessing Rates of False Positives and False Negatives for Other Qualitative Methods Using a Decision Point as the Limit of Detection

K.3.1 A sample shall be prepared in a blank matrix fortified with the decision point concentration of the analyte and analyzed at least three times to establish the average response for the decision point concentration.

NOTE 1 "Analyzed" refers to samples independently extracted or prepared and tested. For instrumental techniques, they are not reinjections of the same sample.

NOTE 2 A single matrix type (e.g., blood) may be used to prepare the decision point concentration sample to validate other matrices (e.g., vitreous, urine, tissues).

K.3.2 A minimum of 10 unique blank matrix sources for each matrix type shall be obtained.

K.3.3 Each of the unique blank matrix sources shall be divided into two subsamples.

K.3.3.1 The first subsample for each unique blank matrix source shall be fortified with the analyte at a concentration that is no less than half of the decision point concentration.

Example:

If a method's decision point concentration is set at 10 ng/mL, the first subsample could be fortified at a concentration of no less than 5 ng/mL.

K.3.3.1.1 The first subsamples shall serve as "negatives."

K.3.3.1.2 Each negative subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently extracted or prepared and tested. For instrumental techniques, they are not reinjections of the same sample.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.3.3.1.2.1 Each data point with a response below the average response of the decision point concentration shall be recorded as a "true negative" (TN).

K.3.3.1.2.2 Each data point with a response above the average response of the decision point concentration shall be recorded as a "false positive" (FP).

K.3.3.2 The second subsample for each unique blank matrix source shall be fortified with the analyte at a concentration no more than 1.5 times the decision point concentration.

Example:

If a method's decision point concentration is set at 10 ng/mL, the second subsample could be fortified at a concentration of no more than 15 ng/mL.

K.3.3.2.1 The second subsamples shall serve as "positives."

K.3.3.2.2 Each positive subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently extracted or prepared and tested. For instrumental techniques, they are not reinjections of the same sample.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.3.3.2.2.1 Each data point with a response above the average response of the decision point concentration shall be recorded as a "true positive" (TP).

K.3.3.2.2.2 Each data point with a response below the average response of the decision point concentration shall be recorded as a "false negative" (FN).

K.4 Assessing Rates of False Positives and False Negatives for Qualitative Methods that Do Not Use Decision Points as the Limit of Detection

K.4.1 A minimum of 10 unique blank matrix sources for each matrix type shall be obtained.

K.4.2 Each of the unique blank matrix sources shall be divided into two subsamples.

K.4.2.1 The first subsample for each unique blank matrix source shall remain blank (unfortified).

K.4.2.2 The first subsamples shall serve as "negatives."

K.4.2.3 Each negative subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently extracted or prepared and tested. For instrumental techniques, they are not reinjections of the same sample.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.4.2.3.1 Each data point not meeting the method's predefined detection and identification criteria shall be recorded as a "true negative" (TN).

K.4.2.3.2 Each data point that meets the method's predefined detection and identification criteria shall be recorded as a "false positive" (FP).

K.4.2.4 The second subsample for each unique blank matrix source shall be fortified with the analyte at a concentration no more than 1.5 times the LOD concentration.

Example:

If a method's LOD concentration is 100 ng/mL, the second subsample could be fortified at a concentration of no more than 150 ng/mL.

K.4.2.4.1 The second subsamples shall serve as "positives."

K.4.2.4.2 Each positive subsample shall be analyzed an approximately equal number of times to reach at least *n* analyses as defined in Table K-1.

NOTE 1 "Analyzed" refers to samples independently extracted or prepared and tested. For instrumental techniques, they are not reinjections of the same sample.

NOTE 2 Per Table K-1, a minimum of 22 samples must be analyzed to claim no more than a 10% false result rate; 45 samples for no more than a 5% false result rate; and 230 samples for no more than a 1% false result rate.

Example:

Ten unique matrix sources analyzed six times each, resulting in 60 data points.

K.4.2.4.2.1 Each data point that meets the method's predefined detection and identification criteria shall be recorded as a "true positive" (TP).

K.4.2.4.2.2 Each data point not meeting the method's predefined detection and identification criteria shall be recorded as a "false negative" (FN).

K.5 False Positive and False Negative Rates

K.5.1 False positive or false negative rates of "no more than F% at the C% confidence level" shall be reported, with F and C established based on the validation experiment carried out (Table K-1).

K.5.2 False positive or false negative rates of 0% shall not be claimed, as they are not statistically supported.

Table K.1—Minimum Validation Design to Claim Different False Result Rates at Different Confidence Levels

Claimed False Result Rate	Confidence Level	Minimum Number of Samples Tested	Accepted Number of Failures
10%	90%	22	≤1
		44	≤2
		66	≤3
		88	≤4
	95%	29	≤1
		58	≤2
		87	≤3
	99%	44	≤1
		88	≤2
5%	90%	45	≤1
		90	≤2
	95%	59	≤1
		118	≤2
	99%	90	≤1
1%	90%	230	≤1
	95%	299	≤1
	99%	459	≤1

Examples:

- *If 1 false positive and 0 false negative results were observed after testing 59 negative and 59 positive samples, the laboratory can claim the method has a "false result rate of no more than 5% at the 95% confidence level".*

- *If 2 false positive results were observed after testing 59 negative samples, the laboratory cannot claim the method has a 5% false positive rate at the 95% confidence level.*

- *If 2 false positive and 1 false negative results were obtained after testing 59 negative and 59 positive samples, the laboratory can claim the method has a false positive rate of no more than 10% at the 95% confidence level and a false negative rate of no more than 5% at the 95% confidence level.*

NOTE The most relevant combinations of false result rates and confidence levels were included in Table K-1. Other minimum study designs may be defined using the relationship log(1-C)/log(1-F), which yields the minimum number of samples to be analyzed, with a maximum of one allowed to fail.

Annex L
(normative)

Requirements for Assessing Recovery

L.1 For Method Development

L.1.1 General

Laboratories shall assess the recovery of the method's target analytes and internal standards during method development.

NOTE Monitoring a single precursor to diagnostic product ion transition for each target analyte and internal standard in LC-MS/MS applications is sufficient for assessing recovery.

L.1.2 Post-Extraction Addition to Assess Recovery

L.1.2.1 Two different sets of samples shall be prepared and analyzed.

L.1.2.1.1 "Set A" shall consist of at least three unique blank matrix sources, per matrix type.

NOTE 1 For LC/MS methods, this approach may be performed in combination with ionization suppression/enhancement assessments. Set A above is the equivalent of Set 2 for Ionization Suppression and Enhancement experiments (Annex F).

NOTE 2 Given the variety of sample conditions typically encountered in postmortem toxicology, additional matrix samples may be needed.

L.1.2.1.1.1 Each sample of Set A shall be extracted, fortified at the low concentration used in Set B (below) for each target analyte and the internal standard, and then analyzed.

L.1.2.1.2 "Set B" shall consist of at least three unique blank matrix sources for each matrix type fortified at a low concentration for each target analyte and at the method's defined concentration for each internal standard.

L.1.2.1.2.1 Each sample of Set B shall be extracted and analyzed.

L.1.2.2 Each sample shall be injected at least once; however, the same number of injections shall be used for all samples.

L.1.2.3 The peak areas shall be averaged for Set A to create two values (i.e., Set A_{Low} and Set $A_{Int\ Std}$).

L.1.2.4 The average peak areas for the Set A samples shall be compared to the average peak areas of the Set B samples, as follows:

$$\text{Recovery (\%)} = \left(\frac{\text{Average Area of } B_X}{\text{Average Area of Set } A_X}\right) \times 100$$

Annex M
(normative)

Requirements for Assessing Upper and Lower Reliability Limits

NOTE Understanding the reliability limits of analytical methods that depend on a decision point concentration to determine whether a result is classified as "positive" versus "negative" is fundamental to the method's overall effectiveness and credibility.

M.1 General

M.1.1 For qualitative methods that use a decision point concentration as the estimated limit of detection, upper and lower reliability limits shall be assessed during method development.

M.1.2 Laboratories shall define appropriate false positive and false negative rates (expressed as percentages) above which the results obtained will be considered unreliable (e.g., 1%, 5%, 10%).

M.1.3 There are two ways a laboratory can assess upper and lower reliability limits:

M.2 Assessment via Fortified Samples

M.2.1 A sample shall be prepared in a blank matrix fortified at the decision point concentration of the analyte and analyzed at least three times to establish the average response for the decision point concentration.

NOTE A single matrix type (e.g., blood) may be used to prepare the decision point concentration sample used to evaluate other matrices (e.g., vitreous, urine, tissues).

M.2.2 Laboratories shall fortify concentration levels surrounding the decision point concentration for each matrix.

Example:

> *For a decision point concentration of 10 ng/mL, a laboratory may choose to assess concentration levels of 5 ng/mL, 8 ng/mL, 12 ng/mL, and 15 ng/mL (for each intended matrix of the method) to determine which concentrations can meet the predefined false positive and false negative rates.*

M.2.3 A minimum of 10 unique blank matrix sources shall be obtained.

M.2.4 Each of the unique blank matrix sources shall be divided into subsamples based on the number of concentrations evaluated.

M.2.4.1 Each subsample shall be fortified with one of the chosen concentration levels.

M.2.4.1.1 The subsamples fortified at concentrations below the decision point shall serve as "negatives" and be analyzed at least once.

NOTE This will result in at least 10 data points for each "negative" concentration.

M.2.4.1.1.1 Each data point with a response below the average response of the decision point concentration shall be recorded as a "true negative" (TN).

M.2.4.1.1.2 Each data point with a response above the average response of the decision point concentration shall be recorded as a "false positive" (FP).

M.2.4.1.2 The subsamples fortified at concentrations above the decision point shall serve as "positives" and be analyzed at least once.

NOTE This will result in at least 10 data points for each "positive" concentration.

M.2.4.1.2.1 Each data point with a response above the average response of the decision point concentration shall be recorded as a "true positive" (TP).

M.2.4.1.2.2 Each data point with a response below the average response of the decision point concentration shall be recorded as a "false negative" (FN).

M.2.5 Calculating False Positive and False Negative Rates:

M.2.5.1 From the data collected, false positive rates shall be calculated for each concentration below the decision point as:

$$\text{False Positive Rate (\%)} = \frac{FP}{FP+TN} \times 100$$

M.2.5.2 From the data collected, false negative rates shall be calculated for each concentration above the decision point as:

$$\text{False Negative Rate (\%)} = \frac{FN}{FN+TP} \times 100$$

M.2.6 The calculated false positive and false negative rates shall be compared to the predefined ("appropriate") false positive and false negative rates.

M.2.7 The highest concentration meeting the pre-established rate for false positives is the lower reliability limit, and the lowest concentration meeting the pre-established rate for false negatives is the upper reliability limit.

M.3 Assessment via Standard Deviation of the Decision Point Signal

M.3.1 A blank matrix fortified with the decision point concentration of the analyte shall be prepared in at least three replicates and analyzed once each to establish the average response and its standard deviation at the decision point concentration.

NOTE There is value in repeating this process with each matrix type (e.g., vitreous, urine, tissues) included in the method scope.

M.3.2 The standard deviation of the signal at the decision point shall be multiplied by a coverage factor (k) corresponding to the one-tailed t-value at the significance level equal to the false positive/negative rate established in Section M.2 and then added to or subtracted from the average decision point signal.

$$\text{Signal}_{\text{Upper}} = \text{Average Decision Point Signal} + (k \times \text{std dev})$$

$$\text{Signal}_{\text{Lower}} = \text{Average Decision Point Signal} - (k \times \text{std dev})$$

NOTE If a laboratory chooses a 5% false positive and negative rate and does the minimum of three replicates, the coverage factor (k) will be 2.920 from a one-tailed Student's t distribution table.

M.3.3 The lower and upper reliability limits (in concentration units) shall be obtained by multiplying the signals obtained in Section M.3.2 by the decision point concentration and dividing by the average response at the decision point concentration.

$$\text{Reliability Limit}_{\text{Upper}} = \frac{\text{Signal}_{\text{Upper}} \times \text{Decision Point Conc}}{\text{Average Response for Decision Point Conc}}$$

$$\text{Reliability Limit}_{\text{Lower}} = \frac{\text{Signal}_{\text{Lower}} \times \text{Decision Point Conc}}{\text{Average Response for Decision Point Conc}}$$

Annex N
(informative)

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