FBI Laboratory
Practices for Validating Chemical Procedures

1 Purpose

These practices provide direction for validating chemical procedures prior to the procedures being introduced into casework in the FBI Laboratory. Validation is the process for determining whether specified requirements are adequate for an intended use. These practices also satisfy the requirements of the FBI Laboratory Quality Assurance Manual and Laboratory Operations Manual (LOM), as well as the applicable accrediting body(ies).

2 Scope

These practices apply to FBI Laboratory personnel who are authorized to validate technical procedures used for chemical analyses. These practices supplement the requirements in the LOM - Practices for Developing Methods and Validating Technical Procedures. For disciplines and/or categories of testing that have validation requirements specified by other authoritative bodies, those requirements may supersede these practices.

3 Practices

3.1 Validation

The following will be conducted and recorded when validating chemical procedures in the FBI Laboratory.

3.1.1 Define the Scope of the Chemical Procedure

The scope of the chemical procedure will be recorded. The scope will declare the targeted matrices and analyte(s), analytical technique(s), specific equipment, intended application of the chemical procedure, and acceptable limits, when possible. The application of a chemical procedure will generally fall into the following categories:

- Screening for the presence or absence of a specified analyte or class of analytes
- Qualitative identification of a specified analyte or class of analytes
- Quantitation of a specified analyte or class of analytes

3.1.2 Identify the Performance Characteristics of the Chemical Procedure

The performance characteristics will vary depending on the scope of the procedure. This decision requires professional judgment. For example, some performance characteristics are not relevant to particular sample types, but when appropriate, the following performance

characteristics must be considered.

3.1.2.1 Performance Characteristics for the Screening of an Analyte or Class of Analytes

- Limit of Detection
- Processed Sample Stability
- Interferences

3.1.2.2 Performance Characteristics for the Qualitative Identification of an Analyte or Class of Analytes

- Limit of Detection
- Processed Sample Stability
- Interferences

3.1.2.3 Performance Characteristics for the Quantitation of a Specified Analyte or Class of Analytes

- Accuracy
- Calibration Model
- Carryover
- Ionization Suppression/Enhancement
- Limit of Detection
- Limit of Quantitation
- Precision
- Processed Sample Stability
- Interferences

3.1.3 Establishing a Validation Plan

Based on the scope, a validation plan will be recorded and technically reviewed prior to initiating the validation study. The validation plan will be generated, reviewed, and approved according to the LOM - Practices for Developing Methods and Validating Technical Procedures. The plan will include the analytical method(s), specific equipment, and sample preparation techniques(s) to be used for the chemical procedure. Further, it will record the validation requirements of the procedure, as well as the limits of the method that will allow it to be fit for use. The validation plan will provide direction for the experiments that will be performed and acceptance criteria for each performance characteristic. The validation study will include the minimum requirements as described in the LOM - Practices for Developing Methods and Validating Technical Procedures.

3.1.4 Conduct Validation Experiments

The following requirements are the minimum for assessing the listed performance characteristics. In certain instances, it may be beneficial to use more samples than indicated to achieve more statistically meaningful results. The experiments are listed alphabetically and not necessarily in procedural order.

3.1.4.1 Accuracy

Accuracy is the closeness of a measured value to the known, or "true" value and is typically reported as a percent difference. The accuracy of an analytical method can be estimated by measuring materials of known concentration or purity (e.g., reference materials) and comparing the result(s) with the true value(s). Matrix-matched reference materials are preferred for estimating accuracy. When practicable, these samples are obtained from an independent source rather than produced by the same person(s) performing the validation. The samples should be evaluated near the extremes of the calibration range but may also include a sample near the middle of the calibration curve.

At a minimum, five data sets consisting of two different concentrations or amounts analyzed in triplicate are collected over multiple days or in successive runs using a new calibration curve with each set. The accuracy is calculated as the percent difference of the grand mean at each concentration level from the respective known value as follows:

$$\text{Accuracy at Concentration}_x = \left[\frac{\text{Grand Mean of Calculated Concentration}_x - \text{Known Concentration}_x}{\text{Known Concentration}_x}\right] \times 100$$

In most instances, the preferred accuracy is ±15% or better but higher values may be unavoidable, especially near the limit of quantitation (Section 3.1.4.6). The acceptable range will depend on the matrix analyzed (e.g., biological, water, solid mixture) and the equipment employed. In any situation, the percent difference must fall within ±30%.

3.1.4.2 Calibration Model

The calibration model must be determined for quantitative methods. This is accomplished by first determining the range of analyte concentrations over which the method may be used. Within this range, there will be a relationship between the signal response and the analyte concentration in the sample. The calibration model is the mathematical model used to describe this relationship. The choice of an appropriate model is necessary for accurate and reliable quantitative results.

To establish the calibration model, analysis of matrix-matched, spiked calibrator samples is carried out. The calibrator samples should span the range of concentrations expected. At least five different non-zero concentration levels must be used to establish the calibration model. The concentration levels should be evenly spaced over the calibration range. A minimum of three replicates per concentration level must be analyzed and the combined data used to establish the calibration model.

The most often used calibration model is the least squares model for linear regression, although it should be noted that this model is only applicable when there is constant variance over the whole concentration range. When there is a significant difference between variances at the lowest and highest concentration levels, a weighted least squares model should be applied. Ultimately, the

simplest calibration model that adequately describes the concentration-response relationship should be used.

A calibration model can be visually evaluated using residual plots. These allow one to check for outliers that should be eliminated if found to be statistically significant. Residual plots also allow one to determine if the variances appear equal across the calibration range (similar degree of scatter at each level). Finally, they give an indication if the chosen model adequately fits the data (random distribution of individual points). It is emphasized that a calibration model cannot be evaluated simply via its coefficient of correlation. More appropriate alternatives are the Analysis of Variance (ANOVA) lack-of-fit test for unweighted models or checking for significance of the second order term in quadratic (second order polynomial) models.

Once the calibration model has been established, fewer calibration levels and replicates may be used for routine analysis and additional validation experiments, provided the lowest and highest calibration samples continue to be used. For example, if nine calibration samples (e.g., 1, 5, 10, 15, 20, 25, 30, 50, 75 ppm) are used to establish the calibration model, it is acceptable to use less calibration samples (e.g., 1, 10, 20, 50, 75 ppm) for routine use of the method.

3.1.4.3 Carryover

Carryover is the appearance of an analyte signal in samples after the analysis of a positive sample. Carryover will be evaluated during method development and its source investigated. This can be accomplished by running matrix blank samples immediately after a high concentration sample or calibration standard. If possible, the analytical procedure will be modified to remove any carryover. In cases when it is not possible to eliminate the carryover, the technical procedure must address how carryover will be managed (e.g., the signal in case sample must be ten times greater than the signal in a blank sample immediately preceding the case sample).

3.1.4.4 Ionization Suppression/Ionization Enhancement

The enhancement or suppression of analyte ionization resulting from the presence of co-eluting matrix components is a phenomenon commonly encountered in liquid chromatography/mass spectrometry (LC/MS). Ionization suppression/enhancement experiments may be performed during the method development phase to ensure extraction and instrumental conditions are properly optimized. It can be further evaluated during the validation phase using either of the following approaches:

3.1.4.4.1 Post-Column Infusion to Assess Ionization Suppression/Enhancement

The post-column infusion approach provides information on retention times where ionization suppression/enhancement occurs. A solution of the analyte is constantly infused with a syringe pump into the mobile phase from the column via a post-column tee-connection and a constant, baseline signal for the analyte of interest is collected in the mass spectrometer using the method parameters. A minimum of five different extracted matrix blanks are injected into the LC/MS. If

there is any considerable suppression or enhancement of the infused analyte signal at the retention time of the analyte, then modification of the chromatographic system or the sample preparation may be required to minimize the effect of ionization suppression or enhancement.

3.1.4.4.2 Post-Extraction Addition Approach to Assess Ionization Suppression/Enhancement

The post-extraction addition approach yields a quantitative estimation of ionization suppression/enhancement. Two different sets of samples are prepared, and the analyte peak areas are compared between sets to evaluate the ionization suppression/enhancement effects. The first set consists of the neat standards at both low and high concentrations run a minimum of two times each. Set two is made of a minimum of five samples extracted from different matrix sources fortified with the analyte(s) of interest after extraction (at both low and high concentrations). The average area of each set ($\overline{X}$) is used to estimate the suppression/enhancement effect at each concentration as follows:

Ionization Suppression/Enhancement (%) = [$\overline{X}$ Area Set 2 / $\overline{X}$ Area Set 1] * 100

Again, the effect of ionization suppression or enhancement should be minimized during the method development phase. In instances when it is not possible to eliminate the enhancement or suppression, the SOP must address how it will be managed.

3.1.4.5 Limit of Detection

The limit of detection (LOD) is an estimate of the lowest concentration, or smallest amount, of an analyte that can be reliably differentiated from the analyte-free matrix and/or the background noise. For methods that incorporate identification criteria (e.g., mass spectral comparison criteria), these criteria should be met in order for the sample(s) to be considered reliably differentiated. In some instances, it may not be necessary to establish the absolute LOD provided it is shown to be less than the lowest concentration required by the method. Because a procedure's LOD incorporates the instrumental performance as well as the sample matrix and inherent procedural limitations, it may be important to assess LOD over multiple days.

The LOD may be determined by one or more of the following approaches:

3.1.4.5.1 Estimating LOD for Screening Methods

Matrix-matched samples at decreasing concentrations are analyzed in duplicate to estimate the LOD for methods that screen for the presence or absence of a specified analyte or class of analytes (e.g., chemical color tests).

3.1.4.5.2 Estimating LOD Using Background Noise

The following approaches may be used for determining the LOD of methods that demonstrate equipment-related background noise.

3.1.4.5.2.1 Estimating LOD Using Reference Materials

For estimating the LOD using reference materials, two or more replicates of matrix-matched reference materials at known concentrations are analyzed. The LOD is defined as the smallest amount (or concentration) of an analyte that reproducibly yields a reliable signal greater than or equal to three times the noise level of the background signal.

3.1.4.5.2.2 Estimating LOD Using Statistics

The LOD may also be determined by statistically comparing results obtained from blank matrix samples and matrix-matched reference materials at known concentrations. At least three blank or negative samples are analyzed. The average signal ($\overline{X}$) and its standard deviation (σ or SD) for these blank samples are calculated. Likewise, matrix-matched reference materials at decreasing concentrations are analyzed in triplicate, however the triplicate signals are evaluated independently and not averaged. The LOD is considered as the lowest concentration of a reference material that consistently yields a signal greater than the average signal of the negative samples plus 3.3 times the standard deviation of the concentrations.

3.1.4.5.3 Estimating LOD Using Calibration Curves

The use of the lowest non-zero calibrator or a linear calibration curve are appropriate approaches for quantitative procedures.

3.1.4.5.3.1 Estimating LOD Using Concentration of Lowest Non-Zero Calibrator

In some instances, it may be sufficient to define the detection limit as the value of the lowest acceptable non-zero calibrator (Section 3.1.4.2). A minimum of three replicates of the lowest calibrator will be analyzed. It is acceptable to use the replicates generated to establish the calibration model.

3.1.4.5.3.2 Estimating LOD Using a Linear Calibration Curve

A linear calibration model is useful for estimating the LOD for quantitative procedures. A minimum of three calibration curves are constructed (Section 3.1.4.2). The LOD can be estimated from the standard deviation of the *y* intercept (σ_y) and the average slope (m_{avg}) as:

$$LOD = (3.3\ \sigma_y)/m_{avg}$$

3.1.4.6 Limit of Quantitation

The limit of quantitation (LOQ) is an estimate of the lowest concentration or smallest amount of an analyte that can be reliably differentiated and quantitated from analyte-free matrix. For methods that incorporate identification and/or quantitation criteria (e.g., mass spectral comparison criteria), these criteria should be met in order for the sample(s) to be considered

reliably differentiated and/or quantitated. In some instances, it may not be necessary to establish the absolute LOQ, provided it is shown to be at least that of the lowest non-zero calibrator. Because a procedure's LOQ incorporates the instrumental performance, as well as the sample matrix and inherent procedural limitations it may be important to assess LOQ over multiple days.

The LOQ may be estimated by one or more of the following approaches:

3.1.4.6.1 Estimating LOQ Using Concentration of Lowest Non-Zero Calibrator

In some instances, it may be sufficient to define the quantitation limit as the value of the lowest acceptable non-zero calibrator (Section 3.1.4.2). A minimum of three replicates of the lowest calibrator will be analyzed. It is acceptable to use the replicates generated to establish the calibration model.

3.1.4.6.2 Estimating LOQ Using Reference Materials

Triplicates of matrix-matched reference materials are analyzed, and the concentrations calculated from a calibration curve constructed over the entire working range. The lowest concentration that is capable of achieving an acceptable accuracy (Section 3.1.4.1) and precision (Section 3.1.4.7) in all three measurements is considered the LOQ.

3.1.4.7 Precision

Precision is a measure of the repeatability of a series of measurements of the same sample. It is expressed as the coefficient of variation (%CV) and two different types of precision studies will be assessed during method validation: within-run precision and intermediate precision.

Matrix-matched reference materials are preferred for estimating precision. When practical, these samples are obtained from an independent source rather than produced by the same person(s) performing the validation. At a minimum, for a quantitative assay, precision will be assessed by using triplicate determinations per concentration, at two different concentrations in the expected range (low and high) over five different days or runs.

Acceptable %CV values may range from 0% to 30%. The acceptable range will depend on the matrix analyzed and the equipment employed. For most methods, 20% or better is preferred, although ≤30% is acceptable near the LOQ.

3.1.4.7.1 Within-Run Precision Calculations

Within-run precisions may be calculated using the data from the first triplicate analyses of the sample sets as:

$$Within-run\ CV(\%) = \frac{SD\ of\ day\ 1\ samples}{mean\ calculated\ value\ of\ day\ 1\ samples} x100$$

3.1.4.7.2 Intermediate Precision Calculations

Intermediate precisions may be calculated using the combined data from the multiple analyses as:

$$Intermediate\ CV(\%) = \frac{SD\ of\ combined\ means\ for\ each\ level}{grand\ mean\ for\ each\ level} x100$$

3.1.4.7.3 One-way ANOVA Approach to Calculating Within-Run and Intermediate Precision

Both within-run and intermediate precisions may be calculated using the one-way ANOVA approach with the varied factor (run number) as the grouping variable. Using this approach, within-run precisions are calculated as:

$$Within-run\ CV(\%) = \left[\frac{\sqrt{MS_{wg}}}{grand\ mean\ for\ each\ level}\right] x100$$

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

Likewise, intermediate precisions are calculated as:

$$Intermediate\ CV = \left[\frac{\sqrt{\frac{MS_{bg}+(n-1)*MS_{wg}}{n}}}{grand\ mean\ for\ each\ level}\right] x100$$

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 when doing triplicate analyses). An example can be found in Appendix A.

3.1.4.8 Processed Sample Stability

Circumstances may arise in which samples that have undergone routine preparation cannot be immediately analyzed. It may be necessary to run the sample the following day or later. In these instances, it is important to evaluate the length of time a prepared sample can be maintained before it undergoes unacceptable changes, preventing reliable detection or quantitation.

Reference materials at low and high concentrations in appropriate matrices are processed and used for stability determinations. It is important to ensure that sufficient quantity is prepared to complete this evaluation, keeping in mind that it may be necessary to split the sample into multiple portions.

The first portion of these samples will be immediately analyzed in triplicate. The remaining portions are analyzed in triplicate at different time intervals and responses are compared. For example, samples in different autosampler vials may be analyzed every 8 hours up to 72 hours.

The average responses for analytes of interest and any internal standards are used to evaluate significant changes over the duration of the study. The analyte or internal standard will be considered as stable until average signal decreases to 80% or increases above 120% of the original average response.

3.1.4.9 Interference Studies

Interference studies are used to assess the selectivity of a method. Selectivity is the extent to which an analytical procedure is free from interferences arising from non-analytes, including matrix components which may be expected to be present. Selectivity can often be improved by modifying instrumental parameters (e.g., using a different column in chromatography or monitoring an alternate emission line in emission spectroscopy).

The use of an alternate analytical procedure for verification of analytical findings is an additional assessment of selectivity. Whenever possible, orthogonal analytical techniques will be employed to respond to different properties of a particular analyte. For example, Fourier Transform Infrared Spectroscopy (FTIR) and mass spectrometry are orthogonal to each other, while FTIR and Raman spectroscopy are complementary, but non-orthogonal.

3.1.4.9.1 Matrix Interferences

Matrix interferences are usually sample specific and will be addressed on a matrix-by-matrix basis. When applicable, analyze a minimum of 10 matrix blanks from different sources to demonstrate the absence of interferences in the matrix.

3.1.4.9.2 Other Interferences

In certain instances, it is necessary to check for possible interferences from other analytes which may be expected to be present in authentic samples. For example, a method for analyzing blood samples for cocaine must be evaluated for interferences caused by the blood matrix, but also evaluated for common drugs of abuse (e.g., opiates, cannabinoids, amphetamines). This is accomplished by analyzing a negative matrix spiked with the potential interference(s) at appropriate concentration(s). Alternatively, neat standards of potentially interfering compounds can also be injected for this evaluation.

3.1.4.9.3 Stable-Isotope Internal Standard Interferences

In methods using stable isotope-labeled analogs, the isotopically labeled compounds may contain the non-labeled compound as an impurity. 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, the peak area of the analyte peak would be overestimated, thus compromising quantitation.

Internal standard interferences are assessed by analyzing a blank sample spiked with the internal standard and monitoring the signal of the analyte(s) of interest. Likewise, a blank sample spiked

with the analyte(s) at the upper limit of the calibration range is analyzed without internal standard, to evaluate if the unlabeled analyte ions appear as isotopically labeled compound fragments.

3.2 Modification of Previously Validated Procedures

Modifications to a validated method require verification that the changes do not have an adverse effect on the method's performance. The decision regarding which performance characteristics require additional validation will be based on logical consideration of the specific parameters likely to be affected by the change(s). These changes may include, but are not limited to:

- Analytical conditions
- Equipment
- Sample processing
- Data software

For example, changes of extraction solvent or a buffer may affect linearity, selectivity, LOQ, precision, and accuracy. A change of the analytical column or mobile phase may affect linearity and selectivity. Further, consideration should be given to conducting parallel studies with known samples utilizing both a previously validated procedure and the modified procedure in order to evaluate the effects of the changes. The goal is to demonstrate the changes do not negatively impact the performance of the previously validated procedure. Any modifications to validated chemical procedures will follow the requirements of the LOM – Practices for Developing Methods and Validating Technical Procedures.

3.3 Technical Review of Validation Records

The technical review and approval of all validation records will be conducted in accordance with the LOM - Practices for Developing Methods and Validating Technical Procedures. The technical review(s) will be recorded on the *Validation of Chemical Procedures Review Form* (7-267) (Appendix B).

3.4 Efficiency with Validation

It is recognized that method validation is a time-consuming endeavor. Personnel should keep in mind that some validation experiments may be conducted concurrently. Appendices C, D, and E present examples to assist in streamlining validation experiments.

4 Records

The data generated during method validation studies must be recorded and available for audits, reviews, or inspections. These records must be easily retrievable. Further, the records must refer to the appropriate technical procedure(s).

Validation records must include a summary of the studies conducted and their results. The records will include the following:

- Scope
- Validation plan
  - Description of all the performance characteristics that have been evaluated. If any of the above required performance characteristics have not been evaluated, then the reason must be stated or justified.
- Sample preparation steps to include concentrations and matrices
- Raw data or reference to where the raw data may be found
- Results and calculations
- Conclusions
- References
- *Validation of Chemical Procedures Review Form*

It is important that the validation records contain specific details regarding the studies conducted, including:

- Personnel involved in the validation
- Specific equipment
- Dates

5 References

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Rev. #	Issue Date	History
5	06/03/19	Updated scope in section 2 to specify that personnel must be authorized to perform validations. Modified section 3.1.3 for consistency with LOM - Practices for Developing Methods and Validating Technical Procedures. Updated list of references in section 5.
6	12/21/20	Minor edits throughout for clarity. 1 - updated definition of validation for consistency with LOM Definitions. 2 – added disciplines 3.1.2 - added when appropriate 3.1.4.1 - change to concentration or purity from composition 3.1.4.3 and 4 - SOP to technical procedure 3.1.4.5.2.2 - deleted LOD equation

Approval Redacted - Signatures on File

Laboratory Director Date: 12/18/2020

Quality Manager Date: 12/18/2020

Appendix A: *Example use of ANOVA table to calculate accuracy (bias), within-run precision, and intermediate precision (Sections 3.1.4.1 and 3.1.4.7.3)*

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Appendix B: *Validation of Chemical Procedures Review Form* (7-267)

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Appendix C: *Example Flowchart of Chemical Procedure Validation Experiments*

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Appendix D: *Table of Example Experiments for Validation of Screening or Qualitative Methods*

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Appendix E: *Table of Example Experiments for Validation of Quantitative Methods*

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