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Guideline for Conducting Test Method Development, Validation, and Verification in Forensic Toxicology

Guideline for Conducting Test Method Development, Validation, and Verification in Forensic Toxicology

ASB Approved XXX 202X

ANSI Approved XXX 202X

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Foreword

This guideline is intended to provide examples to assist the user in the application of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology.* Three scenarios are presented: one for method development, one for method validation, and a third for method verification. Additionally, guidance is provided for the method of standard addition, as well as creating an efficient validation workflow.

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: *ASB Standard 036, method development, method validation, method verification, forensic toxicology*

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Guideline for Conducting Test Method Development, Validation, and Verification in Forensic Toxicology

1 Scope

This document provides examples for developing, validating, and verifying test methods in conformance with ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*.

2 Normative References

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

ANSI/ASB Standard 036, *Standard Practices for Method Validation in Forensic Toxicology,* 2nd edition, 2024[a].

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

3 Terms and Definitions

For purposes of this document, applicable terms are defined in ANSI/ASB Technical Report 208, *Forensic Toxicology: Terms and Definitions* and ANSI/ASB Standard 036, *Standard Practices for Method Validation in Forensic Toxicology,* 2nd edition, 2024.

4 Background

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 may use a non-standard test method, including one developed in-house. These options allow laboratories to maintain flexibility and adaptability in their testing approaches to meet their analytical needs.

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

Method validation is the process of performing experiments to establish objective evidence that a developed method is fit for purpose and to identify limitations.

Method verification is an assessment of an unmodified standard test method. Method verification experiments demonstrate a laboratory's ability to meet (or exceed) published parameters of a standard test method.

Revalidation is necessary when modifications are made to a previously validated method, such as adding compounds to a method's scope, adjusting the calibration range or model, or upgrading

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

instrumentation. Full revalidation is typically necessary unless an abbreviated validation is justified.

ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology,* delineates minimum requirements for selecting, developing, validating, and verifying test methods used in forensic toxicology that target specific analytes or analyte classes. This guidance document provides useful examples for implementing those requirements.

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ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology* allows multiple experiments to be conducted with the same fortified samples. Annex N provides an example to efficiently conduct method validation experiments.

5.7 Summaries

ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology* requires a summary of method validation and method verification experiments conducted and their results. Annex O provides examples of how these summaries may be prepared.

Annex A
(informative)

Examples of Plans for Method Development, Validation, and Verification

A.1 Example Method Development Plan

A laboratory plans to develop a quantitative method to analyze daridorexant (a sedative-hypnotic) in blood samples. While daridorexant reference materials are available, there are no commercially available reference materials for its metabolites; therefore, the method will only be developed for the parent drug.

The laboratory will rely on several published methods. Sample preparation will be a protein precipitation technique with analysis by LC-MS/MS. Since an isotopically labeled formulation of daridorexant is unavailable, the laboratory will evaluate another orexin receptor antagonist (suvorexant-D_6) as the internal standard.

Per Section 5.2 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a development plan in the form of a memorandum to address the questions to be answered by the test method and specific experiments that will be performed (Figure A-1).

102 **Figure A.1—Example Method Development Plan in the Form of a Memorandum**

Memorandum

To: *Toxicology Staff*
From: *Toxicology Technical Leader*
CC: *Laboratory Director*

Approval Date: *Date of Approval*
Authorization Name and Date: *Name of Authorizer / Date of Authorization*
Individuals Assigned: *Names of all individuals who will work on the development of this method*

RE: Laboratory ABC's Plan for Development of LC-MS/MS Method to Analyze Blood Samples for Daridorexant

The following plan has been approved and authorized for initiation.

- *Analytes:* Daridorexant
- *Matrix:* Blood (Antemortem and Postmortem)
- *Desired Concentration Range:* 100 to 5000 ng/mL
- *Internal Standard*: Suvorexant-D_6
- *Sample Preparation Technique:* Protein precipitation with cold acetonitrile
- *Instrumentation*: Company XYZ – LC-MS/MS
- *Customer Needs:* Ability to quantitate therapeutic and lethal concentrations of daridorexant

Method Development Phases of Work:

1) *Development and Optimization of Instrumental Parameters*: Instrumental parameters on the XYZ LC-MS/MS system will be determined and optimized by analyzing a purchased certified reference material of daridorexant. Using typical temperatures and gas flows, appropriate voltages and cycle/dwell times will be established. Initial evaluation of MS and MS/MS diagnostic ions will be determined with direct infusion into the system using electrospray ionization with ions selected based on published references. Once the MRM transitions are optimized, the chromatographic parameters will be optimized utilizing mobile phase composition/columns currently used for similar methods in the laboratory. Typical injection volumes (1 and 2 microliters) and temperature (4° C) will be evaluated on the system's autosampler.

2) *Defining Observations, Data Processing, and Calculations*: Using the daridorexant CRM, chromatography will be evaluated over at least 10 runs to ensure standard requirements can be met (gaussian peak shape, retention times within 0.1 min of the mean of the 10 runs, signal-to-noise of at least 10). A minimum of two MRM transitions for both the analyte and internal standard will be determined, with ion ratios meeting the established requirements of ANSI/ASB Std 098. Peak smoothing parameters will be evaluated to determine appropriate settings. A preliminary calibration model will be evaluated using diluted reference materials. A 5-point calibration curve will be used based on peak area ratios to the internal standard for quantitation. Bias and precision acceptance criteria will be established to follow ANSI/ASB Std 036. The number of identification points (per ANSI/ASB Std 113) equals 5 points [1 (chromatography) + 2 for each low-resolution precursor product ion transition (x2)], exceeding the minimum requirement of 4 points for identification. Data presentation templates will be developed.

3) *Development and Optimization of Sample Preparation Steps*: A protein precipitation with cold acetonitrile will be used. Different sample volumes of blank blood fortified with various concentrations of daridorexant will be evaluated. Reconstitution solvent and volumes will be evaluated. Ionization suppression and enhancement will be evaluated using the Post-Column Infusion technique with a minimum of three unique blank blood sources, daridorexant solutions at low and high concentrations, and the internal standard. Should ionization suppression and enhancement appear significant for the analyte or internal standard, modifications to the chromatographic system will be made before reassessment, or an additional assessment will be performed using the Post-Extraction Addition technique. If this continues to indicate a concern with ionization suppression or enhancement, an alternative sample preparation technique will be explored. Recovery will be evaluated as defined in ANSI/ASB Std 036. The calibration range will be evaluated over three runs using processed blood samples fortified with daridorexant at varying concentrations. Processed sample stability experiments will not be conducted as part of method development, as these experiments will be included in the validation plan.

Metrological Traceability: As this will be a quantitative test method, metrological traceability will be established with the CRM and calibrated equipment used to prepare the calibration samples.

A.2 Example Validation Plan

A laboratory has recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method uses isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis is performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). In some instances (e.g., quantitative failures of QCs), the laboratory will allow for results to be reported qualitatively.

Per Section 6.4 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a validation plan in the form of a table that details the sample preparation steps, instrumental parameters, validation experiments to be conducted (based on the method's scope), and the acceptance requirements for each experiment to demonstrate the method is fit-for-purpose (Table A-1).

Table A.1—Sample Validation Plan for a Laboratory-Developed Test Method

Validation Plan for ABC Laboratory		
Method: Fentanyl and norfentanyl in antemortem blood using solvent extraction and LC-MS/MS *NOTE: Printouts of the draft sample extraction procedure and instrumental parameters that will be used are attached.*[b]		
Parameter:	***Minimum Number of Samples:***	***Acceptance Requirements:***
Interferences	*Matrix Interferences:* — No less than ten (10) sources of blank antemortem blood *Internal Standard Interferences:* — One blank antemortem blood sample fortified with fentanyl-D_5 and norfentanyl-D_5 — One blank antemortem blood sample fortified with fentanyl and norfentanyl *Interferences from Common Analytes:* — Common recreational drugs of abuse and metabolites — Common prescription medications and metabolites — Common OTC drugs and metabolites	Interfering signals that will impact detection (e.g., retention time, peak shape, mass spectrometry ratios) and quantitation (>20% of the area of the lowest calibrator) must be addressed through laboratory quality assurance practices.

[b] For the purposes of this example, the extraction procedure and instrumental parameters are not attached to this validation plan.

Parameter:	***Minimum Number of Samples:***	***Acceptance Requirements:***
Calibration Model	At least six non-zero fentanyl and norfentanyl calibrators – between (and including) 1 and 200 ng/mL – prepared in blank antemortem blood over five runs. Fresh calibrators will be prepared daily using a different source of blank antemortem blood.	The calibration range must be at least 1 – 200 ng/mL for fentanyl and norfentanyl. A linear model is desired but not required. The appropriate calibration model will be determined through the F-test and weighted residual plots
Ionization Suppression/ Enhancement	Post-extraction addition approach will be used. At least 10 unique blank antemortem blood sources will be used to evaluate at two concentrations: 3 ng/mL and 160 ng/mL for fentanyl and norfentanyl. The internal standards will also be evaluated at 50 ng/mL.	Significant suppression or enhancement will be considered an average instrumental response that drops to less than 75%, increases to more than 125%, or has a % CV exceeding 20%. If significant suppression/enhancement occurs, the impact on LOD, LLOQ, bias, and precision will be assessed by at least tripling the number of unique sources of blank matrices used for their evaluation.
Bias	At least one source of blank antemortem blood will be used to prepare the following concentration pools: low (3 ng/mL), medium (90 ng/mL), and High (180 ng/mL). Each concentration pool will be analyzed in triplicate daily over at least five days.	Must be ± 20% or less for fentanyl and norfentanyl
Precision (within-run and between-run)		% CV must not exceed 20% for fentanyl and norfentanyl.
Dilution Integrity	Samples from the high-concentration pool (180 ng/mL) prepared from at least one of the blank sources of antemortem blood will be diluted at two different ratios (1:2 and 1:10) and analyzed.	Samples prepared with each dilution ratio must meet the above bias and within-run precision requirements for the dilution ratio to be considered acceptable for use.
Carryover	An extracted blank antemortem blood sample will be analyzed immediately following the highest extracted fentanyl and norfentanyl calibrator (200 ng/mL). This will be repeated daily for five days.	For both fentanyl and norfentanyl, any carryover observed after the highest calibrator (200 ng/mL) cannot exceed 10% of the signal (relative peak area) of the lowest calibrator (1 ng/mL) and have all detection criteria met (e.g., retention time, peak shape, mass spectrometry ratios). If carryover is observed, quality assurance practices will be implemented to mitigate.
Limit of Detection	The lowest non-zero calibrator (at least 1 ng/mL) will be assigned as the LOD. At least three unique sources of blank antemortem blood will be used to prepare different samples fortified at the same lowest non-zero concentration. Each calibrator sample (n=3) will be analyzed over three different runs (n=9).	All detection and identification criteria must be met in at least 95% of the replicates.

Parameter:	***Minimum Number of Samples:***	***Acceptance Requirements:***
Lower Limit of Quantitation	The lowest non-zero calibrator (at least 1 ng/mL) will be assigned as the LLOQ. At least three unique sources of blank antemortem blood will be used to prepare different samples fortified at the same lowest non-zero concentration.(n=3) will be analyzed over three different runs (n=9).	Bias (±20%) and precision (≤ 20%) requirements must be met.
Processed Sample Stability	12 different aliquots of the 3 ng/mL low-concentration pool (prepared from at least one of the blank sources of antemortem blood) used for the bias and precision studies will be freshly extracted. The extracts will be combined, mixed, and divided into 12 autosampler vials. The first vial will be immediately analyzed in triplicate. The second vial will be analyzed in triplicate after 6 hours at autosampler temperature (4°C). The third vial after 12 hours, etc., for up to 66 hours. The same experiment will be conducted with the high-concentration pool (180 ng/mL).	Relative peak areas of extracted samples of fentanyl and norfentanyl stored on the autosampler must remain stable (remain within ±20%) when compared to time zero for 12 hours or more.
Rates of False Positives and False Negatives	At least 10 unique sources of blank antemortem blood will be used. Each source of antemortem blood will be divided into two subsamples. The first subsample ("negatives") from each antemortem blood source will be extracted and analyzed 6 times each (n=60 or more). The second subsample ("positives") from each antemortem blood source will be fortified with fentanyl and norfentanyl at 1.5 ng/mL. Each fortified antemortem blood subsample will be extracted and analyzed 6 times each (n=60 or more). Rates of false results will be based on Table K-1 of ASB 036.	Assess False Negative and False Positive Rates at a 95% confidence level with a minimum of 60 data points. The rate will not exceed 10% for the method to be considered acceptable.
The above validation plan is approved for use:	*Name and signature of approver*	*Date of approval*
The following individuals are authorized to initiate the above validation plan:	*Names of all authorized individuals* *Name and signature of authorizer*	*Date of authorization*

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A.3 Example Verification Plan

A laboratory will implement an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual-column gas chromatograph with flame ionization detectors. The method relies on the use of an n-propanol internal standard. The standard test method declares that it can meet the following validation parameters.

- *Interferences:* No interferences from antemortem blood or other common volatile compounds.
- *Calibration Range and Model:* The standard test method uses a curve using six calibrators between 10 mg/dL and 400 mg/dL; however, the calibration model is not defined.
- *Limit of Detection:* 5 mg/dL.
- *Lower Limit of Quantitation:* 10 mg/dL.
- *Bias:* ± 10% or less.
- *Precision (within-run):* <10%.
- *Precision (between-run):* <10%.
- *Carryover:* None observed at 400 mg/dL.
- *Processed Sample Stability:* Stable for up to 72 hours after preparation.
- *False Positive Rates (if used qualitatively):* Not greater than 5% at a 99% confidence level.
- *False Negative Rates (if used qualitatively):* Not greater than 5% at a 99% confidence level.

Per Section 7.5 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a verification plan in the form of a bulleted list that summarizes the experiments they will conduct (based on the method's scope) and the acceptance requirements for each experiment to verify their ability to use the standard test method within the defined parameters (Figure A-2).

Figure A.2—Sample Verification Plan for a Standard Test Method Used Without Modification

Verification Plan for Laboratory ABC

Method: Standard Test Method 123-25 for Postmortem Blood

The following will be verified for this standard test method, as described:

- Calibration Model: The following calibrators will be used: 10 mg/dL; 50 mg/dL; 100 mg/dL; 200 mg/dL; 300 mg/dL; and 400 mg/dL. The matrix of the calibrators will be aqueous, as within the standard test method. A separate calibration curve will be prepared once per day over at least five days to establish the appropriate calibration model.

- Limit of Detection: Blank postmortem blood samples from five unique sources will be fortified with ethanol at 5 mg/dL. Each 5 mg/dL postmortem blood sample will be analyzed in triplicate over three runs (a total of 45 analyses). The results for each sample will be evaluated to determine if at least 43 of the 45 analyses (≥95%) achieve the appropriate detection criteria (e.g., retention time, peak shape, signal-to-noise).

- Lower Limit of Quantitation: Blank postmortem blood samples from five unique sources will be fortified with ethanol at 10 mg/dL. Each 10 mg/dL postmortem blood sample will be analyzed in triplicate over three runs (a total of 45 analyses). The results for each sample will be evaluated to determine if the appropriate bias (± 10% or less) and precision (%CV ≤10%) are met.

- Bias and Precision: Three concentration pools will be prepared, each with blank postmortem blood: Low Pool (25 mg/dL); Medium Pool (150 mg/dL); and High Pool (350 mg/dL). Each concentration pool will be analyzed five times per run for three runs (a total of 15 analyses for each concentration pool). The results will be used to verify that the calculated bias for each concentration pool is no more than ±10% for all samples analyzed, as defined in the standard test method. The results will also verify that the calculated within-run and between-run precision for each concentration pool is no more than 10% for all samples analyzed, per the standard test method.

- Carryover: Three blank postmortem blood samples (from the same source) will be prepared. Each will be analyzed sequentially following a high calibrator sample (400 mg/dL). This will be repeated over two additional runs. The results from the blank matrix will be evaluated to determine if any carryover occurs that meets the detection criteria for ethanol (proper retention time, appropriate peak shape, appropriate signal-to-noise).

- False Positive and False Negative Rates: A blank postmortem blood sample will be fortified with ethanol at 5 mg/dL (the standard method's LOD) and analyzed three times to establish an average response ratio to the internal standard. Then, fifteen additional unique sources of blank postmortem blood will be divided into two subsamples. The first subset of each unique blank postmortem blood source will be fortified with ethanol at 3 mg/dL and analyzed six times each and serve as the "negative" samples (total of 90 "negative" analyses). The second subset of each unique blank blood source will be fortified with ethanol at 7 mg/dL and analyzed six times each and serve as the "positive" samples (total of 90 "positive" analyses). The results will be used to calculate false positive and false negative rates.

I, _____, approve of the use of the above method verification plan.

The following individuals are authorized to participate in this method verification:
a. [NAME #1]
b. [NAME #2]
c. [NAME #3]

A.4 Example Revalidation Plan

A laboratory would like to add two new designer benzodiazepines – bromazolam and etizolam – to its existing, validated quantitative benzodiazepine assay. The method uses isotopically-labeled internal standards for each benzodiazepine, so bromazolam-D_5 and etizolam-D_3 will also be added to the test method. Samples are prepared by solid-phase extraction in the current procedure, and analysis is performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Sections 8.4 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, a full revalidation of the method is required unless the laboratory can logically deduce that some of the validation parameters are not significantly impacted with the introduction of additional analytes. Following Section 6.4 of the standard, the laboratory creates a validation plan in the form of a memorandum that details the sample preparation steps, instrumental parameters, validation experiments to be conducted (based on the method's scope), and the acceptance requirements for each. The plan will also detail the validation experiments that will not be performed and the rationale for their omission (Figure A-3).

159 **Figure A.3—Sample Revalidation Plan**

Memorandum

To: *Toxicology Staff*
From: *Toxicology Technical Leader*
CC: *Laboratory Director*

Approval Date: *Date of Approval*
Individuals Assigned: *Names of all individuals who will work on the revalidation of this method*

RE: Laboratory ABC's Revalidation Plan for Addition of Bromazolam and Etizolam to SOP 203-4: Benzodiazepines in Blood and Urine by LC-MS/MS

The following plan has been approved for initiation.

Evaluate the impact of adding bromazolam, etizolam, and additional internal standards to the existing SOP 203-4 for both blood and urine matrices. This is a quantitative test method for blood and qualitative for urine. No changes are planned for the solid-phase extraction steps and instrumental analysis parameters.

Method Validation Experiments:

Interferences:

Matrix Interferences: Follow ANSI/ASB Std 036 for all benzodiazepines in the method, including bromazolam, etizolam, and their respective internal standards. Both blood and urine matrices will be evaluated.

Internal Standard Interferences: Follow ANSI/ASB Std 036 for bromazolam, etizolam, and their respective internal standards. As these experiments were already conducted for all other benzodiazepines and associated internal standards, the historical validation materials will be used in lieu of repeating those experiments (sufficient chromatographic separation with the existing analytes has been demonstrated).

Interferences from Common Analytes: Per ANSI/ASB Std 036, injection standards of common recreational drugs of abuse, prescription medications, OTC drugs, and metabolites will be analyzed for an interfering signal impacting the detection of bromazolam, etizolam, or their respective internal standards. Potential interference from common analytes was evaluated during the original validation but will be re-evaluated for all of the method's benzodiazepines and metabolites during the current experiment.

There should be no interfering signal from or with the matrix samples, internal standards, or common drugs of abuse, OTC drugs, and prescription medications that will prevent all required detection criteria (e.g., retention time, peak shape, mass spectrometry ratios) from being met for bromazolam or etizolam or their internal standards, as well as the method's existing benzodiazepines and metabolites. If an interference is detected, laboratory reporting procedures must address how it will be mitigated.

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Calibration Model: Per ANSI/ASB Std 036, at least six non-zero bromazolam and etizolam calibrators will be prepared in blank blood within the range of 20 to 500 ng/mL and analyzed for five days. The simplest calibration model will be used, as determined through the F-test.

The calibration models for all other benzodiazepines used in this method do not require revalidation. The existing historical data from the initial validation, as well as the data from the five random runs will be included in the validation material to support the use of their current calibration models.

Ionization Suppression and Enhancement: The impact of ionization suppression and enhancement will be evaluated for both blood and urine matrices using the post-extraction addition approach of ANSI/ASB Std 036. Low concentrations of bromazolam and etizolam will be at 50 ng/mL and high concentrations will be at 400 ng/mL. Their respective internal standards will also be evaluated. Peak areas from the sets will be compared and evaluated per ANSI/ASB Std 036. If these requirements are exceeded, additional blank matrices will be included in the LOD, LLOQ, bias, and precision studies, per ANSI/ASB Std 036. Historical validation data will be used for all other benzodiazepines, in lieu of repeating those experiments.

Bias and Precision: ANSI/ASB Std 036 will be followed to assess bias and precision for bromazolam and etizolam at 50 ng/mL, 200 ng/mL, and 400 ng/mL in fortified blank blood. Bias cannot exceed ±20%. Within-run and between-run precisions cannot exceed 20%. Previously determined bias and precision data will be used in lieu of repeating these studies for the other benzodiazepines.

Dilution Integrity: This will be evaluated only for bromazolam and etizolam in blood following ANSI/ASB Std 036. Existing data for other benzodiazepines will be used, instead of repeating those experiments.

Carryover: This will be limited to only bromazolam and etizolam in both blood and urine, as earlier validation work demonstrated no carryover for the other benzodiazepines. ANSI/ASB Std 036 will be followed.

Limit of Detection: The LOD for bromazolam and etizolam will be estimated in both blood and urine. For blood, the LOD will be estimated following the ANSI/ASB Std 036 Annex G.1.3.5.3 – Using a Linear Calibration Curve approach. For urine, the LOD will be estimated using the Reference Materials approach described in ANSI/ASB Std 036 Annex G.1.3.5.1. All other benzodiazepines will rely on their previously estimated LODs, and experiments will not be repeated.

Lower Limit of Quantitation: The LLOQ for bromazolam and etizolam will be determined in blood. This will be done using the Lowest Non-Zero Calibrator approach described in ANSI/ASB Std 036 Annex H.1.3.1. This will not be repeated for all other benzodiazepines.

Processed Sample Stability: ANSI/ASB Std 036 will be followed to determine the stability of bromazolam and etizolam in extracted blood and urine samples for up to 96 hours at 12-hour intervals. This will not be repeated for all other benzodiazepines. Bromazolam and etizolam must remain stable (remain within ±20%) for at least 12 hours.

Rates of False Positives and False Negatives: ANSI/ASB Std 036 will be followed to determine the false positive and false negative rates for both blood and urine matrices from bromazolam and etizolam. Assessment will be made at 95% confidence levels or higher.

A.5 Example Validation Plan for Method of Standard Addition

A laboratory needs to quantify phenazepam in a submitted postmortem blood specimen but does not have a validated method for this analyte. Since they anticipate that this request will be rare, the laboratory will not perform a full quantitative method validation using a traditional external calibration curve. Instead, the laboratory will use the method of standard addition (MSA).

NOTE 1 MSA is a non-standard method where all validation and sample analysis may be encapsulated within a single analytical run. It is uniquely suited for situations such as:

— a laboratory anticipates that quantification of the analyte will be a rare occurrence (e.g., only one case or a minimal number of cases over time);

— blank matrix is difficult to acquire, is especially complex, and/or generates a high level of matrix effects, complicating or preventing accurate quantification by a traditional external calibration curve approach.

NOTE 2 In MSA, a portion of the test specimen is divided into multiple aliquots of the same volume to perform internal quantitation. One aliquot of the test specimen remains unfortified (i.e., "zero level"), while the others are fortified with a known and increasing quantity of reference material. A regression is performed on the resulting signals (or peak area ratios) plotted against the fortified concentrations. The concentration of the analyte in the unfortified aliquot is calculated as the absolute value of the x-intercept.

Some specific aspects of MSA justify modifications to the traditional validation requirements of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*. First, matrix effects are inherently compensated for by their equal presence in all samples. This includes matrix interferences on the analyte and internal standard, as well as ionization suppression and enhancement. Second, encapsulating all samples in a single batch means that any validation parameter targeting the method's behavior over time (e.g., between-run precision) is moot. Therefore, no specimen-specific validation aspect (e.g., calibration model) is transferable to a future MSA analysis performed on a different sample. Finally, LOD and LLOQ are inherently established through the test specimen analyzed by MSA.

The laboratory's benzodiazepines extraction method (protein precipitation) and LC-MS/MS chromatography will be used for phenazepam. Phenazepam-D_4 will be used as the internal standard. Transitions for the analyte and the internal standard will be determined during method development using a reference material in solution.

Per Section 6.4 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a validation plan that details the sample preparation steps, instrumental parameters, validation experiments they will conduct (based on the method's scope), and the acceptance requirements for each experiment so that the method can be considered fit-for-purpose (Table A.2). A validation plan will be developed for each iteration of MSA use within the laboratory; however, an abbreviated plan may be employed to reduce the experiments performed when they are not dependent on the test specimen (e.g., interferences).

200 **Table A.2—Sample Validation Plan for a Laboratory-Developed Test Method Using Standard**
201 **Addition**

Validation Plan for ABC Laboratory		
Method: Phenazepam in postmortem blood by protein precipitation and LC-MS/MS NOTE Printouts of the draft sample extraction procedure and instrumental parameters that will be used are included.		
Parameter:	***Minimum Number of Samples:***	***Acceptance Requirements:***
Interferences	*Matrix Interferences:* — N/A, corrected by using MSA *Internal Standard Interferences:* — One blank postmortem blood sample fortified with phenazepam-D_4 — One blank postmortem blood sample fortified with phenazepam *Interferences from Common Analytes:* — Common recreational drugs of abuse and metabolites — Common prescription medications and metabolites — Common OTC drugs and metabolites	Interfering signals that will impact detection (e.g., retention time, peak shape, mass spectrometry ratios) and quantitation must be addressed through laboratory procedures.
Calibration Model	The test specimen will be divided into multiple aliquots and fortified with 5, 10, 25, 40, 50 ng/mL. One aliquot of the test specimen will remain unfortified. Each extracted calibrator will be injected in triplicate. A regression will be performed on the resulting peak area ratios plotted against the fortified concentrations. The concentration of the analyte in the unfortified aliquot will be calculated as the absolute value of the x-intercept.	The calibration model will be assessed using a linear regression model.
Bias	An aliquot of the test specimen will be fortified at 15 ng/mL, extracted, and analyzed in triplicate.	Must be ± 20% or less for phenazepam
Precision		% CV must not exceed 20% for phenazepam NOTE: Given that all analyses will occur in a single run, between-run precision will not be calculated.
Dilution Integrity	N/A	N/A
Carryover	An extracted blank postmortem blood sample will be analyzed immediately following the extracted authentic aliquot with the highest fortified phenazepam concentration (50 ng/mL).	If phenazepam is detected in the blank postmortem blood sample, the signal cannot exceed 10% of that of the unfortified authentic aliquot. If carryover is observed, quality assurance practices will be implemented to mitigate.

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Parameter:	***Minimum Number of Samples:***	***Acceptance Requirements:***
Limit of Detection and Lower Limit of Quantitation	N/A – LOD and LLOQ are inherently met when using MSA	N/A
Processed Sample Stability	N/A – all samples will be analyzed within 24 hours of preparation	N/A
The above validation plan is approved for use:	*Name and signature of approver*	*Date of approval*
The following individuals are authorized to initiate the above validation plan:	*Names of all authorized individuals* *Name and signature of authorizer*	*Date of authorization*

203
204

Annex B
(informative)

Examples of Bias Assessment

B.1 Example of Bias Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section A.2 of Annex A in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated bias during method validation of the non-standard test method. The data acquired for the evaluation of bias was also utilized for the evaluation of within-run and between-run precision. The laboratory assessed bias using fortified antemortem blood concentration pools at low, medium, and high concentrations. Given the ionization suppression that was noted within the method, the number of unique antemortem blood sources was increased by the laboratory to perform bias experiments. Thus, each concentration was assessed with three (3) unique sources of blank antemortem blood. The calibration range for the method was between 1 and 200 ng/mL. Therefore, the fortified antemortem blood concentration pools were prepared at 3 ng/mL, 90 ng/mL, and 180 ng/mL.

Each fortified antemortem blood concentration pool (a total of three for each concentration) was extracted in triplicate alongside an extracted calibration curve. This experiment was repeated over five days using independently calibrated analytical runs. The data obtained from the analysis of the three concentration pools for fentanyl and norfentanyl are shown in Table B.1.

229 **Table B.1—Results of Fentanyl and Norfentanyl for Bias**

Concentration Pool	Source	Replicate	Analyte	Run 1	Run 2	Run 3	Run 4	Run 5
Low (3 ng/mL)	1	A	Fentanyl	2.9	2.8	2.8	3.0	3.1
			Norfentanyl	3.1	3.0	2.8	2.9	3.0
		B	Fentanyl	2.8	2.9	2.7	2.8	2.8
			Norfentanyl	2.8	2.8	3.0	2.8	2.9
		C	Fentanyl	3.0	2.8	2.7	2.7	2.7
			Norfentanyl	2.7	2.6	2.6	2.8	2.8
	2	A	Fentanyl	3.1	3.2	3.0	3.1	3.2
			Norfentanyl	2.9	3.3	3.3	3.1	3.2
		B	Fentanyl	3.1	3.0	3.1	2.9	3.3
			Norfentanyl	3.1	2.7	3.4	2.5	3.3
		C	Fentanyl	3.2	3.2	3.1	3.0	3.2
			Norfentanyl	3.4	3.4	3.5	3.3	3.2
	3	A	Fentanyl	3.2	2.9	2.7	2.9	2.4
			Norfentanyl	3.1	2.9	2.9	3.1	2.8
		B	Fentanyl	3.0	3.0	2.8	2.7	2.9
			Norfentanyl	3.0	3.0	2.8	2.9	2.9
		C	Fentanyl	2.7	2.8	2.8	2.6	2.8
			Norfentanyl	2.9	3.1	3.0	3.0	2.8
Medium (90 ng/mL)	1	A	Fentanyl	92	100	95	93	105
			Norfentanyl	79	82	90	86	88
		B	Fentanyl	98	102	97	102	91
			Norfentanyl	82	90	89	92	92
		C	Fentanyl	87	97	90	96	94
			Norfentanyl	88	89	92	85	85
	2	A	Fentanyl	98	88	95	95	97
			Norfentanyl	98	90	99	92	95
		B	Fentanyl	100	82	90	88	90
			Norfentanyl	92	95	97	95	88
		C	Fentanyl	102	89	82	94	98
			Norfentanyl	95	87	98	94	82
	3	A	Fentanyl	79	87	84	90	88
			Norfentanyl	92	98	95	98	92
		B	Fentanyl	95	99	94	97	93
			Norfentanyl	95	99	94	97	93
		C	Fentanyl	80	82	80	85	85
			Norfentanyl	91	92	98	97	95
High (180 ng/mL)	1	A	Fentanyl	150	149	182	195	192
			Norfentanyl	155	147	180	198	199
		B	Fentanyl	185	152	175	187	199
			Norfentanyl	175	154	176	185	193
		C	Fentanyl	168	169	174	188	201
			Norfentanyl	168	162	175	192	195
	2	A	Fentanyl	149	159	145	150	146
			Norfentanyl	193	192	213	146	200
		B	Fentanyl	162	146	156	158	152
			Norfentanyl	197	201	206	152	201
		C	Fentanyl	156	152	150	151	149
			Norfentanyl	205	196	197	155	198
	3	A	Fentanyl	192	180	200	198	192
			Norfentanyl	205	199	192	205	207
		B	Fentanyl	198	192	205	184	180
			Norfentanyl	210	201	200	213	207
		C	Fentanyl	200	194	197	188	197
			Norfentanyl	203	205	195	200	208

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The laboratory calculated the bias by first determining the grand mean for each concentration pool for each individual antemortem blood source. The calculated means for fentanyl and norfentanyl in each concentration pool (3 ng/mL, 90 ng/mL, and 180 ng/mL) are shown in Table B.2.

Table B.2—Grand Mean Concentrations per Source for Bias Calculations

Source	Analyte	Calculated Grand Mean		
		Low Pools (3 ng/mL)	Medium Pools (90 ng/mL)	High Pools (180 ng/mL)
1	Fentanyl	2.83	96.0	178
	Norfentanyl	2.84	87.3	177
2	Fentanyl	3.11	92.5	152
	Norfentanyl	3.17	93.1	190
3	Fentanyl	2.81	87.9	193
	Norfentanyl	2.95	95.1	203

The data were analyzed by calculating the bias for each source's concentration pool using the following formula:

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

The bias for Source 1 Low Concentration Pool (3 ng/mL) was calculated for fentanyl.

$$\text{Bias (\%) 3 ng/mL} = \left[\frac{2.83 - 3.00}{3.00}\right] \times 100$$

$$\text{Bias (\%) 3 ng/mL} = -5.7\%$$

The bias for Source 1 Low Concentration Pool (3 ng/mL) was calculated for norfentanyl.

$$\text{Bias (\%) 3 ng/mL} = \left[\frac{2.80 - 3.00}{3.00}\right] \times 100$$

$$\text{Bias (\%) 3 ng/mL} = -6.7\%$$

The bias for each source's concentration pool is shown in Table B.3.

Table B.3—Bias of Fentanyl and Norfentanyl

Source	Analyte	Calculated Bias		
		Low Pools (3 ng/mL)	Medium Pools (90 ng/mL)	High Pools (180 ng/mL)
1	Fentanyl	-5.7%	6.7%	-1.1%
	Norfentanyl	-5.3%	-3.0%	-1.7%
2	Fentanyl	3.7%	2.8%	-15.6%
	Norfentanyl	5.7%	3.4%	5.6%
3	Fentanyl	-6.3%	-2.3%	7.2%
	Norfentanyl	-1.7%	5.7%	12.7%

The acceptance criteria for bias within the validation plan for fentanyl and norfentanyl were defined as ±20% (or less) for each concentration pool. The bias did not exceed ±20% for the concentrations evaluated from each unique source of antemortem blood.

B.2 Example of Bias Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual column gas chromatograph with flame ionization detectors.

Per Section A.3 of Annex A in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory verified their bias using the standard test method. The standard test method lists a bias of within ±10%. Therefore, the laboratory had to demonstrate their ability to at least meet this bias value when using the method for postmortem blood. The data acquired for the evaluation of bias were also utilized for the evaluation of within-run and between-run precision.

The laboratory assessed bias using fortified postmortem blood concentration pools at low, medium, and high concentrations. The calibration range for the standard test method was between 10 mg/dL and 400 mg/dL. Therefore, the fortified postmortem blood concentration pools were prepared at 25 mg/dL, 150 mg/dL, and 350 mg/dL.

Each fortified postmortem blood concentration pool was extracted with five replicates alongside an extracted calibration curve. This experiment was repeated over three independently calibrated analytical runs. The data obtained from the analysis of the low, medium, and high concentration pools for ethanol is shown in Table B.4. Table B.4 also shows the calculated mean for each concentration pool.

The bias was calculated for each concentration using the following formula:

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

For example, the bias for the low concentration pool (25 mg/dL) was calculated for ethanol as:

$$\text{Bias (\%) 25 mg/dL} = \left[\frac{23.6 - 25}{25}\right] \times 100$$

$$\text{Bias (\%) 3 ng/mL} = \text{-5.6\%}$$

The bias results for each concentration pool are shown in Table B.4.

275 **Table B.4—Ethanol Bias Results**

Concentration Pool	Replicate	Run 1 (mg/dL)	Run 2	Run 3	Mean Result	Bias (%)
Low (25 mg/dL)	A	22.4	23.6	22.7	23.6	-5.6
	B	23.5	23.8	24.2		
	C	23.4	24.4	23.8		
	D	24.8	23.5	22.9		
	E	22.6	24.8	23.6		
Medium (150 mg/dL)	A	153	155	148	155	3.3
	B	155	160	151		
	C	150	163	153		
	D	151	162	152		
	E	153	160	152		
High (350 mg/dL)	A	332	320	375	344	-1.7
	B	346	320	367		
	C	342	320	368		
	D	350	322	350		
	E	352	323	370		

276 The standard test method denotes a ±10% bias within the standard test method. The bias obtained
277 by the laboratory met the predefined criteria.

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Annex C
(informative)

Examples of Calibration Model Assessment

C.1 Example of Calibration Model Assessment for Method Development

C.1.1 General

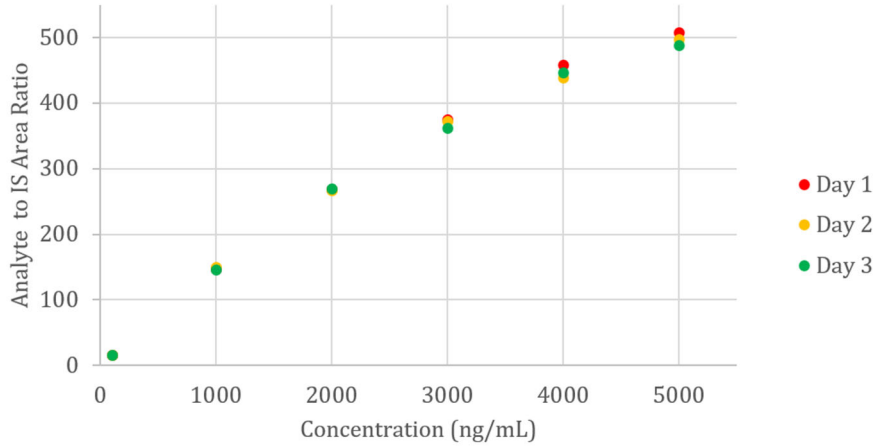
A laboratory developed a quantitative method to analyze daridorexant in blood samples. A protein precipitation technique was used for sample clean-up and extracts were analyzed by LC-MS/MS. Another orexin receptor antagonist, suvorexant-D_6, served as the internal standard.

Per Sections B.1 and B.2 of Annex B in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory performed a preliminary assessment of the calibration model. Six non-zero calibrators spanning the desired concentration range of 100 to 5000 ng/mL were used. Calibrator samples were extracted and analyzed in three separate runs spanning an interval of ten days. The data obtained were reported in Table C-1 and displayed as a plot in Figure C.1.

Table C.1—Daridorexant Calibration Model Assessment Data

Concentration (ng/mL)	Peak Area Ratio		
	Day 1	Day 2	Day 3
100	16.3	15.6	15.6
1000	146.9	150.3	146.5
2000	267.9	267.0	269.9
3000	375.6	372.9	361.9
4000	458.1	439.7	447.5
5000	508.3	497.9	489.0

Figure C.1—Daridorexant Peak Area Ratio as a Function of Concentration



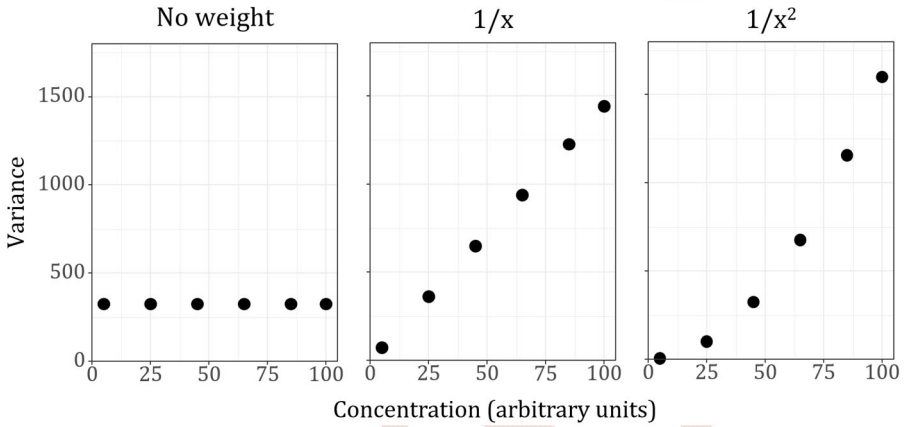
Section B.1 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology* presents several options for assessing the weighting and linearity of the calibration. For the preliminary determination of the calibration model, the

laboratory chose to use the simpler, graphical techniques. The data were analyzed using Microsoft Excel.

C.1.2 Weighting

A variance plot was used to evaluate the presence of heteroscedasticity. For each calibration level, variance of the peak area ratios was calculated. Variance was then plotted against the concentration. A few common patterns were observed in these variance plots (Figure C-2), each pointing towards the adequate weighting factor to use in the calibration model.

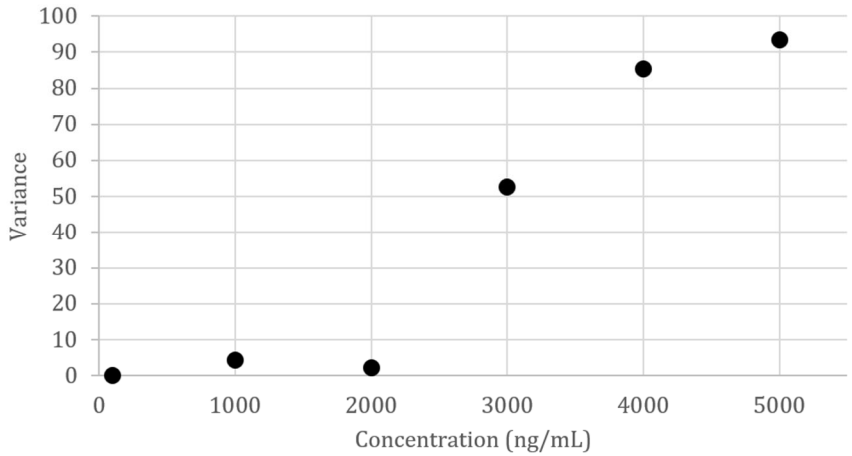
Figure C.2—Common Patterns Observed in Variance Plots



Unweighted calibration (i.e., weight of 1) is appropriate when the variance plot displays no increase of variance with concentration (i.e., when the data is homoscedastic). A 1/x weighting is appropriate when the variance plot displays a linear increase of variance with concentration. A $1/x^2$ weighting is appropriate when the variance plot displays a parabolic increase of variance with concentration.

The variance plot obtained for daridorexant is shown in Figure C.3.

Figure C.3—Variance of Daridorexant Peak Area Ratios as a Function of Concentration



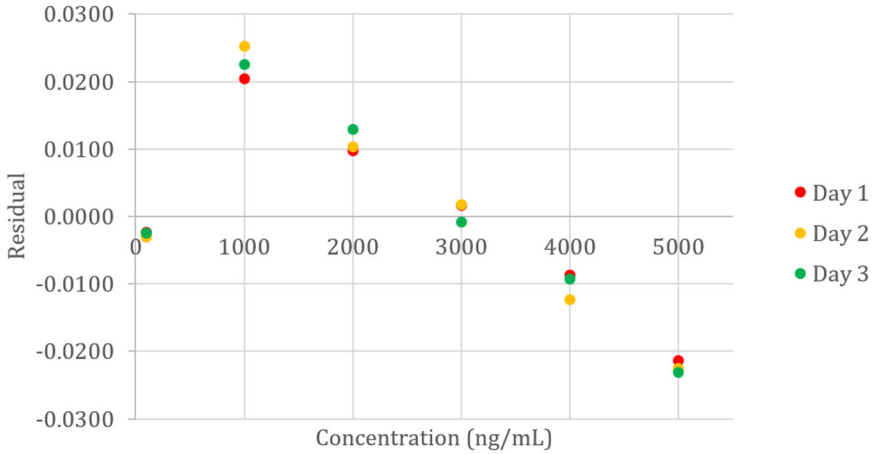
The variance increased across the concentration range: the data were therefore heteroscedastic and a weighted regression was used. The data obtained were ambiguous with regards to weighting factor, likely due to the imprecision of the standard deviation estimated from only three measurements. Nonetheless, a $1/x^2$ weighting was selected by the laboratory. This weighting scheme is more likely on LC-MS/MS instrumentation, and the variance graph fit a parabolic increase slightly better than a linear increase.

C.1.3 Linearity

The presence of non-linearity was evaluated via a weighted residuals plot. A linear, $1/x^2$ calibration model was used to fit each of the 3 calibration curves collected. Using the regression parameters obtained, weighted residuals were calculated. Weighted residuals were then plotted against the standards' concentrations, as shown in Figure C.4.

NOTE Full equations and stepwise results are not within the scope of this document and can be found in the referenced literature. Nonetheless, an Excel workbook accompanies this Guideline including a calculation template and example for each statistical evaluation.

Figure C.4—Weighted Residuals Plot for Daridorexant (Linear, $1/x^2$ Calibration)

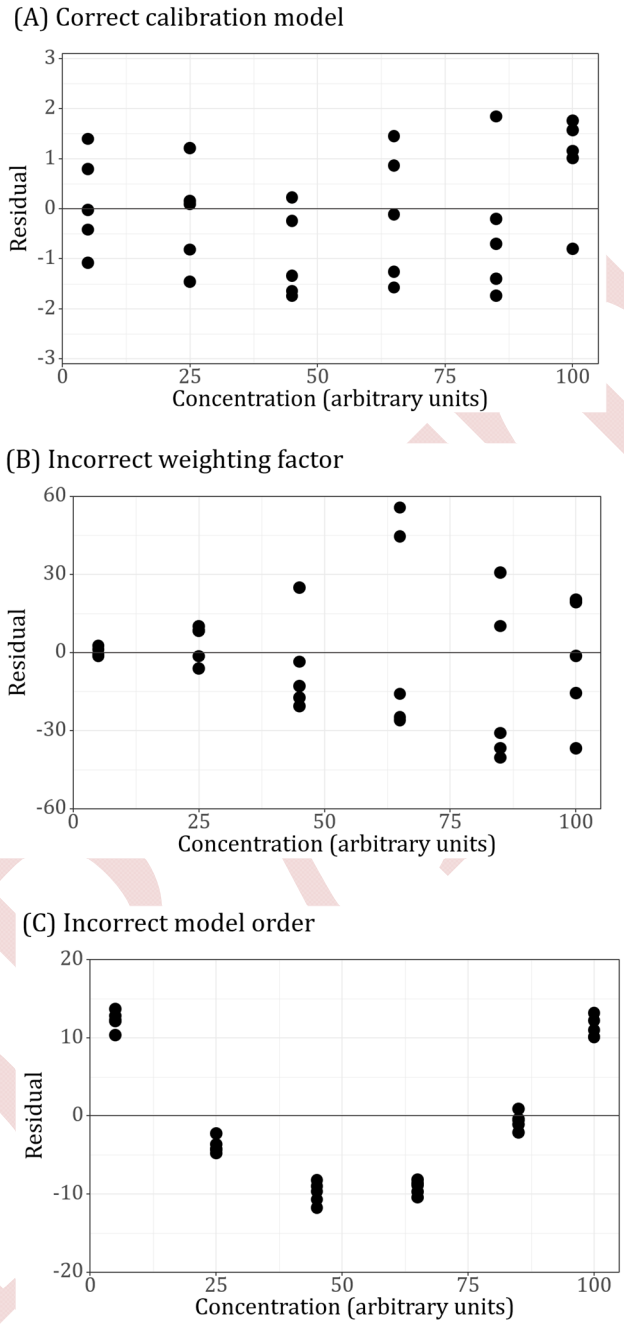


The weighted residuals plot displayed a clear curvature, indicating that quadraticity existed in the original data which was not accounted for by the linear regression. A quadratic calibration model was therefore chosen by the laboratory.

C.1.4 Goodness of fit

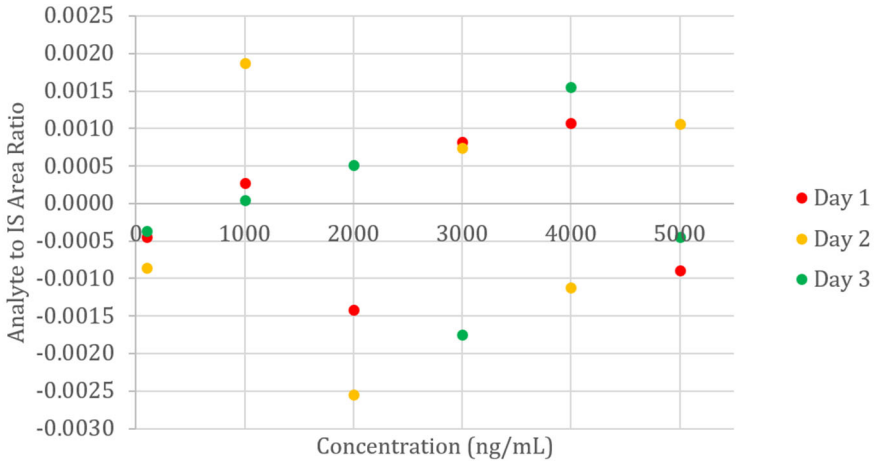
The preliminary calibration model for daridorexant was a quadratic, $1/x^2$ weighted regression. Goodness of fit was evaluated using a weighted residuals plot. The selected calibration model was used to fit each of the calibration curves collected. Using the regression parameters obtained, weighted residuals were calculated and plotted against the standards' concentrations. If the calibration model selected is adequate, residuals should be randomly distributed around the x-axis, with no clear pattern, as in Figure C.5 (A). Residuals with a fan-shaped pattern, as in Figure C.5 (B), typically indicate the weighting factor used is not adequate (e.g., an unweighted regression was used where a 1/x weighting would have been appropriate). Residuals displaying a curved pattern, as in Figure C.5 (C), typically indicate that curvature in the data is not taken into account by the calibration model used (e.g., a linear calibration is used where a quadratic one would be adequate).

Figure C.5—Common Patterns Observed in Residuals Plots



The weighted residuals plot obtained for daridorexant is shown in Figure C.6.

Figure C.6—Weighted Residuals Plot for Daridorexant (Quadratic, $1/x^2$ Calibration)



Residuals appeared to be randomly distributed around the x-axis, with no clear pattern to the residuals. This indicated that the calibration model (quadratic, $1/x^2$) properly fits the daridorexant data. This will be further assessed during method validation using additional replicates.

C.2 Example of Calibration Model Assessment for Method Validation

C.2.1 General

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards (fentanyl-D_5 and norfentanyl-D_5) and solvent extraction with a back-extraction clean-up step. Analysis used liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

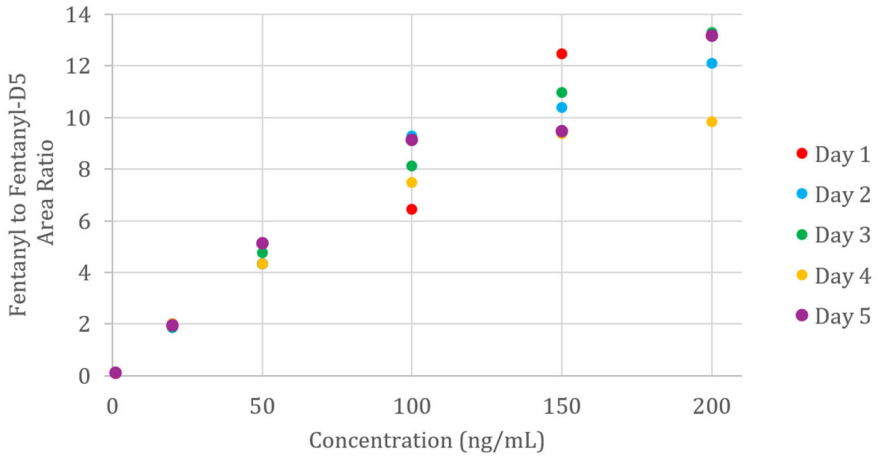
Per Sections B.1 and B.3 of Annex B in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory must determine the appropriate calibration model. Six non-zero calibrators spanning the desired concentration range of 1 to 200 ng/mL were used. Calibrator samples were extracted and analyzed in five separate runs spanning an interval of two weeks. The data obtained were reported in Table C.2 and displayed as plots in Figure C.7.

Table C.2—Fentanyl and Norfentanyl Calibration Model Assessment Data

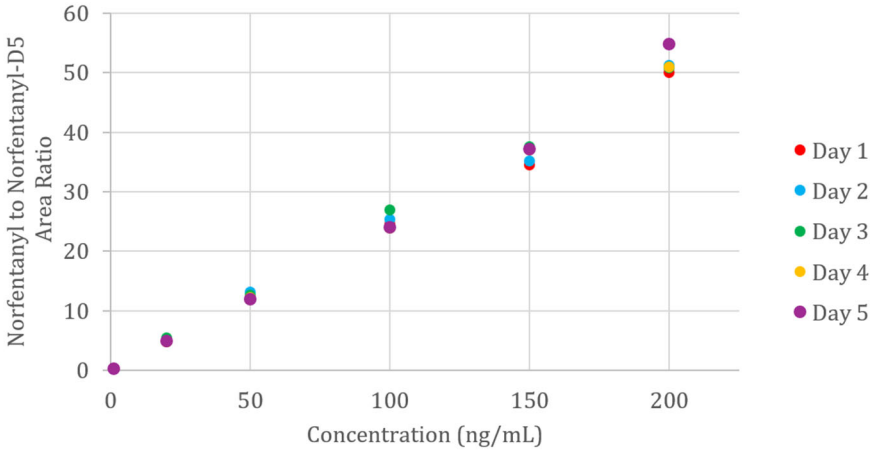
Concentration (ng/mL)	Fentanyl to Fentanyl-D_5 Peak Area Ratio				
	Day 1	Day 2	Day 3	Day 4	Day 5
1	0.1242	0.1069	0.1043	0.0974	0.1134
20	2.0081	1.8689	1.8968	1.9999	1.9401
50	4.3224	4.3375	4.7709	4.3224	5.1177
100	6.4441	9.2775	8.1336	7.4740	9.1417
150	12.4801	10.3958	10.9590	9.3803	9.4861
200	13.2078	12.0949	13.2781	9.8418	13.1599
Concentration (ng/mL)	**Norfentanyl to Norfentanyl-D_5 Peak Area Ratio**				
	Day 1	Day 2	Day 3	Day 4	Day 5
1	0.2539	0.2369	0.2441	0.2509	0.2485
20	4.9458	5.1702	5.3815	4.8398	4.9079
50	12.2642	13.1675	12.6365	12.2518	11.9322
100	24.5613	25.2847	27.0047	23.8460	23.9602
150	34.5352	35.2091	37.4951	37.0222	37.1484
200	49.9855	51.2577	50.8660	50.9256	54.8113

Figure C.7—Fentanyl and Norfentanyl Peak Area Ratios as a Function of Concentration

(A) Fentanyl



(B) Norfentanyl



Section B.1 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology* presents several options for assessing the weighting and linearity of the calibration. For the validation, the laboratory chose to use statistical tests with a significance level of 5% (0.05). The data were analyzed using Microsoft Excel.

C.2.2 Weighting

C.2.2.1 Evaluation of the Presence of Heteroscedasticity

The presence of heteroscedasticity was evaluated using a one-tailed F-test on peak area ratios of the highest and lowest calibration levels.

For fentanyl, a p-value of 6.85×10^{-9} was obtained, well below the 0.05 significance level. It was unlikely that the variances were the same at the low and high end of the calibration range. The laboratory concluded that heteroscedasticity was present, and weighting was required for fentanyl's calibration model.

For norfentanyl, a p-value of 4.75×10^{-10} was obtained, well below the 0.05 significance level. It was unlikely that the variances were the same at the low and high end of the calibration range. The laboratory concluded that heteroscedasticity was present, and weighting was required for norfentanyl's calibration model.

C.2.2.2 Selection of a Weighting Factor

The appropriate weighting factor was selected by compiling the total weighted normalized variance for three potential weighting factors: no weight, $1/x$, and $1/x^2$. While the unweighted calibration was ruled out by the previous test (C.2.2.1), it was still considered here for confirmation purposes.

For fentanyl, the total normalized weighted variance was 7.0×10^{-4} for a weight of 1 (no weight), 5.5×10^{-6} for a weight of $1/x$, and 1.4×10^{-9} for a weight of $1/x^2$. The laboratory selected $1/x^2$ as the calibration model weight since it yielded the smallest normalized weighted variance.

For norfentanyl, the total normalized weighted variance was 1.5×10^{-3} for a weight of 1 (no weight), 8.2×10^{-6} for a weight of $1/x$, and 1.3×10^{-9} for a weight of $1/x^2$. The laboratory selected a weight of $1/x^2$ for the calibration model since it yielded the smallest normalized weighted variance.

C.2.3 Linearity

The presence of non-linearity was evaluated for both analytes using a partial F-test.

A p-value of 1.52×10^{-8} was obtained for fentanyl, well below the 0.05 significance level. When switching from a linear to a quadratic calibration model, the magnitude of the residuals was reduced, and it was not likely that this improvement was attributed to noise alone. The laboratory concluded that a quadratic model was indicated for fentanyl.

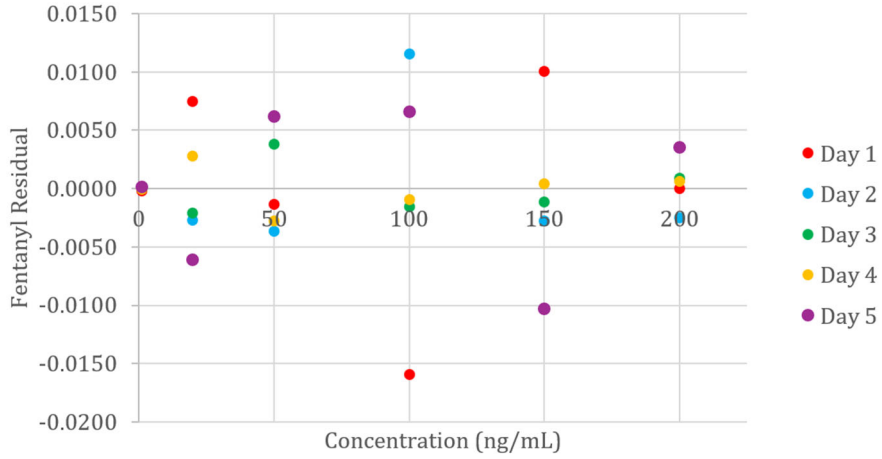
A p-value of 0.64 was obtained for norfentanyl, above the 0.05 significance level. If there was a reduction in the magnitude of the residuals when switching from a linear to a quadratic calibration model, there was a high probability that it was attributed to noise alone. The laboratory concluded that the linear model was the simplest calibration model that best fits the concentration-response relationship.

C.2.3 Goodness of Fit

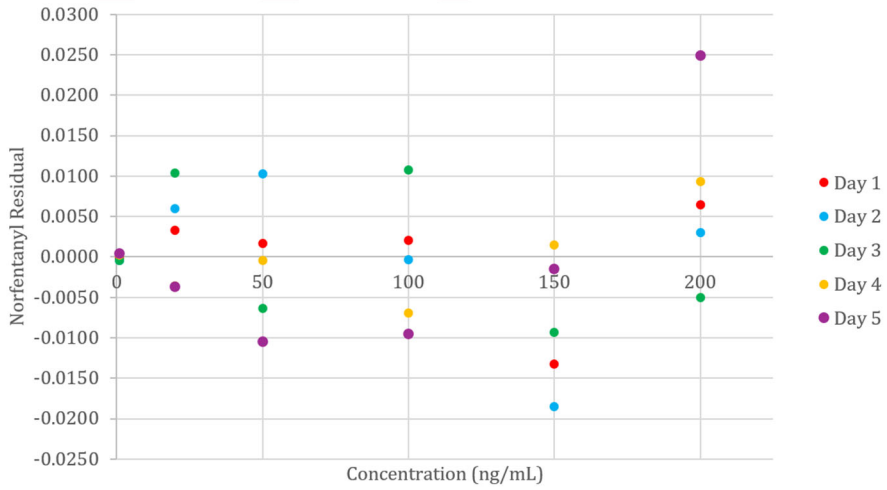
A quadratic, $1/x^2$ weighted calibration model was selected for fentanyl; and a linear, $1/x^2$ weighted calibration model was selected for norfentanyl. The goodness of fit was evaluated using a weighted residuals plot. The selected calibration model was used to fit each of the five calibration curves collected for each analyte. Using the regression parameters obtained, weighted residuals were calculated. Weighted residuals were then plotted against the standards' concentration. Figure C.8 shows the weighted residuals plot obtained for fentanyl (A) and norfentanyl (B).

Figure C.8—Weighted Residuals Plot for Fentanyl and Norfentanyl

(C) Fentanyl (Quadratic, $1/x^2$ Calibration)



(D) Norfentanyl (Linear, 1/x2 Calibration)



In both cases, residuals appeared to be randomly distributed around the x-axis, with no clear pattern to the residuals. This indicated that the selected calibration model (quadratic, $1/x^2$ for fentanyl; linear, $1/x^2$ for norfentanyl) is appropriate for the collected data.

Since norfentanyl followed a linear calibration model, the laboratory could have decided to reduce its number of calibrators from six to four for the other validation experiments. However, since the

laboratory will prepare both fentanyl and norfentanyl calibrators together, they chose to keep all six calibration points for both analytes.

C.3 Example of Calibration Model Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual column gas chromatograph with flame ionization detectors. The method used an undefined calibration model using six calibrators between 10 mg/dL and 400 mg/dL.

Per Section B.3 of Annex A in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory determined the most appropriate calibration model for the working calibration range identified in the standard test method. The approach for determining the appropriate calibration model during method verification is very similar to the approach for method validation. For more details, the reader is referred to example C.2.

The laboratory determined that a linear, unweighted calibration model was most appropriate for the calibration range of 10 mg/dL to 400 mg/dL.

Annex D
(informative)

Examples of Carryover Assessment

D.1 Example of Carryover Experiments for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, spiked at 50 ng/mL and solvent extraction with a back-extraction clean-up step. Analysis used liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section C.1 of Annex C in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated carryover during method validation activities for the non-standard test method. The laboratory limited the carryover study to the highest calibrator sample in their method (200 ng/mL) for both fentanyl and norfentanyl.

Using the method under validation, a blank antemortem blood sample was extracted. Likewise, a set of calibrator samples was extracted to establish a calibration curve. The instrument sequence was created so that the extract of the highest calibrator sample (200 ng/mL) was immediately followed by a blank antemortem blood sample extract. This experiment was repeated over five different days.

The data were analyzed for indications of the presence (e.g., correct retention time window, peak shape, and ion ratios) of fentanyl or norfentanyl in the blank blood samples (Table D.1). Where all detection and identification criteria were met, the relative peak areas of the analyte to internal standard were compared to the lowest calibrator sample (1 ng/mL) to determine if any of the unintended peaks exceeded 10% of the lowest calibrator.

Table D.1—Results of Carryover Experiments

Day	Analyte:	Presence of Analyte in Blank Sample?	Relative Peak Area of Analyte in Blank Sample	Relative Peak Area of Analyte in Low Calibrator (1 ng/mL)	Percent of Lowest Calibrator
1	Fentanyl	No	N/A	N/A	N/A
	Norfentanyl	No	N/A	N/A	N/A
2	Fentanyl	Yes	0.0013	0.0245	5.3%
	Norfentanyl	No	N/A	N/A	N/A
3	Fentanyl	No	N/A	N/A	N/A
	Norfentanyl	No	N/A	N/A	N/A
4	Fentanyl	No	N/A	N/A	N/A
	Norfentanyl	No	N/A	N/A	N/A
5	Fentanyl	Yes	0.0031	0.0207	**15.0%**
	Norfentanyl	No	N/A	N/A	N/A

Since fentanyl carryover on the fifth day exceeded 10% of the relative peak area of the 1 ng/mL calibrator, the laboratory initiated a requirement for extracted blank samples to be analyzed after each test specimen within a batch when the method was placed into service.

D.2 Example of Carryover Experiments for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual-column gas chromatograph with flame ionization detectors. The standard test method declared no carryover at concentrations as high as 400 mg/dL.

Per Section C.2 of Annex C in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated carryover during method verification using the standard test method.

Three blank postmortem blood samples from the same source were prepared using the standard test method. Likewise, a set of calibrators was prepared to establish a calibration curve. The instrument sequence was designed so the highest calibrator (400 mg/dL) was immediately followed by the blank blood sample. This experiment was repeated in two additional runs.

The data were analyzed for indications (e.g., correct retention time window, peak shape, and predefined signal-to-noise ratio) of ethanol in the blank blood samples. Where all detection and identification criteria were met, relative peak areas of the analyte to internal standard were to be compared to the lowest calibrator (10 mg/dL) to determine if any of the unintended peaks exceeded 10% of the lowest calibrator.

The results showed no peaks in the blank postmortem blood samples that immediately followed the 400 mg/dL calibrator, verifying the laboratory's ability to achieve the same performance for carryover, as defined within the standard test method.

Annex E
(informative)
Examples of Dilution Integrity Assessment

E.1 Example of Dilution Integrity Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Annex D in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated the need for sample dilution to be performed. This assessment determined that sample dilutions would be required.

The laboratory chose to evaluate the impact of 1:2 and 1:10 dilutions of fortified antemortem blood samples using water as the dilution matrix. Acceptance criteria were the same as those of the method's bias and within-run precision experiments.

The high concentration pool of blank blood fortified at 180 ng/mL with fentanyl and norfentanyl from the earlier bias and precision experiments was used for the dilution integrity assessment. Aliquots of this sample pool were prepared in triplicate. The method's prescribed sample volume was 0.5 mL. For the 1:2 dilution, 250 µL of sample was mixed with 250 µL of water. For the 1:10 dilution, 50 µL of sample was mixed with 450 µL of water. Samples were then fortified with internal standards and prepared, per the method's specifications, alongside a standard calibration curve.

The measured quantitative results corrected with the dilution factors were compared to the target concentration (180 ng/mL). The process was repeated over five runs for a total of 15 replicates analyzed for each dilution. Bias and within-run precision measurements were then calculated.

The prescribed bias and precision criteria were met for fentanyl at the 1:2 and 1:10 dilutions. For norfentanyl, the bias and precision criteria were met at the 1:2 dilution; however, within-run criteria for precision were not met for norfentanyl at the 1:10 dilution (Table E.1). The laboratory accepted the performance of the method except for 1:10 dilutions for norfentanyl. Therefore, the laboratory will only allow case samples to be diluted 1:2 for norfentanyl quantitations.

522 **Table E.1—Results of Dilution Integrity Experiments (Target Concentration 180 ng/mL)**

Run	Replicate	Fentanyl				Norfentanyl			
		1:2 (ng/mL)	Within-Run Precision	1:10 (ng/mL)	Within-Run Precision	1:2 (ng/mL)	Within-Run Precision	1:10 (ng/mL)	Within-Run Precision
1	A	177	1.7 %	182	2.7%	178	1.2%	112	**24.2%**
	B	180		189		179		179	
	C	183		192		182		175	
2	A	179	2.2%	196	1.6%	183	0.8%	196	16.0%
	B	176		197		182		144	
	C	184		202		180		160	
3	A	178	1.4%	205	6.8%	177	0.9%	250	**22.4%**
	B	180		195		178		179	
	C	175		179		180		168	
4	A	180	1.0%	182	0.8%	178	1.2%	159	18.2%
	B	177		184		182		145	
	C	180		181		181		204	
5	A	182	1.1%	179	2.8%	178	1.5%	256	16.8%
	B	184		186		183		279	
	C	180		176		182		199	
Bias (%)		-0.2%		4.6%		0.1%		3.9%	
Between-Run Precision (%CV)		1.5%		4.8%		1.2%		**24.4%**	

523

Annex F
(informative)

Examples of Interference Assessment

F.1 Example of Interference Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method uses isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Annex E of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated potential interferences during method validation of the non-standard test method. The laboratory assessed interferences from blank matrices, target analytes, and internal standards, as well as drugs and metabolites routinely encountered in casework. If interfering peaks were observed that were greater than 20% of the area of the lowest calibrator, the laboratory would need to address how the interferences would impact the data and develop a mitigation plan.

A low calibrator sample was prepared at 1 ng/mL of fentanyl and norfentanyl and analyzed to establish the 20% area threshold. The average peak area for the 1 ng/mL fentanyl calibrator was 1000, while the 1 ng/mL norfentanyl calibrator averaged 500. Therefore, significant interferences would need to satisfy all identification criteria and have a peak area above 200 (20% of 1000) for fentanyl and 100 (20% of 500) for norfentanyl.

To demonstrate the absence of interferences from the antemortem blood matrix, ten unique antemortem blank blood sources were analyzed without the addition of fentanyl-D_5 and norfentanyl-D_5. Interferences affecting fentanyl, norfentanyl, and their internal standards were not observed from the blank matrix.

Interferences from the internal standards were assessed by analyzing single blank antemortem blood samples, one each fortified with fentanyl-D_5 or norfentanyl-D_5 at 50 ng/mL and evaluated by monitoring the signals for fentanyl and norfentanyl. A signal was observed for norfentanyl in the sample fortified with only the internal standards; however, the response was 4% of the low calibrator sample and, therefore, deemed insignificant.

Interferences from the analytes of interest were assessed by analyzing separate single blank antemortem blood samples that were fortified with fentanyl or norfentanyl at 200 ng/mL and evaluated by monitoring the signals for fentanyl-D_5 and norfentanyl-D_5. Signals for the internal standards were not observed.

Routinely encountered drugs and metabolites were evaluated by analyzing blank antemortem blood samples fortified with analyte mixtures, but without the addition of internal standard (see Table F.1).

560 **Table F.1—List of Analytes Evaluated for Interferences**

Drug Class	Analyte (Concentration, ng/mL)
Stimulants	Amphetamine (1,000), Methamphetamine (1,000), MDMA (100), MDA (100), Phentermine (100), Cocaine (100), BZE (1,000)
Benzodiazepines	Alprazolam (50), Hydroxyalprazolam (50), Diazepam (200), Nordiazepam (200), Oxazepam (200), Temazepam (100), Etizolam (50), Bromazolam (50)
Opiates/Opioids	6-AM (50), Morphine (100), Codeine (100), Hydrocodone (100), Oxycodone (100)
	Methadone (500), EDDP (500), Buprenorphine (50), Norbuprenorphine (50), Tramadol (500), O-Desmethyltramadol (500)
Cannabinoids	THC (100), THC-COOH (500), CBD (100)
Therapeutics and Incidentals	Fluoxetine (200), Norfluoxetine (200), Amitriptyline (200), Nortryptyline (200), Trazodone (200), Imipramine (200), Desipramine (200), Carisoprodol (200), Meprobamate (200), Cyclobenzaprine (200), Norcyclobenzaprine (200), Diphenhydramine (200), Pseudoephedrine (200), Acetaminophen (1000), Doxylamine (200), Ibuprofen (1000), Caffeine (1000)
Fentanyl Analogs	Acetylfentanyl (100), Alfentanil (50), Carfentanil (50), para-Fluorofentanyl (100), Furanylfentanyl (100), Methylacetylfentanyl (100), ortho-Methylfentanyl (100), Sufentanil (50), Thienylfentanyl (100 ng/mL)

561 None of the tested analytes interfered with norfentanyl. A signal for fentanyl that satisfied all
562 identification criteria was present in the blank antemortem blood sample fortified with fentanyl
563 analogs (Table F.2).

564

565

566 **Table F.2—Results from Interference Studies**

Interference Test		Interfering Signal Detected (%)			
		Fentanyl	*Norfentanyl*	*Fentanyl-D_5*	*Norfentanyl-D_5*
Matrix Interferences					
Antemortem Blank Blood	Source 1	No	No	No	No
	Source 2	No	No	No	No
	Source 3	No	No	No	No
	Source 4	No	No	No	No
	Source 5	No	No	No	No
	Source 6	No	No	No	No
	Source 7	No	No	No	No
	Source 8	No	No	No	No
	Source 9	No	No	No	No
	Source 10	No	No	No	No
Internal Standard Interferences					
Antemortem Blank Blood Fortified With:	Fentanyl-D_5 (50 ng/mL)	No	No	N/A	No
	Norfentanyl-D_5 (50 ng/mL)	No	Yes (4%)*	No	N/A
	Fentanyl (200 ng/mL)	N/A	No	No	No
	Norfentanyl (200 ng/mL)	No	N/A	No	No
Common Analytes					
Antemortem Blank Blood Fortified With:	Stimulants Mix	No	No	No	No
	Benzodiazepines Mix	No	No	No	No
	Opiates/Opioids Mix	No	No	No	No
	Cannabinoids Mix	No	No	No	No
	Therapeutics & Incidentals Mix	No	No	No	No
	Fentanyl Analogs Mix	**Yes (46%)**	No	No	No

567 * Insignificant as it does not exceed 20% peak area threshold established using the 1 ng/mL norfentanyl
568 calibrator.

569 The analytes in the fentanyl analogs mixture were re-evaluated individually in blank antemortem
570 blood to determine the specific interfering compound(s) (Table F.3). It was determined that
571 methylacetylfentanyl was the only analyte that contributed to the signal for fentanyl and met all
572 identification criteria.

573

574 **Table F.3—Results from Fentanyl Analogs Interference Studies**

Interference Test		Interfering Signal Detected (%)			
		Fentanyl	*Norfentanyl*	*Fentanyl-D5*	*Norfentanyl-D5*
Fentanyl Analogs					
Antemortem Blank Blood Fortified With:	Acetylfentanyl (100 ng/mL)	No	No	No	No
	Alfentanil (50 ng/mL)	No	No	No	No
	Carfentanyl (50 ng/mL)	No	No	No	No
	p-Fluorofentanyl (100 ng/mL)	No	No	No	No
	Furanylfentanyl (100 ng/mL)	No	No	No	No
	Methylacetylfentanyl (100 ng/mL)	**Yes (51%)**	No	No	No
	o-Methylfentanyl (100 ng/mL)	No	No	No	No
	Sufentanil (50 ng/mL)	No	No	No	No
	Thienylfentanyl (100 ng/mL)	No	No	No	No

575 To assess the impact of methylacetylfentanyl interference on fentanyl, the laboratory explored
576 different options for resolving this issue.

577 1. Investigate whether new ion transitions for fentanyl could be used to eliminate the contribution
578 of methylacetylfentanyl.

579 2. Redevelop the chromatographic separation method to resolve methylacetylfentanyl from
580 fentanyl, while maintaining the lack of contribution from other analytes.

581 3. Clearly explain this method limitation when reporting fentanyl positive results (e.g., report as
582 "fentanyl/methylacetylfentanyl", add a note on the report about the potential interference of
583 methylacetylfentanyl).

584 4. Develop a secondary method for additional analysis that would accurately differentiate fentanyl
585 from methylacetylfentanyl.

586 The laboratory chose to provide a statement concerning methylacetylfentanyl interference when
587 reporting fentanyl (Option #3), as the other two options would require further method
588 development and subsequent method validation.

589

590

Annex G
(informative)

Examples of Ionization Suppression and Enhancement Assessment

G.1 Example of Ionization Suppression and Enhancement Assessment for Method Development

A laboratory developed a quantitative method to analyze daridorexant in blood samples. A protein precipitation technique was used for sample clean-up and extracts were analyzed by LC-MS/MS. Another orexin receptor antagonist, suvorexant-D_6, served as the internal standard.

Per Section F.1 of Annex F in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory assessed the impact of ionization suppression/enhancement on the method's target analyte and internal standard during method development. The laboratory chose the post-column infusion method to assess ionization suppression/enhancement at the retention times of the analyte and internal standard.

A neat solution of daridorexant at 300 ng/mL and suvorexant-D_6 at the method's defined concentration (2000 ng/mL) was prepared in a reconstitution solution. Blank antemortem and postmortem blood sources, three of each, were processed following the method's sample preparation technique. The daridorexant/suvorexant-D_6 solution was infused into the eluent from the column using a syringe pump and a post-column T-connector. Baseline signals for the analyte and internal standard were monitored. The method's reconstitution solution was injected to evaluate the impact of the solution on the baseline signals. The prepared blank blood samples were sequentially injected into the LC-MS/MS, and the change in baseline signals at the retention times of the analyte and internal standard were monitored. Changes greater than 25% of those observed from the solvent blank were considered as an indication of significant ionization suppression/enhancement.

Ionization suppression/enhancement was not considered significant for daridorexant in antemortem blood extracts (Figure G.1). Ionization suppression/enhancement was also not considered significant in both antemortem and postmortem blood extracts for suvorexant-D_6 (data not shown). However, significant ionization enhancement was observed for daridorexant from postmortem blood extracts (Figure G.2).

Figure G.1—Example of Daridorexant Ionization Suppression/Enhancement Assessment in Processed Antemortem Blood

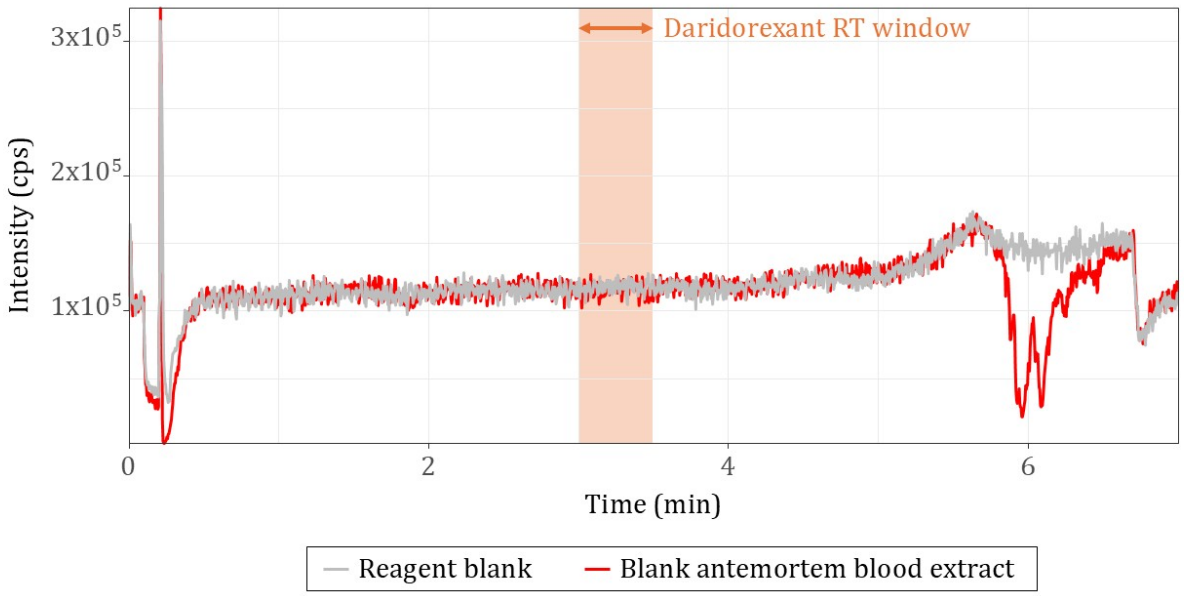
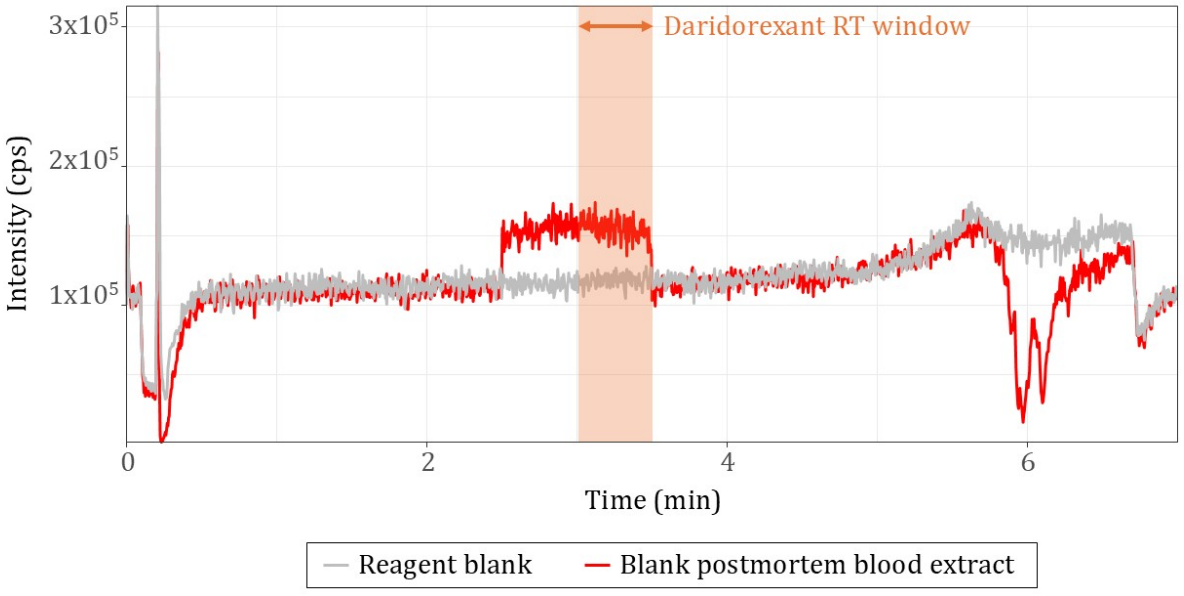


Figure G.2—Example of Daridorexant Ionization Suppression/Enhancement Assessment in Processed Postmortem Blood



Therefore, the laboratory modified the chromatographic system by adjusting the solvent gradient. After the modification, the experiment was repeated. The modified chromatographic system minimized the impact of ionization enhancement for daridorexant (Figure G.3) without impacting the suvorexant-D_6 (Figure G.4).

Figure G.3—Example of Daridorexant Ionization Suppression/Enhancement Assessment in Processed Postmortem Blood after Modification of Solvent Gradient

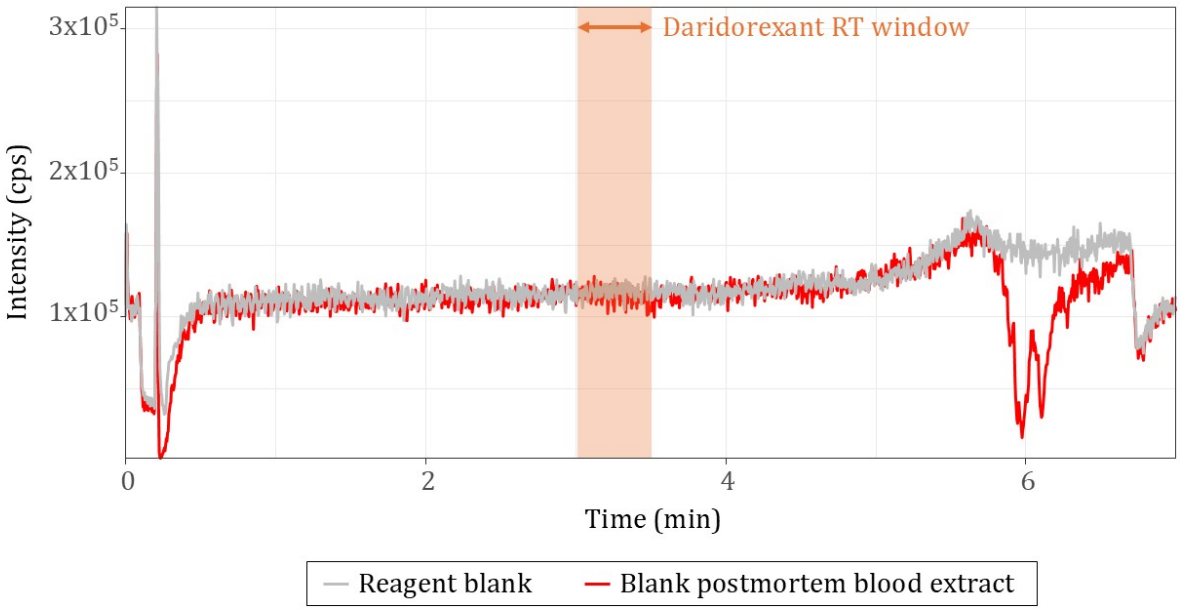
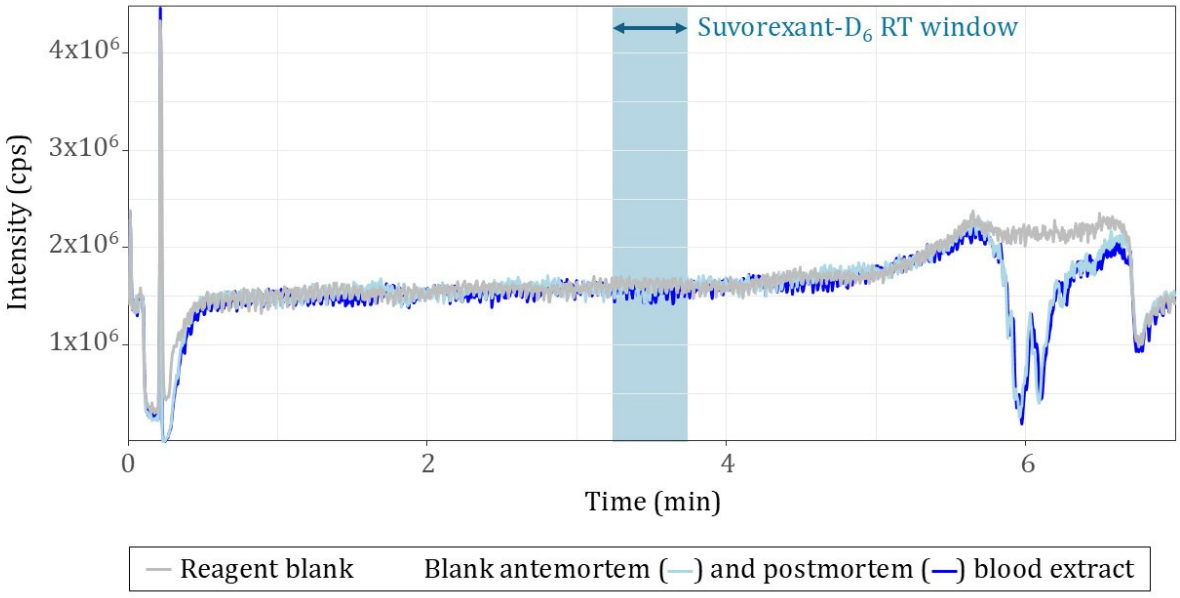


Figure G.4—Example of Suvorexant Ionization Suppression/Enhancement Assessment in Processed Antemortem and Postmortem Blood after Modification of Solvent Gradient



Modifying the solvent gradient forced the laboratory to ensure other method development experiments were not impacted by this change.

G.2 Example of Ionization Suppression and Enhancement Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section F.2 of Annex F in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated ionization suppression/enhancement during method validation for the non-standard test method. The laboratory used the post-extraction addition assessment approach. Fentanyl and norfentanyl were evaluated at low (3 ng/mL) and high (160 ng/mL) concentrations. Fentanyl-D_5 and norfentanyl-D_5 were also evaluated at the method's defined internal standard concentration (50 ng/mL). A single precursor to diagnostic product ion transition for each target analyte and internal standard were evaluated.

"Set 1" consisted of two neat standards. The first contained fentanyl and norfentanyl at 3 ng/mL with 50 ng/mL of fentanyl-D_5 and norfentanyl-D_5. The second contained fentanyl and norfentanyl at 160 ng/mL with 50 ng/mL of fentanyl-D_5 and norfentanyl-D_5. Each neat standard was injected six times.

"Set 2" consisted of ten unique blank antemortem blood sources. Each source was extracted in duplicate. One replicate from each extracted source was reconstituted with the low concentration standard and internal standards, and the other replicate with the high concentration standard and internal standards. The effects of dilutions were taken into account to ensure that the absolute concentrations evaluated matched those in Set 1. Each sample was injected once.

The average peak areas of Set 1 were compared to the peak of the individual Set 2 samples, as follows:

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

For example, the average peak area for the Fentanyl Set 1 samples at the low concentration (3 ng/mL) was 7856. The first matrix sample fortified with fentanyl at 3 ng/mL (Set 2) resulted in a peak area of 7104.

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{7104}{7856}\right) \times 100 = 90.4\%$$

All data and calculations are shown in Table G.3.

In general, the method demonstrated ionization suppression within 25% for all tested analytes and internal standards at all concentrations, except for norfentanyl at the high concentration. All %CVs were less than 20%.

As a result of the significant norfentanyl ionization suppression, the number of unique sources of blank antemortem blood was tripled for the evaluation of LOD, LLOQ, bias, and precision.

678 **Table G.3—Ionization Suppression/Enhancement Results for Fentanyl, Norfentanyl, and**
679 **Internal Standards**

		Low (3 ng/mL)				High (160 ng/mL)			
		Fentanyl (337.2 m/z → 188.2 m/z)	**Norfentanyl (233.2 m/z → 84.1 m/z)**	**D5-Fentanyl (342.2 m/z → 188.2 m/z)**	**D5-Norfentanyl (238.2 m/z → 84.1 m/z)**	**Fentanyl (337.2 m/z → 188.2 m/z)**	**Norfentanyl (233.2 m/z → 84.1 m/z)**	**D5-Fentanyl (342.2 m/z → 188.2 m/z)**	**D5-Norfentanyl (238.2 m/z → 84.1 m/z)**
Set 1 (Neat)	1	7535	8564	130933	140586	415685	416368	136865	124586
	2	7935	8614	145156	139458	410658	426588	141558	139548
	3	8229	8259	138458	129458	403158	410158	129568	142586
	4	7973	7968	128568	133158	420156	425648	139548	145548
	5	7692	9015	139458	135458	432201	418548	142586	138486
	6	7772	8459	131588	145256	410120	422588	139125	139658
	Avg.	*7856*	*8480*	*135694*	*137229*	*415330*	*419983*	*138208*	*138402*
	S.D.	*243*	*353*	*6350*	*5670*	*10052*	*6232*	*4679*	*7244*
	%CV	*3.1%*	*4.2%*	*4.7%*	*4.1%*	*2.4%*	*1.5%*	*3.4%*	*5.2%*
Set 2 (Matrix)	1	7104	8249	128458	135648	405158	300158	138548	132518
	2	5767	7925	129548	136458	410215	315486	129468	135136
	3	7456	7844	130125	129586	399158	320158	137458	138454
	4	7578	7950	127589	128458	412258	299158	128458	137458
	5	7136	8210	128468	127458	408123	320158	132156	129458
	6	7251	8354	126458	130125	409521	316158	134586	135125
	7	7904	8025	129158	135128	401215	314886	136485	133125
	8	7190	7954	131278	129158	410288	302987	138458	130125
	9	7270	8348	126158	137595	411002	298483	129586	131125
	10	7564	8025	128458	136586	407586	303897	131586	132185
Matrix Effects Calculations	1	90.4%	97.3%	94.7%	98.8%	97.6%	71.5%	100.2%	95.7%
	2	73.4%	93.5%	95.5%	99.4%	98.8%	75.1%	93.7%	97.6%
	3	94.9%	92.5%	95.9%	94.4%	96.1%	76.2%	99.5%	100.0%
	4	96.5%	93.8%	94.0%	93.6%	99.3%	71.2%	92.9%	99.3%
	5	90.8%	96.8%	94.7%	92.9%	98.3%	76.2%	95.6%	93.5%
	6	92.3%	98.5%	93.2%	94.8%	98.6%	75.3%	97.4%	97.6%
	7	100.6%	94.6%	95.2%	98.5%	96.6%	75.0%	98.8%	96.2%
	8	91.5%	93.8%	96.7%	94.1%	98.8%	72.1%	100.2%	94.0%
	9	92.5%	98.4%	93.0%	100.3%	99.0%	71.1%	93.8%	94.7%
	10	96.3%	94.6%	94.7%	99.5%	98.1%	72.4%	95.2%	95.5%
	Avg.	*91.9%*	*95.4%*	*94.7%*	*96.6%*	*98.1%*	***73.6%***	*96.7%*	*96.4%*
	S.D.	*7.2%*	*2.2%*	*1.2%*	*2.9%*	*1.0%*	*2.1%*	*2.8%*	*2.2%*
	%CV	*7.9%*	*2.3%*	*1.2%*	*3.0%*	*1.1%*	*2.9%*	*2.9%*	*2.3%*

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Annex H
(informative)

Examples of Limit of Detection Assessment

H.1 Example of Limit of Detection Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section G.1 of Annex G in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated the limit of detection (LOD) during method validation of the non-standard test method.

The calibration range for the method was previously established to be 1 – 200 ng/mL. The lowest non-zero calibrator (at least 1 ng/mL) was the desired the LOD.

For the evaluation of the LOD, the laboratory utilized the method validation requirements from G.4 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*. They evaluated the LOD using fortified antemortem blood to prepare multiple samples at the concentration of the low calibrator. Since ionization suppression had been observed for norfentanyl in earlier validation experiments, the number of unique antemortem blood sources was increased threefold for the LOD determination. Therefore, nine antemortem blood samples were fortified at a concentration of 1 ng/mL with both fentanyl and norfentanyl. Since the lowest non-zero calibrator was also used as the LLOQ for this method, the same samples and data were used for both LOD and LLOQ (see Annex I) evaluations.

Each of the nine fortified samples was analyzed in three separate runs (n = 27). Acceptance criteria (peak shape, retention time, and ion ratios) were met for all except for one norfentanyl sample (Table H.1), in which the ion ratios failed.

Table H.1—Results of LOD Studies for Fentanyl and Norfentanyl Using Lowest Calibrator Concentration (1 ng/mL)

Analyte	Run	Fortified Antemortem Blood Source									Pass Rate
		1	2	3	4	5	6	7	8	9	
Fentanyl	A	Y	Y	Y	Y	Y	Y	Y	Y	Y	100%
	B	Y	Y	Y	Y	Y	Y	Y	Y	Y	
	C	Y	Y	Y	Y	Y	Y	Y	Y	Y	
Norfentanyl	A	Y	Y	Y	Y	Y	Y	Y	Y	Y	96.3%
	B	Y	Y	Y	Y	Y	Y	Y	Y	Y	
	C	Y	Y	Y	Y	Y	**N**	Y	Y	Y	

All detection and identification criteria were met in at least 95% of the replicate results, so the lowest calibrator concentration was established as the LOD for both fentanyl and norfentanyl.

H.2 Example of Limit of Detection Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual-column gas chromatograph with flame ionization detectors. The standard test method specified a 5 mg/dL LOD.

Per Section G.5 of Annex G in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated that their implementation of the standard test method could reliably detect ethanol at 5 mg/dL.

Blank postmortem blood samples from five unique sources were fortified with ethanol at 5 mg/dL. Each 5 mg/dL postmortem blood sample was analyzed in triplicate over three separate runs, resulting in a total of 45 analyses. The results for each sample were evaluated to determine if at least 95% (43 of the 45 analyses) met the appropriate detection criteria (e.g., retention time, peak shape, signal-to-noise).

All detection and identification criteria were met in all replicate results (Table H.2). Therefore, the verification met the LOD requirements established in the standard test method.

Table H.2—Results of LOD Studies for Ethanol (5 mg/dL) Using a Standard Test Method

Run	Replicate	Ethanol Fortified Postmortem Blood Source (5 mg/dL)					Pass Rate
		1	2	3	4	5	
1	A	Y	Y	Y	Y	Y	100%
	B	Y	Y	Y	Y	Y	
	C	Y	Y	Y	Y	Y	
2	A	Y	Y	Y	Y	Y	
	B	Y	Y	Y	Y	Y	
	C	Y	Y	Y	Y	Y	
3	A	Y	Y	Y	Y	Y	
	B	Y	Y	Y	Y	Y	
	C	Y	Y	Y	Y	Y	

Annex I
(informative)

Examples of Lower Limit of Quantitation Assessment

I.1 Example of Lower Limit of Quantitation Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section Annex H.1 of Annex H in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated the lower limit of quantitation (LLOQ) during method validation of the non-standard test method.

The calibration range for the method was previously established to be 1 – 200 ng/mL. The lowest non-zero calibrator (at least 1 ng/mL) was the desired LLOQ.

For the evaluation of the LLOQ, the laboratory utilized the method validation requirements from H.2 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*. They evaluated the LLOQ using fortified antemortem blood to prepare multiple samples at the concentration of the low calibrator. Since ionization suppression had been observed for norfentanyl in earlier validation experiments, the number of unique antemortem blood sources was increased threefold for the LLOQ determination. Therefore, nine antemortem blood samples were fortified at a concentration of 1 ng/mL with both fentanyl and norfentanyl. Since the lowest non-zero calibrator was also used as the LOD for this method, the same samples and data from the LOD experiment were used in the evaluation of the LLOQ.

Each of the nine fortified samples was analyzed in three separate runs, each with an independent calibration curve (n = 27). For all samples in which the detection and identification criteria were met, the bias (±20%) and precision (≤ 20%) requirements were also met (Table I.1). This allowed the lowest calibrator to serve as the method's LLOQ.

Table I.1—Results of LLOQ Studies for Fentanyl and Norfentanyl Using Lowest Calibrator Concentration (1 ng/mL)

Analyte	Run	Measured Concentration from Fortified Antemortem Blood Source (ng/mL)									Bias	Precision
		1	2	3	4	5	6	7	8	9		
Fentanyl	A	0.9	0.8	1.0	0.8	1.0	1.0	1.1	1.0	1.0	-7%	11.6%
	B	0.8	0.9	0.8	1.0	0.9	1.1	0.9	1.1	0.9		
	C	0.8	0.7	0.7	1.0	1.1	0.9	0.9	1.0	0.9		
Norfentanyl	A	1.1	0.9	0.9	1.0	1.0	0.9	1.0	0.9	0.9	-6%	8.5%
	B	0.8	0.9	0.9	1.1	0.9	0.9	1.0	0.9	1.0		
	C	0.9	0.8	1.0	0.9	0.9	**0.9***	1.1	0.8	1.0		

* This replicate was omitted from the evaluation of LLOQ as it did not meet the detection and identification criteria (see LOD example).

I.2 Example of Lower Limit of Quantitation Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual-column gas chromatograph with flame ionization detectors. The standard test method specified a 10 mg/dL LLOQ.

Per Section H.3 of Annex H in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory verified that their implementation of the standard test method could reliably quantitate ethanol at 10 mg/dL, which is considered an administratively-defined decision point.

Blank postmortem blood samples from five unique sources were fortified with ethanol at 10 mg/dL. Each 10 mg/dL postmortem blood sample was analyzed in triplicate over three separate runs, resulting in a total of 45 analyses. For all samples in which the detection and identification criteria were met, the bias (±10%) and precision (≤ 10%) requirements were also met (Table I.2). This allowed the decision point concentration of 10 mg/dL to serve as the method's LLOQ.

Table I.2—Results of LLOQ Studies for Ethanol (10 mg/dL) Using a Standard Test Method

Run	Replicate	Measured Ethanol Concentration from Fortified Postmortem Blood Source (mg/dL)					Bias	Precision
		1	2	3	4	5		
1	A	11.2	10.6	11.3	9.5	10.5	10%	6.7%
	B	10.3	10.2	10.2	10.2	10.3		
	C	9.9	9.4	9.6	10.0	10.1		
2	A	11.6	9.8	9.0	9.9	10.1		
	B	10.2	10.1	10.0	11.7	9.8		
	C	9.7	9.8	10.3	10.7	9.4		
3	A	10.3	10.3	10.2	9.9	10.9		
	B	11.0	8.3	10.1	8.9	10.3		
	C	10.7	9.8	9.7	9.5	9.1		

Annex J
(informative)

Examples of Precision Assessment

J.1 Example of Precision Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Section I.2 of Annex I in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated precision during method validation of the non-standard test method. The data acquired to evaluate precision was also utilized to evaluate bias. The laboratory assessed precision using fortified antemortem blood concentration pools at low, medium, and high concentrations. Given the ionization suppression noted within the method, the laboratory increased the number of unique antemortem blood sources for precision experiments. Thus, each concentration was assessed with three (3) unique sources of blank antemortem blood. The calibration range for the method was 1 to 200 ng/mL. Therefore, the fortified antemortem blood concentration pools were prepared at 3 ng/mL, 90 ng/mL, and 180 ng/mL.

Each fortified antemortem blood concentration pool (a total of three for each concentration) was extracted in triplicate alongside a single extracted calibration curve. This experiment was repeated over five days using independently calibrated analytical runs. The data obtained from the analysis of the three concentration pools for fentanyl and norfentanyl are shown in Table J.1.

798 **Table J.1—Results of Fentanyl and Norfentanyl for Precision Runs**

Concentration Pool	Source	Replicate	Analyte	Run 1	Run 2	Run 3	Run 4	Run 5
Low (3 ng/mL)	1	A	Fentanyl	2.9	2.8	2.8	3.0	3.1
			Norfentanyl	3.1	3.0	2.8	2.9	3.0
		B	Fentanyl	2.8	2.9	2.7	2.8	2.8
			Norfentanyl	2.8	2.8	3.0	2.8	2.9
		C	Fentanyl	3.0	2.8	2.7	2.7	2.7
			Norfentanyl	2.7	2.6	2.6	2.8	2.8
	2	A	Fentanyl	3.1	3.2	3.0	3.1	3.2
			Norfentanyl	2.9	3.3	3.3	3.1	3.2
		B	Fentanyl	3.1	3.0	3.1	2.9	3.3
			Norfentanyl	3.1	2.7	3.4	2.5	3.3
		C	Fentanyl	3.2	3.2	3.1	3.0	3.2
			Norfentanyl	3.4	3.4	3.5	3.3	3.2
	3	A	Fentanyl	3.2	2.9	2.7	2.9	2.4
			Norfentanyl	3.1	2.9	2.9	3.1	2.8
		B	Fentanyl	3.0	3.0	2.8	2.7	2.9
			Norfentanyl	3.0	3.0	2.8	2.9	2.9
		C	Fentanyl	2.7	2.8	2.8	2.6	2.8
			Norfentanyl	2.9	3.1	3.0	3.0	2.8
Medium (90 ng/mL)	1	A	Fentanyl	92	100	95	93	105
			Norfentanyl	79	82	90	86	88
		B	Fentanyl	98	102	97	102	91
			Norfentanyl	82	90	89	92	92
		C	Fentanyl	87	97	90	96	94
			Norfentanyl	88	89	92	85	85
	2	A	Fentanyl	98	88	95	95	97
			Norfentanyl	98	90	99	92	95
		B	Fentanyl	100	82	90	88	90
			Norfentanyl	92	95	97	95	88
		C	Fentanyl	102	89	82	94	98
			Norfentanyl	95	87	98	94	82
	3	A	Fentanyl	79	87	84	90	88
			Norfentanyl	92	98	95	98	92
		B	Fentanyl	95	99	94	97	93
			Norfentanyl	95	99	94	97	93
		C	Fentanyl	80	82	80	85	85
			Norfentanyl	91	92	98	97	95
High (180 ng/mL)	1	A	Fentanyl	150	149	182	195	192
			Norfentanyl	155	147	180	198	199
		B	Fentanyl	185	152	175	187	199
			Norfentanyl	175	154	176	185	193
		C	Fentanyl	168	169	174	188	201
			Norfentanyl	168	162	175	192	195
	2	A	Fentanyl	149	159	145	150	146
			Norfentanyl	193	192	203	164	200
		B	Fentanyl	162	146	156	158	152
			Norfentanyl	197	201	206	152	201
		C	Fentanyl	156	152	150	151	149
			Norfentanyl	205	196	197	155	198
	3	A	Fentanyl	192	180	200	198	192
			Norfentanyl	205	199	192	205	207
		B	Fentanyl	198	192	205	184	180
			Norfentanyl	210	201	200	213	207
		C	Fentanyl	200	194	197	188	197
			Norfentanyl	203	205	195	200	208

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The data were analyzed by calculating the within-run precision for each source's concentration pool by first determining the mean and standard deviation for each analytical run (Table J.2)

Table J.2—Within-Run Mean (ng/mL) and Standard Deviations for Concentration Pools Prepared from Unique Sources of Antemortem Blood

Low Conc Pools (3 ng/mL)	Analyte	Run 1		Run 2		Run 3		Run 4		Run 5	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Source 1	Fentanyl	2.90	0.10	2.83	0.06	2.73	0.06	2.83	0.15	2.87	0.21
	Norfentanyl	2.87	0.21	2.80	0.20	2.80	0.20	2.83	0.06	2.87	0.10
Source 2	Fentanyl	3.13	0.06	3.13	0.12	3.07	0.06	3.00	0.10	3.23	0.06
	Norfentanyl	3.13	0.25	3.13	0.38	3.40	0.10	2.97	0.42	3.23	0.06
Source 3	Fentanyl	2.97	0.25	2.90	0.10	2.77	0.06	2.73	0.15	2.70	0.26
	Norfentanyl	3.00	0.10	3.00	0.10	2.90	0.10	3.00	0.10	2.83	0.06
Med Conc Pools (90 ng/mL)	**Analyte**	**Run 1**		**Run 2**		**Run 3**		**Run 4**		**Run 5**	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Source 1	Fentanyl	92.3	5.5	99.7	2.5	94.0	3.6	97.0	4.6	96.7	7.4
	Norfentanyl	83.0	4.6	87.0	4.4	90.3	1.5	87.7	3.8	88.3	3.5
Source 2	Fentanyl	100.0	2.0	86.3	3.8	89.0	6.6	92.3	3.8	95.0	4.4
	Norfentanyl	95.0	3.0	90.7	4.0	98.0	1.0	93.0	1.5	88.3	6.5
Source 3	Fentanyl	84.7	9.0	89.3	8.7	86.0	7.2	90.7	6.0	88.7	4.0
	Norfentanyl	92.7	2.1	96.3	3.8	95.7	2.1	97.3	0.6	93.3	1.5
High Conc Pools (180 ng/mL)	**Analyte**	**Run 1**		**Run 2**		**Run 3**		**Run 4**		**Run 5**	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Source 1	Fentanyl	168	17.5	157	10.8	177	4.4	190	4.4	197	4.7
	Norfentanyl	166	10.1	154	7.5	177	2.6	192	6.5	196	3.1
Source 2	Fentanyl	156	6.5	152	6.5	150	5.5	153	4.4	149	3.0
	Norfentanyl	198	6.1	196	4.5	202	4.6	157	6.2	200	1.5
Source 3	Fentanyl	197	4.2	189	7.6	201	4.0	190	7.2	190	8.7
	Norfentanyl	206	3.6	202	3.1	196	4.0	206	6.6	207	0.6

The within-run precision was calculated for each analytical run using the following formula:

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

For example, the within-run precision for Source 1 Low Concentration Pool (3 ng/mL) was calculated for fentanyl.

$$\text{Within-run CV}(\%) = \frac{0.10}{2.90} \times 100$$

$$\text{Within-run CV}(\%) = 3.4\%$$

Likewise, the within-run precision for Source 1 Low Concentration Pool (3 ng/mL) was calculated for norfentanyl.

$$\text{Within-run CV}(\%) = \frac{0.21}{2.87} \times 100$$

$$\text{Within-run CV}(\%) = 7.3\%$$

The within-run precision for each analytical run for each source's concentration pool is shown in Table J.3.

The acceptance criteria for within-run precision (%CV) in the validation plan for fentanyl and norfentanyl were defined as 20% (or less) for each concentration pool. The largest within-run precision results for each unique concentration pool are indicated (bold font) in Table J.3. None exceeded 20%.

Table J.3—Within-Run Precision for Three Concentration Pools Calculated per Antemortem Blood Source for Each Analytical Run

Low Conc Pools (3 ng/mL)	Analyte	Run 1	Run 2	Run 3	Run 4	Run 5
Source 1	Fentanyl	3.4%	2.1%	2.2%	5.3%	***7.3%***
	Norfentanyl	***7.3%***	7.1%	7.1%	2.1%	3.5%
Source 2	Fentanyl	1.9%	***3.8%***	2.0%	3.3%	1.9%
	Norfentanyl	8.0%	12.1%	2.9%	***14.1%***	1.9%
Source 3	Fentanyl	8.4%	3.4%	2.2%	5.5%	***9.6%***
	Norfentanyl	3.3%	3.3%	***3.4%***	3.3%	2.1%
Med Conc Pools (90 ng/mL)	**Analyte**	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**
Source 1	Fentanyl	6.0%	2.5%	3.8%	4.7%	***7.7%***
	Norfentanyl	***5.5%***	5.1%	1.7%	4.3%	4.0%
Source 2	Fentanyl	2.0%	4.4%	***7.4%***	4.1%	4.6%
	Norfentanyl	3.2%	4.4%	1.0%	1.6%	***7.4%***
Source 3	Fentanyl	***10.6%***	9.7%	8.4%	6.6%	4.5%
	Norfentanyl	2.3%	***3.9%***	2.2%	0.6%	1.6%
High Conc Pools (180 ng/mL)	**Analyte**	**Run 1**	**Run 2**	**Run 3**	**Run 4**	**Run 5**
Source 1	Fentanyl	***10.4%***	6.9%	2.5%	2.3%	2.4%
	Norfentanyl	***6.1%***	4.9%	1.5%	3.4%	1.6%
Source 2	Fentanyl	4.2%	***4.3%***	3.7%	2.9%	2.0%
	Norfentanyl	3.1%	2.3%	2.3%	***3.9%***	0.8%
Source 3	Fentanyl	2.1%	4.0%	2.0%	3.8%	***4.6%***
	Norfentanyl	1.7%	1.5%	2.0%	***3.2%***	0.3%

The laboratory also calculated the between-run precision for the method. They did so by first determining the fentanyl and norfentanyl mean concentration and standard deviation for all observations of each concentration pool (Table J.4).

Table J.4—Mean Concentrations and Standard Deviation for Between-Run Precision Calculations

		3 ng/mL		90 ng/mL		180 ng/mL	
Concentration Pool	**Analyte**	Calculated Mean	Standard Deviation	Calculated Mean	Standard Deviation	Calculated Mean	Standard Deviation
1	Fentanyl	2.83	0.12	95.9	5.0	178	17.3
	Norfentanyl	2.84	0.15	87.3	4.0	177	17.0
2	Fentanyl	3.11	0.11	92.5	6.1	152	5.1
	Norfentanyl	3.17	0.28	93.1	4.7	191	18.0
3	Fentanyl	2.81	0.19	87.9	6.5	193	7.4
	Norfentanyl	2.95	0.11	95.1	2.7	203	5.6

The between-run precision was calculated for all observations of each concentration pool using the following formula:

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

For example, the between-run precision for Source 1 Low Concentration Pool at 3 ng/mL was calculated for fentanyl.

$$\text{Within-run CV}(\%) = \frac{0.12}{2.83} \times 100$$

$$\text{Within-run CV}(\%) = 4.2\%$$

Likewise, the between-run precision for Source 1 Low Concentration Pool at 3 ng/mL was calculated for norfentanyl.

$$\text{Within-run CV}(\%) = \frac{0.16}{2.80} \times 100$$

$$\text{Within-run CV}(\%) = 5.7\%$$

The between-run precision for each source's concentration pool is shown in Table J.5.

Table J.5—Between-Run Precision Results

Concentration Pool	Analyte	Precision (3 ng/mL)	Precision (90 ng/mL)	Precision (180 ng/mL)
1	Fentanyl	4.4%	5.2%	9.8%
	Norfentanyl	5.1%	4.6%	9.6%
2	Fentanyl	3.4%	6.6%	3.4%
	Norfentanyl	8.8%	5.1%	9.4%
3	Fentanyl	6.7%	7.4%	3.8%
	Norfentanyl	3.6%	2.8%	2.8%

The validation plan requires the between-run precision not to exceed 20% for fentanyl and norfentanyl. The results demonstrated acceptable results for the between-run precision.

J.2 Example of Precision Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual column gas chromatograph with flame ionization detectors.

Per Section I.6 of Annex I in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory verified their precision using the standard test method. The standard test method lists a within-run and between-run precision of 10%. Therefore, the laboratory had to demonstrate their ability to meet these precision values when using the method for postmortem blood. The data acquired to evaluate precision were also utilized to evaluate bias.

The laboratory assessed precision using fortified postmortem blood concentration pools at low, medium, and high concentrations. The calibration range for the standard test method was 10 to 400 mg/dL. Therefore, the fortified postmortem blood concentration pools were prepared at 25 mg/dL, 150 mg/dL, and 350 mg/dL.

Each fortified postmortem blood concentration pool was extracted with five replicates alongside a single extracted calibration curve. This experiment was repeated over three independently calibrated analytical runs. The data obtained from the analysis of the low, medium, and high concentration pools for ethanol is shown in Table J.6.

The laboratory calculated the within-run precision. First, they determined the mean and standard deviation for each concentration pool for each analytical run (Table J.6).

The within-run precision was calculated for each analytical run using the following formula:

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

For example, the within-run precision for the 25 mg/dL concentration pool was calculated for ethanol as:

$$\text{Within-run CV}(\%) = \frac{0.9}{23.3} \times 100$$

$$\text{Within-run CV}(\%) = 3.9\%$$

Table J.6—Ethanol Within-Run Precision

Concentration Pool	Replicate	Run 1	Run 2	Run 3
Low (25 mg/dL)	A	22.4	23.6	22.7
	B	23.5	23.8	24.2
	C	23.4	24.4	23.8
	D	24.8	23.5	22.9
	E	22.6	24.8	23.6
	Mean	*23.3*	*24.0*	*23.4*
	SD	*0.9*	*0.6*	*0.6*
	Within-Run Precision	**3.9%**	2.5%	2.6%
Medium (150 mg/dL)	A	153	155	148
	B	155	160	151
	C	150	163	153
	D	151	162	152
	E	153	160	152
	Mean	*153*	*160*	*151*
	SD	*2.0*	*3.0*	*1.9*
	Within-Run Precision	*1.3%*	***1.9%***	*1.3%*
High (350 mg/dL)	A	332	320	375
	B	346	320	367
	C	342	320	368
	D	350	322	350
	E	352	323	370

	Mean	*344*	*321*	*366*
	SD	*8.1*	*1.5*	*9.4*
	Within-Run Precision	*8.1%*	*1.5%*	***9.4%***

Table J.6 shows the within-run precision for each concentration. The largest within-run precision for each concentration pool is indicated (bold font). None exceeds the standard test method's 10% within-run precision limits.

Next, the laboratory calculated the between-run precision. They first determined the mean and standard deviation for all observations of each concentration pool (Table J-7).

The between-run precision was calculated for all observations of each concentration pool using the following formula:

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

For example, the between-run precision for the 25 ng/dL concentration pool was calculated for ethanol as:

$$\text{Within-run CV}(\%) = \frac{0.17}{23.6} \times 100$$

$$\text{Within-run CV}(\%) = 0.7\%$$

Table J.7 shows between-run precision results for each concentration pool.

Table J.7—Ethanol Between-Run Precision

Concentration Pool	Replicate	Run 1	Run 2	Run 3	Mean	SD	Between-Run Precision
Low (25 mg/dL)	A	22.4	23.6	22.7	23.6	0.17	*0.7%*
	B	23.5	23.8	24.2			
	C	23.4	24.4	23.8			
	D	24.8	23.5	22.9			
	E	22.6	24.8	23.6			
Medium (150 mg/dL)	A	153	155	148	155	0.61	*0.4%*
	B	155	160	151			
	C	150	163	153			
	D	151	162	152			
	E	153	160	152			
High (350 mg/dL)	A	332	320	375	344	4.2	*1.2%*
	B	346	320	367			
	C	342	320	368			
	D	350	322	350			
	E	352	323	370			

The standard test method requires ethanol's between-run precision not to exceed 10%. The laboratory demonstrated that it could successfully use the method within these precision requirements.

Annex K
(informative)

Examples of Processed Sample Stability Assessment

K.1 Example of Processed Sample Stability Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method uses isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Per Annex J in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory evaluated the stability of samples in order to determine the length of time a sample may be stored before it undergoes unacceptable changes.

From the concentration pools prepared for the bias and precision experiments, the laboratory chose one low concentration pool (3 ng/mL) and one high concentration pool (180 ng/mL) for the processed sample stability assessment. The laboratory extracted 12 different aliquots from each concentration pool. After extraction, the 12 low aliquots were combined, mixed, and divided into 12 autosampler vials; the same was done for the high. All aliquots were placed into the autosampler at 4°C. One set of aliquots (low and high) was injected in triplicate immediately after extraction (time zero). Individual aliquots were injected in triplicate at 6 hours, 12 hours, 24 hours, 48 hours, and 66 hours after extraction. Results were deemed acceptable if the average relative peak area changed by no more than 20% from the average time zero relative peak area for at least 24 hours.

Table K.1 shows the average relative peak areas for each time point for the two concentration pools. The average change is also indicated in this table.

Table K.1—Experiments performed to evaluate processed sample stability

Time	Conc Pool	Fent	Avg Change (%)	Norfent	Avg Change (%)	Fent-D_5	Avg Change (%)	Norfent-D_5	Avg Change (%)
Zero	Low	4,850	N/A	5,100	N/A	49,500	N/A	48,600	N/A
	High	98,500	N/A	102,400	N/A	49,250	N/A	49,500	N/A
6 hrs	Low	5,200	+7.2	5,025	-1.5	50,100	+1.2	49,600	+2.1
	High	101,000	+2.5	99,570	-2.8	49,350	+0.2	48,200	-2.6
12 hrs	Low	4,970	+2.5	4,850	-4.9	51,000	+3.0	50,250	+3.4
	High	99,700	+1.2	98,600	-3.7	50,400	+2.3	50,200	+1.4
24 hrs	Low	4,850	0.00	4,950	-2.9	50,200	+1.4	50,100	+3.1
	High	98,200	-0.3	99,300	-3.0	50,100	+1.7	49,900	+0.8
48 hrs	Low	4,500	-7.2	4,500	-11.8	45,000	-9.1	45,000	-7.4
	High	90,000	-8.6	90,000	-12.1	45,000	-8.6	45,000	-9.1
66 hrs	Low	4,100	-15.5	4,080	-20.0	41,050	-17.1	42,300	-13.0
	High	82,090	-16.7	83,040	-18.9	42,070	-14.6	42,900	-13.3

The laboratory determined that fentanyl, norfentanyl, fentanyl-D_5, and norfentanyl-D_5 were stable after extraction from antemortem blood for at least 66 hours, the longest interval tested, after storage in 4°C. Therefore, the laboratory will not, under any circumstances, analyze samples more than 66 hours after extraction.

Annex L
(informative)

Examples of Assessing Rates of False Positive and False Negative

L.1 Example of False Positive and False Negative Rates Assessment for Method Validation

A laboratory recently developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method uses isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS).

Since the laboratory will allow qualitative reporting of results in some instances (e.g., quantitative failures of QCs), Annex K in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, required the laboratory to assess the method's rates of false positives and false negatives. Section K.3 was followed since a decision point concentration was not used as the method's limit of detection. The laboratory requires that the method has a claimed false result rate of no more than 10% at a 95% (requiring at least 29 samples to be tested) or better confidence level for it to be fit for purpose; however, they will evaluate at least 59 samples in hopes to achieve a 5% false result rate at a 95% confidence level.

Ten (10) unique sources of blank antemortem blood were obtained. Each source was divided into two subsamples – "N" and "P". Subsample N from each unique source served as the "negative" samples. Six aliquots of each negative sample were extracted and analyzed, producing results for 60 extracted negative samples.

"True negatives" (TN) were those results that a) did not contain a peak within the established retention time window for fentanyl (F) or norfentanyl (NF) or b) did not have the appropriate corresponding mass spectral product ions at the established ion ratios.

In contrast, "false positives" (FP) were those results that a) did contain a peak within the established retention time window for fentanyl or norfentanyl and b) had the appropriate corresponding mass spectral product ions at the established ion ratios.

All "negative" fentanyl samples were true negatives, and one of the "negative" norfentanyl samples were false positives. See Table L.1.

Table L.1—Results for "Negative" Subsamples

Subsample	Aliquot											
	1		2		3		4		5		6	
	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF
1N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
2N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
3N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
4N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
5N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
6N	TN	TN	TN	**FP**	TN	TN	TN	TN	TN	TN	TN	TN
7N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
8N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
9N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN
10N	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN	TN

Each of the P subsamples was fortified with fentanyl and norfentanyl at 1.5 ng/mL (50% above the method's limit of detection of 1.0 ng/mL) and served as the "positive" samples. Six aliquots from each positive sample were extracted and analyzed, producing results for 60 extracted positive samples.

"True positives" (TP) were those results that a) did contain a peak within the established retention time window for fentanyl or norfentanyl and b) had the appropriate corresponding mass spectral product ions at the established ion ratios.

In contrast, "false negatives" (FN) were those results that a) did not contain a peak within the established retention time window for fentanyl or norfentanyl or b) did not have the appropriate corresponding mass spectral product ions at the established ion ratios.

Two "positive" fentanyl samples were false negatives, and one of the "positive" norfentanyl samples was falsely negative. See Table L.2.

Table L.2—Results for "Positive" Subsamples

Subsample	Aliquot											
	1		2		3		4		5		6	
	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF
1P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
2P	**FN**	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
3P	TP	TP	TP	TP	TP	TP	TP	TP	**FN**	TP	TP	TP
4P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
5P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
6P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
7P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	**FN**
8P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
9P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP
10P	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP	TP

According to Table K.1 of ANSI/ASB Standard 036, with no false positive results for fentanyl after analyzing 60 "negative" samples, the laboratory can claim the method has a fentanyl false positive rate of no more than 5% at a 95% confidence level.

With one false positive result for norfentanyl after analyzing 60 "negative" samples, the laboratory can claim the method has a norfentanyl false positive rate of no more than 5% at a 95% confidence level.

Likewise, with one false negative result for norfentanyl after analyzing 60 "positive" samples, the laboratory can claim the method has a norfentanyl false negative rate of no more than 5% at a 95% confidence level.

However, since the laboratory had two false negative results for fentanyl after analyzing 60 "positive" samples, the best claim they can make is that the method has a fentanyl false negative rate of no more than 10% at a 95% confidence level.

All false result rates comply with the performance required by the laboratory.

L.2 Example of False Positive and False Negative Rates Assessment for Method Verification

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual column gas chromatograph with flame ionization detectors.

Per Annex K in ANSI/ASB Standard 036*, Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory verified its false positive and negative rates using the standard test method. The standard test method declares that, when used qualitatively, the technique is capable of achieving false positive and false negative rates that do not exceed 5% at a 99% confidence level. The method's published limit of detection is 5 mg/dL. Since the LOD of a standard test method is used as an administratively-defined decision point, Section K.3 of ANSI/ASB Standard 036 was followed.

First, a single blank postmortem blood sample was fortified with ethanol at 5 mg/dL and analyzed three times to establish the average response ratio to the internal standard for the decision point concentration.

Next, fifteen (15) additional unique sources of blank postmortem blood were obtained. Each source was divided into two subsamples – "N" and "P". Subsample N from each source was fortified with ethanol at a concentration of 3 mg/dL (~50% below the method's limit of detection of 5 mg/dL) and served as the "negative" samples. Six aliquots of each negative sample were extracted and analyzed using the standard test method, producing results for 90 negative samples.

"True negatives" (TN) were those results that did not contain a peak within the established retention time window for ethanol (EtOH) with a response ratio to the internal standard that exceeded the average response ratio for the decision point concentration.

In contrast, "false positives" (FP) were those results that contained a peak within the established retention time window for ethanol with a response ratio to the internal standard that exceeded the average response ratio for the decision point concentration.

One "negative" ethanol sample gave a false positive result. All others were true negatives. See Table L.3.

Table L.3—Results for "Negative" Subsamples

Subsample	Aliquot					
	1	2	3	4	5	6
1N	TN	TN	TN	TN	TN	TN
2N	TN	TN	TN	TN	TN	TN
3N	TN	TN	TN	TN	TN	TN
4N	TN	TN	TN	TN	TN	TN
5N	TN	TN	TN	TN	TN	TN
6N	TN	TN	TN	TN	TN	TN
7N	TN	TN	**FP**	TN	TN	TN
8N	TN	TN	TN	TN	TN	TN
9N	TN	TN	TN	TN	TN	TN
10N	TN	TN	TN	TN	TN	TN
11N	TN	TN	TN	TN	TN	TN
12N	TN	TN	TN	TN	TN	TN
13N	TN	TN	TN	TN	TN	TN
14N	TN	TN	TN	TN	TN	TN
15N	TN	TN	TN	TN	TN	TN

Each of the P subsamples was fortified with ethanol at 7 mg/dL (~50% above the method's limit of detection of 5 mg/dL) and served as the "positive" samples. Six aliquots from each positive sample were extracted and analyzed using the standard test method, producing results for 90 extracted positive samples.

"True positives" (TP) were those results that contained a peak within the established retention time window for ethanol with a response ratio to the internal standard that exceeded the average response ratio for the decision point concentration.

In contrast, "false negatives" (FN) were those results that did not contain a peak within the established retention time window for ethanol with a response ratio to the internal standard that exceeded the average response ratio for the decision point concentration.

None of the "positive" ethanol samples provided false negative results. All were true positives. See Table L.4.

Table L.4—Results for "Positive" Subsamples

Subsample	Aliquot					
	1	2	3	4	5	6
1P	TP	TP	TP	TP	TP	TP
2P	TP	TP	TP	TP	TP	TP
3P	TP	TP	TP	TP	TP	TP
4P	TP	TP	TP	TP	TP	TP
5P	TP	TP	TP	TP	TP	TP
6P	TP	TP	TP	TP	TP	TP
7P	TP	TP	TP	TP	TP	TP
8P	TP	TP	TP	TP	TP	TP
9P	TP	TP	TP	TP	TP	TP
10P	TP	TP	TP	TP	TP	TP
11P	TP	TP	TP	TP	TP	TP
12P	TP	TP	TP	TP	TP	TP
13P	TP	TP	TP	TP	TP	TP
14P	TP	TP	TP	TP	TP	TP
15P	TP	TP	TP	TP	TP	TP

According to Table K.1 of ANSI/ASB Standard 036, with one false positive result after analyzing 90 "negative" ethanol samples, the laboratory verified a false positive rate of no more than 5% at a 99% confidence level. Likewise, with no false negative results after analyzing 90 "positive" ethanol samples, they verified a false negative rate of no more than 5% at a 99% confidence level. These results demonstrated that the laboratory could successfully use the standard test method with acceptable false result rates.

Annex M
(informative)

Examples of Recovery Assessment

M.1 Example of Assessing Recovery for Method Development

A laboratory developed a quantitative method to analyze daridorexant in antemortem and postmortem blood samples. The method used an isotopically labeled internal standard, suvorexant-D_6, and a protein precipitation technique for sample clean-up. Analysis used liquid chromatography tandem mass spectrometry (LC-MS/MS).

Per Section L.1 of Annex L in ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory assessed recovery of the method's target analyte and internal standard during method development using the post-extraction addition technique. Antemortem blood and postmortem blood were treated as different matrix types.

Two different sets of samples were prepared.

"Set A" consisted of three unique blank sources of antemortem blood and nine unique blank sources of postmortem blood extracted in duplicate before fortification. The laboratory tripled the number of unique postmortem blank blood sources because of the variety of sample conditions typically encountered in postmortem toxicology. One replicate from each of the extracted "Set A" samples was then fortified at the low concentration (300 ng/mL) and the other replicate at the high concentration (4000 ng/mL). Suvorexant-D_6 was added to all "Set A" samples at the method's defined concentration (2000 ng/mL). Each sample was evaporated and reconstituted for LC-MS/MS analysis. The evaporation and reconstitution steps ensured consistency in the final reconstitution volume between Set A and Set B samples.

"Set B" consisted of the same three unique blank sources of antemortem blood and nine unique blank sources of postmortem blood fortified at the low (300 ng/mL) and high (4000 ng/mL) concentrations. Suvorexant-D_6 was added to all "Set B" samples at the method's defined concentration (2000 ng/mL). "Set B" samples were then extracted. After extraction, the samples were evaporated and reconstituted for LC-MS/MS analysis.

All samples from Set A and Set B were injected once. The peak areas of the low concentration, high concentration, and internal standard for each matrix set were averaged.

Percent recoveries were calculated using the following equation:

$$\text{Recovery (\%)} = \left(\frac{\text{Average Area of Set B}_X}{\text{Average Area of Set A}_X}\right) \times 100$$

Example calculation of daridorexant recovery at the low concentration (300 ng/mL) in Antemortem Blood:

$$\text{Recovery (\%)} = \left(\frac{\text{Average Area of Daridorexant Set B}_{\text{Low Antemortem}}}{\text{Average Area of Daridorexant Set A}_{\text{Low Antemortem}}}\right) \times 100$$

$$\text{Recovery (\%)} = \left(\frac{34466}{37981}\right) \times 100$$

$$\text{Recovery (\%)} = 90.7\%$$

Results are presented in Table M.1.

Table M.1—Recovery of Daridorexant and Suvorexant-D_6

Matrix Source	Analyte	Concentration	Average Area		Recovery
			Set A	Set B	
Antemortem Blood	Daridorexant	Low (300 ng/mL)	37981	34466	90.7%
		High (4000 ng/mL)	495716	439295	88.6%
	Suvorexant-D_6	2000 ng/mL	249164	234354	94.1%
Postmortem Blood	Daridorexant	Low (300 ng/mL)	41154	38012	92.4%
		High (4000 ng/mL)	531913	486510	91.5%
	Suvorexant-D_6	2000 ng/mL	254505	241577	94.9%

Percent recoveries ranged from 88.6% to 94.9%, which was deemed suitable by the laboratory.

Annex N
(informative)

Example of Efficient Method Validation Workflow

APRIL						
1	2	3	4	5	6	7
	Perform Experiments for Interferences	Review Data from Experiments for Interferences	Perform Ionization Suppression/ Enhancement Experiments	Review Data from Ionization Suppression/ Enhancement Experiments	Perform Calibration Model and Carryover Experiments (Set 1)	
8	9	10	11	12	13	14
	Perform Calibration Model and Carryover Experiments (Sets 2 and 3)	Perform Calibration Model and Carryover Experiments (Sets 3 and 4)	Review Data from the Calibration Model and Carryover Experiments	Prep for Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments	Perform Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments (Set 1)	
15	16	17	18	19	20	21
	Perform Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments (Set 2)	Perform Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments (Set 3)	Perform Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments (Set 4)	Perform Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments (Set 5)	Review Bias; Precision; LOD; LLOQ; and Dilution Integrity Experiments	
22	23	24	25	26	27	28
	Perform Processed Sample Stability Experiments	Perform Processed Sample Stability Experiments	Perform Processed Sample Stability Experiments	Review Data from Processed Sample Stability Experiments		
29	30					

Annex O
(informative)

Example Summaries of Experiment Results

O.1 Example Summary of Method Validation Results

A laboratory developed a quantitative method for extracting and analyzing fentanyl and norfentanyl in antemortem blood. The method used isotopically-labeled internal standards, fentanyl-D_5 and norfentanyl-D_5, and a solvent extraction with a back-extraction clean-up step. Analysis was performed using liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). In some instances (e.g., quantitative failures of QCs), the laboratory allows for results to be reported qualitatively.

Per Section 9.2 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a summary of the validation experiments conducted and their results in the form of a table that reiterates the method's scope, original validation plan, references where the validation raw data is stored, provides the validation results, and a statement as to the method's fitness for intended use, as well as any identified limitations. It also provides documentation of management review and approval (Table O.1).

1088 **Table O-1: Sample Summary of Method Validation Experiment Results for a Laboratory-Developed Test Method**

Validation Summary for ABC Laboratory			
Method: Fentanyl and norfentanyl in antemortem blood using solvent extraction and LC-MS/MS NOTE Printouts of the sample extraction procedure and instrumental parameters are attached.[3]			
Parameter:	***Number of Samples:***	***Acceptance Requirements:***	***Results and Limitations:***
Interferences	*Matrix Interferences:* — Ten (10) sources of blank antemortem blood *Internal Standard Interferences:* — One blank antemortem blood sample fortified with fentanyl-D_5 and norfentanyl-D_5 — One blank antemortem blood sample fortified with fentanyl and norfentanyl *Interferences from Common Analytes:* • Common recreational drugs of abuse and metabolites • Common prescription medications and metabolites • Common OTC drugs and metabolites	Interfering signals that will impact detection (e.g., retention time, peak shape, mass spectrometry ratios) and quantitation (>20% of the area of the lowest calibrator) must be addressed through laboratory procedures.	Interfering signals that impact detection (e.g., retention time, peak shape, mass spectrometry ratios) and quantitation were not observed for the blank antemortem blood sources analyzed, the deuterated internal standards, or the analytes of interest. Other common analytes tested did not interfere with norfentanyl; however, a methylacetylfentanyl interference was noted for fentanyl. As a result, a statement concerning this interferent will be included when reporting fentanyl positive results.

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[3] For the purposes of this example, the extraction procedure, instrumental parameters, and hardcopies of data are not attached to this validation summary.

Parameter:	*Number of Samples:*	*Acceptance Requirements:*	*Results and Limitations:*
Calibration Model	Six non-zero fentanyl and norfentanyl calibrators – between (and including) 1 and 200 ng/mL – prepared in blank antemortem blood over five runs. Fresh calibrators were prepared daily using a different source of blank antemortem blood.	The calibration range must be at least 1 – 200 ng/mL for fentanyl and norfentanyl. A linear model is desired but not required. The appropriate calibration model will be determined through the F-test and weighted residual plots.	A quadratic, $1/x^2$ calibration model was determined as the most appropriate calibration model for fentanyl. A linear, $1/x^2$ calibration model was most appropriate for norfentanyl.
Ionization Suppression/ Enhancement	Post-extraction addition approach was used. Ten unique blank antemortem blood sources were used to evaluate at two concentrations: 3 ng/mL and 160 ng/mL for fentanyl and norfentanyl. The internal standards were also be evaluated at 50 ng/mL.	Significant suppression or enhancement will be considered an average instrumental response that drops to less than 75%, increases to more than 125%, or has a % CV exceeding 20%. If significant suppression/enhancement occurs, the impact on LOD, LLOQ, bias, and precision will be assessed by at least tripling the number of unique sources of blank matrices used for their evaluation.	The method demonstrated ionization suppression within 25% for all tested analytes and internal standards at all concentrations, except for norfentanyl at the high concentration. All %CVs were less than 20%. The impact was further evaluated by tripling the unique sources of blank antemortem blood used to evaluate LOD, LLOQ, bias, and precision.
Bias	Three sources of blank antemortem blood were used to prepare the following concentration pools: low (3 ng/mL), medium (90 ng/mL), and high (180 ng/mL). Each concentration pool was analyzed in triplicate daily over five days.	Must be ± 20% or less for fentanyl and norfentanyl	The bias did not exceed ±20% for the concentrations evaluated from each unique source of antemortem blood.
Precision (within-run and between-run)		% CV must not exceed 20% for fentanyl and norfentanyl.	The within-run and between-run precisions did not exceed 20% for fentanyl and norfentanyl.
Dilution Integrity	Samples from the high-concentration pool (180 ng/mL) prepared from at least one of the blank sources of antemortem blood were diluted at two different ratios (1:2 and 1:10) and analyzed.	Samples prepared with each dilution ratio must meet the above bias and within-run precision requirements for the dilution ratio to be considered acceptable for use.	The bias and precision criteria were met for fentanyl at the 1:2 and 1:10 dilutions. For norfentanyl, the bias and precision criteria were met at the 1:2 dilution, but not at the 1:10 dilution. While 1:2 and 1:10 dilutions for fentanyl will be allowed, case samples may only be diluted 1:2 for norfentanyl quantitations.

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Parameter:	*Minimum Number of Samples:*	*Acceptance Requirements:*	*Results and Limitations:*
Carryover	An extracted blank antemortem blood sample was analyzed immediately following the highest extracted fentanyl and norfentanyl calibrator (200 ng/mL). This was repeated daily for five days.	For both fentanyl and norfentanyl, any carryover observed after the highest calibrator (200 ng/mL) cannot exceed 10% of the signal (relative peak area) of the lowest calibrator (1 ng/mL) and have all detection criteria met (e.g., retention time, peak shape, mass spectrometry ratios). If carryover is observed, QA measures will be implemented to mitigate.	Evidence of carryover with fentanyl was observed; therefore, extracted blank samples will be analyzed after each test specimen within a batch. There was no evidence of norfentanyl carryover observed.
Limit of Detection	The lowest non-zero calibrator (at least 1 ng/mL) was assigned as the LOD. Nine unique sources of blank antemortem blood were used to prepare different samples fortified at the same lowest non-zero concentration. Each calibrator sample (n=3) was analyzed over three different runs (n=9).	All detection and identification criteria must be met in at least 95% of the replicates.	All detection and identification criteria were met in 100% of the fentanyl replicates and 96.3% of the norfentanyl replicates, so the lowest calibrator concentration was established as the LOD for both analytes.
Lower Limit of Quantitation	The lowest non-zero calibrator (at least 1 ng/mL) was assigned as the LLOQ. Nine unique sources of blank antemortem blood was used to prepare samples fortified at the same lowest non-zero concentration. Each calibrator sample (n=3) was analyzed over three different runs (n=9).	Bias (±20%) and precision (≤ 20%) requirements must be met.	At the low calibrator concentration, the bias was -7% and -6% for fentanyl and norfentanyl, respectively. The precision was 11.6% and 8.5%, respectively. Therefore, the lowest calibrator can serve as the method's LLOQ for both analytes.

Parameter:	*Minimum Number of Samples:*	*Acceptance Requirements:*	*Results and Limitations:*
Processed Sample Stability	12 different aliquots of the 3 ng/mL low-concentration pool (prepared from at least one of the blank sources of antemortem blood) used for the bias and precision studies were freshly extracted. The extracts were combined, mixed, and divided into 12 autosampler vials. The first vial was immediately analyzed in triplicate. The second vial was analyzed in triplicate after 6 hours at autosampler temperature (4°C). The third vial after 12 hours, etc., for up to 66 hours. The same experiment was conducted with the high-concentration pool (180 ng/mL).	Relative peak areas of extracted samples of fentanyl and norfentanyl stored on the autosampler must remain stable (remain within ±20%) when compared to time zero for 12 hours or more.	Fentanyl, norfentanyl, fentanyl-D5, and norfentanyl-D5 were stable after extraction from antemortem blood for at least 66 hours at 4°C. Samples will not be analyzed more than 66 hours after extraction.
Rates of False Positives and False Negatives	At least 10 unique sources of blank antemortem blood were used. Each source of antemortem blood was divided into two subsamples. The first subsample ("negatives") from each antemortem blood source was extracted and analyzed 6 times each (n=60 or more). The second subsample ("positives") from each antemortem blood source was fortified with fentanyl and norfentanyl at 1.5 ng/mL. Each fortified antemortem blood subsample was extracted and analyzed 6 times each (n=60 or more). Rates of false results were based on Table K-1 of ASB 036.	Assess False Negative and False Positive Rates at a 95% confidence level with a minimum of 60 data points. The rate will not exceed 10% for the method to be considered acceptable.	The false positive rates for both fentanyl and norfentanyl were not greater than 5% at a 95% confidence level. The false negative rate for norfentanyl was also not greater than 5% at a 95% confidence level. The false negative rate for fentanyl was determined to be not greater than 10% at a 95% confidence level.
Method's Fitness for Use:	*With the above limitations noted, this method has been determined to be fit for its intended use.*		
I have reviewed this summary and approve the method for use:		*Name and signature of approver*	*Date of approval*

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O.2 Example Summary of Method Verification Results

A laboratory implemented an unmodified standard test method to quantitate ethanol in postmortem blood using a headspace autosampler attached to a dual-column gas chromatograph with flame ionization detectors. The method relied on the use of an n-propanol internal standard. The standard test method declared that it could meet the following validation parameters.

- *Interferences:* No interferences from antemortem blood or other common volatile compounds
- *Calibration Range and Model:* Undefined calibration model using six calibrators between 10 mg/dL and 400 mg/dL.
- *Limit of Detection:* 5 mg/dL.
- *Lower Limit of Quantitation:* 10 mg/dL.
- *Bias:* ± 10% or less.
- *Precision (within-run):* <10%.
- *Precision (between-run):* <10%.
- *Carryover:* None observed at 400 mg/dL.
- *Processed Sample Stability:* Stable for up to 72 hours after preparation.
- *False Positive Rates (if used qualitatively):* Not greater than 5% at a 99% confidence level.
- *False Negative Rates (if used qualitatively):* Not greater than 5% at a 99% confidence level.

Per Section 9.2 of ANSI/ASB Standard 036, *Standard for Test Method Selection, Development, Validation, and Verification in Forensic Toxicology*, the laboratory creates a summary of the verification experiments conducted and their results in the form of a bulleted list that reiterates the method's scope, original verification plan, references where the verification raw data is stored, provides the verification results, and a statement as to the method's fitness for intended use, as well as any identified limitations. It also provides documentation of management review and approval (Table O.1).

Figure O.2—Sample summary of method verification experiment results for a standard test method used without modification

Verification Summary for Laboratory ABC
Method: Standard Test Method 123-25 for Postmortem Blood
As described below, the following were determined or verified for this standard test method. Results are provided. • Calibration Model: The following calibrators were used: 10 mg/dL; 50 mg/dL; 100 mg/dL; 200 mg/dL; 300 mg/dL; and 400 mg/dL. The matrix of the calibrators were aqueous, as within the standard test method. A separate calibration curve was prepared once per day over five days to establish the appropriate calibration model. o **A linear, unweighted calibration model was most appropriate for the calibration range of 10 mg/dL to 400 mg/dL.** • Limit of Detection: Blank postmortem blood samples from five unique sources were fortified with ethanol at 5 mg/dL. Each 5 mg/dL postmortem blood sample were analyzed in triplicate over three runs (a total of 45 analyses). The results for each sample were evaluated to determine if at least 43 of the 45 analyses (≥95%) achieved the appropriate detection criteria (e.g., retention time, peak shape, signal-to-noise). o **All of the 5 mg/dL samples analyzed were detected; the LOD requirements for the standard test method were met.** • Lower Limit of Quantitation: Blank postmortem blood samples from five unique sources were fortified with ethanol at 10 mg/dL. Each 10 mg/dL postmortem blood sample were analyzed in triplicate over three runs (a total of 45 analyses). The results for each sample were evaluated to determine if the appropriate bias (± 10% or less) and precision (%CV ≤10%) were met. o **A bias of 10% and a precision of 6.7% were determined. The LLOQ requirements for the standard test method were met.** • Bias and Precision: Three concentration pools were prepared, each with blank postmortem blood: Low Pool (25 mg/dL); Medium Pool (150 mg/dL); and High Pool (350 mg/dL). Each concentration pool were analyzed five times per run for three runs (a total of 15 analyses for each concentration pool). The results were used to verify that the calculated bias for each concentration pool was no more than ±10% for all samples analyzed, as defined in the standard test method. The results also verified that the calculated within-run and between-run precision for each concentration pool was no more than 10% for all samples analyzed, per the standard test method. o **Bias ranged between -5.6 % and 3.3% at the three concentration pools tested. The highest within-run precision was 9.4% and the highest between-run precision was 1.2%. The bias and precision requirements for the standard test method were met.** • Carryover: Three blank postmortem blood samples (from the same source) were be prepared. Each was analyzed sequentially following a high calibrator sample (400 mg/dL). This was repeated over two additional runs. The results from the blank matrix were evaluated to determine if any carryover occured that meets the detection criteria for ethanol (proper retention time, appropriate peak shape, appropriate signal-to-noise). o **No peaks in the blank postmortem blood samples that immediately followed the 400 mg/dL calibrator were observed, verifying the same performance for carryover, as defined within the standard test method.** • False Positive and False Negative Rates: A blank postmortem blood sample was fortified with ethanol at 5 mg/dL (the standard method's LOD) and analyzed three times to establish an average response ratio to the internal standard. Then, fifteen additional unique sources of blank postmortem blood were divided into two subsamples. The first subset of each unique blank postmortem blood source was fortified with ethanol at 3 mg/dL and analyzed six times each and served as the "negative" samples (total of 90 "negative" analyses). The second subset of each unique blank blood source was fortified with ethanol at 7 mg/dL and analyzed six times each and served as the "positive" samples (total of 90 "positive" analyses). The results were used to calculate false positive and false negative rates. o **False positive and false negative rates for ethanol were not greater than 5% at a 99% confidence level. These values agreed with those listed in the standard test method.** **The method is fit for its intended use. Hard copies of data are included with this summary document.** I, _____, have reviewed this summary and approve of the use of the above standard test method.

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