National Laboratory Certification Program
DRUG TESTING MATTERS

2023

Validating Gas Chromatography–Mass Spectrometry for Urine and Oral Fluid Drug Testing

*This is the updated third part of a five-part **Drug Testing Matters** series on urine and oral fluid drug testing method validation, originally published in 2019. This part covers validating the gas chromatography–mass spectrometry testing method. The first part covered validating immunoassay methods; the second part covered validating specimen validity tests (SVTs); the fourth part will cover validating additional mass spectrometry methods; and the fifth part will cover validation of oral fluid collection devices.*

Gas Chromatography–Mass Spectrometry Overview

Mass spectrometry (MS) is a technique to identify and quantify compounds based on measuring the mass-to-electric charge ratio (*m/z*) of their ionized molecules. The origins of MS date to the discoveries of two early pioneers of particle analysis, Eugen Goldstein and Wilhelm Wien, in the late 1800s. Goldstein observed that electrical and magnetic fields deflect canal rays (i.e., beams of positive ions), and Wien defined particle *m/z* ratios in comparison to hydrogen (1, 2). The acceleration of ions in an electrical field separates ionized particles of different masses. Acceleration is proportional to the molecule's charge and the electrical field strength and inversely proportional to the molecular mass, as follows (3):

$$a = E \times z / m$$

where a = acceleration, E = instrument electric field strength, z = molecular electrical charge, and m = molecular mass.

Today's mass spectrometers consist of the same basic components as their early predecessors, including ion source, mass analyzer, and detector. In a contemporary mass spectrometer, the ion source creates either positive- or negative-charged particles, which are then extracted, focused, and accelerated into a beam. The mass analyzer filters the beam using programmable electrical fields that deflect user-defined charged particles and prevent them from entering the mass detector. Finally, the mass detector records the abundance of each charged particle. The *m/z* values of the ion particles and their relative abundances generate a unique mass spectrum that is used for analyte identification.

MS instrumentation platforms vary based on the type of front-end or chromatography system, the method of ionization, and the mass analyzer. Chromatography systems include gas chromatography (GC) and liquid chromatography (LC). These systems can be customized by varying the flow rate, column size, and column matrix. MS ionization methods depend on the system configuration, and examples include atmospheric-pressure chemical ionization, chemical ionization, electron impact ionization, and electrospray ionization (ESI).

Over the years, many instrument options have evolved for mass analyzers, including quadrupole (single or multiple), ion trap, tandem, and time of flight. Single-quadrupole system operating modes include either full-scan or selected ion monitoring (SIM). Tandem MS (MS/MS) operating modes include full-scan or multiple reaction monitoring (MRM). Tandem mass spectrometers commonly include three quadrupoles (Q_1, Q_2, and Q_3), where Q_1 acts as a filter, only allowing user-defined precursor ions of a particular *m/z* value to pass; Q_2 is a collision cell that fragments the selected ions; and Q_3 scans or selects for the programmed product ion *m/z* transitions (ion fragments). In full-scan mode, data are acquired for all ion particles, whereas SIM and MRM monitor only user-defined ions and transitions, respectively.

GC-MS was invented in 1959 when researchers used GC to introduce samples into a mass spectrometer (4). For urine drug testing, sample preparation for GC requires adding internal standard(s), removing the aqueous phase, and—depending on the analyte—derivatizing to increase volatility. Internal standards typically consist of stable isotope-labeled analyte analogs, which are used to monitor recovery and quantify the analytes. The GC inlet vaporizes the prepared sample and introduces it into a stream of gas within the chromatography column. The sample analytes then partition between the column gas and liquid phases and elute from the column at separate and characteristic column retention times. The column output is coupled to the mass spectrometer inlet, and upon elution, the measurands undergo mass spectrometric analysis (*m/z* and abundance). For drug testing, drug or drug metabolite concentrations in the sample are the measurands.

When using single-quadrupole methods, mass detectors require chromatography to separate measurands from non-target interferences prior to full-scan or SIM mass filtering by the quadrupole. MS/MS methods use both chromatography and the Q_1 quadrupole precursor selection to separate the measurands from interferences before the Q_2 quadrupole collision and subsequent Q_3 MRM transition mass filtering and mass detection.

Drug testing laboratories use initial testing to eliminate "negative" specimens from consideration and to identify specimens that require confirmation or further testing. Initial testing may be performed using immunoassay or alternate technology initial tests (e.g., chromatographic mass spectrometric methods) for accurate and reliable identification of drugs of abuse or their metabolites. Positive initial tests are confirmed using chromatographic mass spectrometric identification tests to report a specimen as positive. Examples of chromatographic and mass spectrometric methods of analysis that may be used for alternate

technology initial tests and confirmatory tests include GC-MS, LC-MS, GC-MS/MS, two-dimensional GC-MS (GC/GC-MS), and LC-MS/MS.

The remainder of this publication focuses on validating GC-MS and GC-MS/MS methods for alternate technology initial tests and confirmatory tests. Validating other chromatographic separation and mass spectrometric methods and instruments will be covered in part four in this series.

GC-MS and GC-MS/MS Validation[a]

Industry Standards

The method validation requirements described in this article are defined by the Department of Health and Human Services (HHS) and the National Laboratory Certification Program (NLCP) (5-8) for HHS-certified laboratories that test donor specimens in compliance with the *Mandatory Guidelines for Federal Workplace Drug Testing Programs using Urine* (UrMG) and the *Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid* (OFMG). HHS-certified laboratories conduct forensic drug testing for federal agencies under Executive Order 12564 and Public Law 100-71 and for specific federally regulated industries. The HHS Mandatory Guidelines for Federal Workplace Drug Testing Programs affect all federal employees in a testing designated position, which is defined by each agency's Drug-Free Workplace Program.

Additional standards and guidance for forensic drug testing applications are published by the American National Standards Institute and the American Academy of Forensic Sciences Standards Board (ANSI/ASB) (9). The ANSI/ASB Standard 036 "Standard Practices for Method Validation in Forensic Toxicology" publication defines forensic toxicology validation practices, such as consensus standards, practice, and protocols, including quality assurance and quality control.

For Clinical Laboratory Improvement Amendments (CLIA) certification of clinical laboratories, the Centers for Medicare & Medicaid Services (CMS) requires laboratories to verify or establish performance specifications for any test system used by the laboratory on or after April 24, 2003 (10).

The goal of validating alternate technology initial test or confirmatory test methods is to provide objective data that (1) demonstrate that the method performs according to its intended use and (2) establish the method limitations under normal operating conditions.

Prior to implementing new or modified methods or instrumentation for use with testing donor specimens, laboratories are required to validate their performance. Some laboratories perform an alternate GC-MS, GC-MS/MS assay (e.g., to eliminate interferences when the desired specificity cannot be obtained using the primary method). These alternate assays are subject to the same method validation requirements as the primary assays. However, if the difference between the primary and alternate methods is relatively minor, studies must focus on assay characteristics that could be affected by the modifications to the primary method. For example, if the alternate method has a different temperature program to resolve an interfering compound, interference studies would be required to document the ability of the alternate assay to resolve the interference, but a precision/accuracy study would not be necessary.

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[a] *For clarity, this series of articles uses "validation" to cover all aspects of laboratory method performance assessments, including verification of unmodified Food and Drug Administration (FDA)-cleared and FDA-approved assays and the validation of laboratory-developed assays.*

Note: If a laboratory chooses to use an alternate technology initial test, the laboratory must contact the NLCP to arrange for NLCP review of the validation records. The laboratory may be required to submit the validation study data and summaries to the NLCP for review or may be required to provide validation records for review by the NLCP inspectors before implementing the method.

Initial tests, including alternate technology initial tests, are used to discriminate between positive and negative samples, whereas confirmatory drug tests are used to identify and quantify positive measurands. Performance characteristic measurements for GC-MS methods include the following:

- The effective linearity of the test
- The limit of detection and limit of quantification
- The precision and accuracy of the test around the cutoff and 40% of the cutoff
- The potential for carryover
- The specificity and potential for interfering substances
- The selection of method parameters including ion/transition selection
- The comparison of results using existing and new/revised procedures (i.e., parallel study)

Documentation

Validation records must include sufficient information to facilitate third-party comprehensive review of studies performed. The study summary and the laboratory's standard operating procedures (SOPs) must describe acceptance criteria for validation study data, agreement of replicate study samples, and defining or excluding true outlier values. Alternate technology initial test and confirmatory test study sample analysis must meet the same qualitative criteria (e.g., retention time, mass ratio, internal standard abundance, chromatography criteria) used for specimen analysis.

At a minimum, validation study records must include the following:

- A stated purpose for the validation
- Description of test methods
- Identity of the instrument(s) used for the study
- A listing of the instrument parameters used for the study
- A description of the study samples
- A summary of the statistical data collected to characterize the assay
- A discussion
- A summary with conclusions
- All raw analytical data from the samples analyzed in the study.

Laboratories must maintain the GC-MS and GC-MS/MS validation study records for an indefinite period. Records for validation studies performed within the last 12 months must be available for review during NLCP inspections.

Each "end-user laboratory" must perform the validation and periodic re-verification studies for its assays and instruments. Off-site validations performed by other entities (e.g., manufacturer, other laboratory) may be used only to provide additional documentation.

Types of Validations

The types of validation studies to be used depend on whether the laboratory is implementing a new test, a new instrument model, or an additional instrument of the same model. Examples of validations include assay, full instrument, abbreviated instrument, and re-verification.

Note: Where multiple instrument models are used for a GC-MS or GC-MS/MS assay, using the most conservative performance limits that can be determined for all models is acceptable, provided that this approach is described in the SOP and the validation study summaries.

Assay

Assay validation studies must be performed prior to use with regulated specimens for the following:

- A new primary or alternate method,
- A revised method,*
- Method calibration scheme changes, or
- Method chromatographic column selection.

**Note: It is usually necessary to perform complete validation studies for revised assays. However, if the modification is relatively minor, the validation studies may focus on those parameters that may have been affected.*

The following studies are required for assay validation for alternate technology initial tests and confirmatory tests using GC-MS, GC-MS/MS:

1. For confirmatory drug tests
   - Linearity and determination of the limit of quantification (LOQ) and upper limit of linearity (ULOL)
   - Determination of the limit of detection (LOD)
   - Precision and accuracy around each cutoff
   - Precision and accuracy around 40% of each cutoff
   - Carryover
   - Specificity and interference
   - Parallel study of NLCP Performance Testing (PT) samples and donor specimens using existing and new/revised methods
   - Method parameters including appropriate ion selection
   - Dilution integrity
   - Hydrolysis (if performed)

   Notes:

   - *Dilution integrity is performed only for confirmatory drug tests with routine specimen dilution of all samples.*
   - *Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s).*

2. For alternate technology initial tests only, studies in addition to confirmatory testing studies include positive and negative sample differentiation versus confirmatory testing.

   Notes:

   - *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drug tests (i.e., all parameters are the same).*
   - *Determining LOD is not required.*

Full Instrument

Full Instrument validation must be performed for at least one instrument *prior to* implementing:

- a new model of an instrument for use with a validated GC-MS, GC-MS/MS assay, or
- a new instrument component that may affect the analysis (e.g., a chromatographic column with a different phase or from a different manufacturer).

The following studies are required for full instrument alternate technology initial tests and confirmatory drug tests using GC-MS, GC-MS/MS:

1. For confirmatory drug tests
   - Linearity and determination of the LOQ and ULOL
   - Determination of the LOD
   - Precision and accuracy around each cutoff
   - Precision and accuracy around 40%
   - Carryover
   - Specificity and interference
   - Parallel study of NLCP PT samples and donor specimens using existing and new/revised methods
   - Parameter optimization
   - Dilution integrity

   Notes:

   - *Dilution integrity performed only for confirmatory drug tests with routine specimen dilution of all samples*
   - *Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s).*
2. For alternate technology initial tests only, studies in addition to confirmatory testing studies include positive and negative sample differentiation versus confirmatory testing.

   Notes:

   - *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drug tests (i.e., all parameters are the same).*
   - *Determining LOD is not required.*

Abbreviated Instrument

Abbreviated Instrument validation must be performed before implementing each additional instrument of the same model that has been previously validated by the laboratory. For an additional confirmatory drug test instrument, the same model has:

- The same chromatographic and mass spectrometric instrument models,
- The same model components (e.g., same model pump and autosampler), and
- The same phase and manufacturer column.

The following studies are required for abbreviated instrument validation for alternate technology initial tests and confirmatory drug tests using GC-MS, GC-MS/MS:

1. For confirmatory drug tests
   - Determination of the LOD, LOQ, and ULOL
   - Carryover
   - Specificity and interference
   - Parameter optimization
2. For alternate technology initial tests only, studies in addition to confirmatory testing studies: Positive and negative sample differentiation versus confirmatory testing.

   Notes:

   - *Positive/negative differentiation studies are not necessary if the laboratory uses the same method for initial and confirmatory drugs tests (i.e., all parameters are the same).*
   - *Determining LOD is not required.*

Re-verification

Re-verification must be performed at least annually. Studies must be performed for each model of the instrument. For confirmatory drug test instrument, the same model has:

- The same chromatographic and mass spectrometric instrument models,
- The same model components (e.g., same model gas chromatograph and autosampler), and
- The same phase and manufacturer of column.

The following studies are required for re-verification for alternate technology initial tests and confirmatory drug tests using GC-MS, GC-MS/MS:

1. For confirmatory and alternate technology initial drug testing
   - Determination of the LOD, LOQ, and ULOL
   - Carryover
   - Specificity/interference
   - Hydrolysis (if performed)

   Note: LOD studies are not required for alternate technology initial drug methods.

Minimum General Study Requirements for Assay Validation, Full Instrument Validation, and Abbreviated Instrument Validation

LOD for Confirmatory Drug Tests

The laboratory must characterize the LOD of the confirmatory assay using *at least five replicates* for decreasing concentrations of the measurand. It is not acceptable to analyze the same sample five times. The LOD is the lowest concentration at which the identity (but not concentration) of the measurand can be accurately established. The LOD must be below 40% of the cutoff. The LOD may be assigned a more conservative value than supported by the validation studies. However, this value must reflect the concentration of a sample analyzed in the linearity studies. Values extrapolated from data or those that do not meet all the acceptance criteria may not be used as an LOD.

Note: The LOD validation is not required for alternate technology initial tests; however, laboratories must be able to quantify drug analytes at or below 40% of the cutoff.

Linearity Studies (LOQ, ULOL)

The laboratory must characterize the linearity of the assay using *at least five replicates* for *at least seven concentrations* of the measurand. It is not acceptable to analyze the same sample five times. The LOQ and ULOL are the lowest and highest concentrations, respectively, at which the identity and concentration of the measurand can be accurately established. The LOQ must be below 40% of the cutoff. Quantitative acceptance criteria for study samples include an acceptance range of ±20% from the target concentration. The laboratory may establish a ULOL or LOQ at a more conservative value than supported by the validation studies. However, these values must reflect the concentration of a sample analyzed in the linearity studies. Values extrapolated from data or those that do not meet all acceptance criteria may not be used as an LOQ or ULOL.

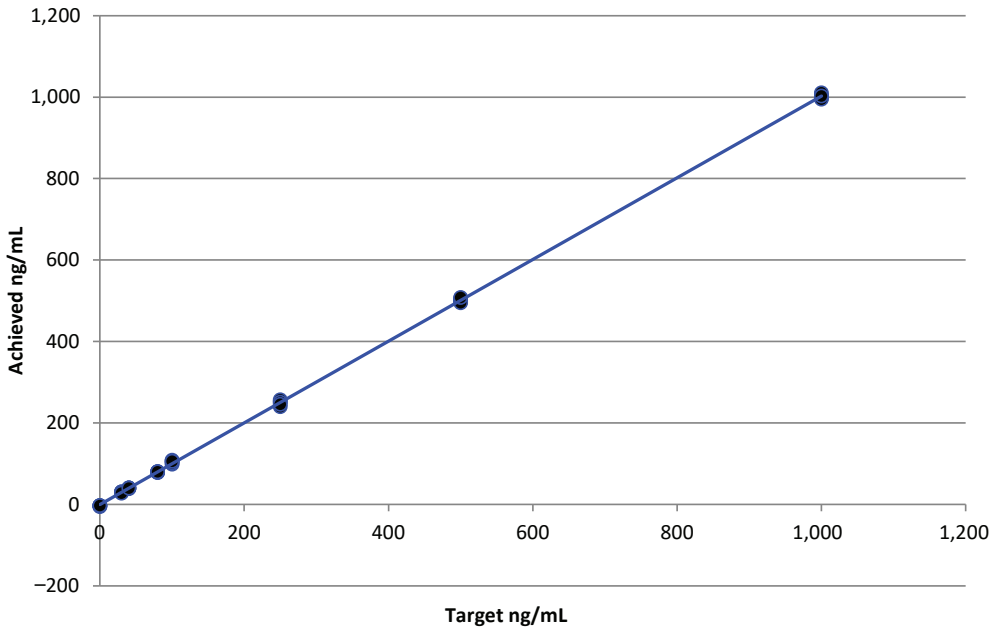
It is not acceptable to use batch calibrators as linearity study samples and to use the linearity of the resulting calibration curve as a demonstration of linearity. All study samples must be analyzed as if they were routine undiluted specimens using the same qualitative criteria (e.g., the same retention time, mass ratio, and chromatography criteria) used for donor specimens.

The concentrations should be distributed as:

- A minimum of three below the cutoff (at least one concentration below 40% of cutoff),
- One equivalent to the cutoff, and
- A minimum of three above the cutoff.

The linearity results must be plotted for review (e.g., achieved concentration units on the Y axis against target concentration on the X axis).

Exhibit 1. Example of GC-MS Linearity Study



Target ng/mL	Linearity 100 ng/mL Cutoff					Results			LOD LOQ ULOL
	1	2	3	4	5	Mean	−2SD	+2SD	
0	−3.0	−3.4	−2.7	−5.2	−2.9	−3.4	−5.5	−1.4	
30	31.3	29.1	30.2	30.5	27.7	29.8	27.0	32.6	LOD, LOQ
40	38.9	39.9	40.7	41.2	39.8	40.1	38.3	41.9	
80	79.9	79.5	78	80.7	79.8	79.6	77.6	81.6	
100	109	99	107	102	106	105	97	113	
250	253	240	257	251	246	249	236	263	
500	502	499	506	495	508	502	492	512	
1,000	1,005	998	995	1,011	1,003	1,002	990	1,015	ULOL

Precision and Accuracy Studies

The laboratory must characterize the precision of the assay using *at least five replicates* for *at least five concentrations* of the measurand at critical concentrations relative to the cutoff. It is not acceptable to analyze the same sample five times. The precision data are evaluated by calculating the mean, standard deviation (SD), and coefficient of variation (CV). The study samples must exhibit the appropriate response relative to one another (i.e., study samples should yield appropriate responses versus the cutoff and relative to the other study samples). The laboratory must also characterize the accuracy (expressed as bias) of the assay by calculating the percentage difference between each analyzed sample result and the target concentration. It is acceptable to use the data from the precision study analysis for the accuracy study. The precision and accuracy studies may be performed using separate batches on multiple days to assess the intra-batch and inter-batch variability.

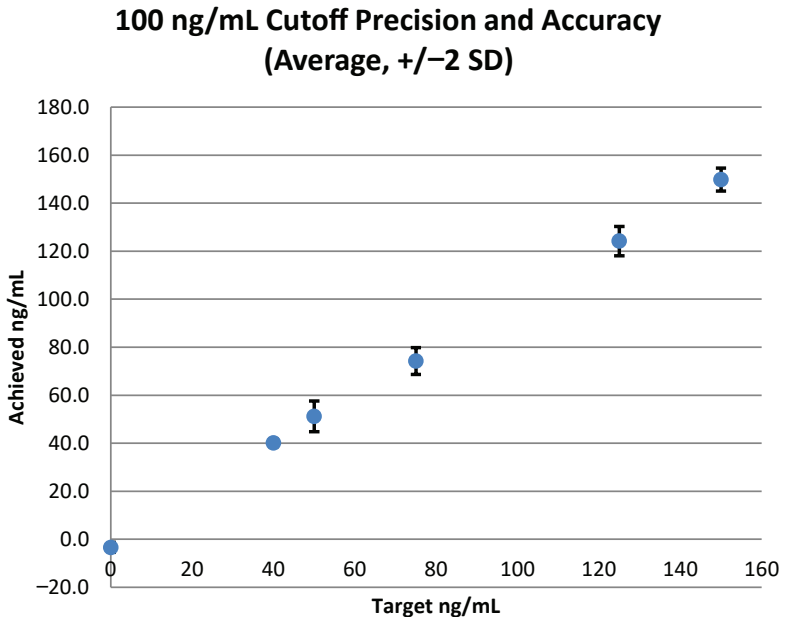
The concentrations relative to the cutoff should be distributed as critical points:

- 0% of cutoff
- 50% of cutoff
- 75% of cutoff
- 125% of cutoff
- 150% of cutoff

  Notes:

  - *Precision and accuracy around 40% of the cutoff of the assay are also required.*
  - *The laboratory must establish criteria for evaluating the statistical analysis. These must be described in the validation study summary.*

Exhibit 2. Example of GC-MS Precision and Accuracy Study



Precision and Accuracy 100 ng/mL Cutoff				
Target ng/mL	**Reps**	**Result ng/mL**	**Accuracy %**	**Precision**
0 ng/mL *0% of cutoff*	1	−2.7	N/A	SD: 1.1 ng/mL
	2	−3.4		
	3	−2.7		
	4	−5.2		
	5	−2.9		
40 ng/mL *40% of cutoff*	1	39.3	−1.8%	SD: 0.8 ng/mL CV: 1.9%
	2	39.7	−0.7%	
	3	40.6	+1.5%	
	4	41.2	+3.0%	
	5	39.8	−0.5%	
50 ng/mL *50% of cutoff*	1	51.1	+2.2%	SD: 3.2 ng/mL CV: 6.3%
	2	52.7	+5.4%	
	3	54.9	+9.8%	
	4	48.8	−2.4%	
	5	47.6	−4.8%	

Precision and Accuracy 100 ng/mL Cutoff				
Target ng/mL	**Reps**	**Result ng/mL**	**Accuracy %**	**Precision**
75 ng/mL *75% of cutoff*	1	72.5	−3.3%	SD: 2.8 ng/mL CV: 3.8%
	2	78.5	+4.7%	
	3	71.2	−5.1%	
	4	74.0	−1.3%	
	5	75.2	+0.3%	
125 ng/mL *125% of cutoff*	1	124	−0.8%	SD: 3.0 ng/mL CV: 2.4%
	2	126	+0.8%	
	3	123	−1.6%	
	4	120	−4.0%	
	5	128	+2.4%	
150 ng/mL *150% of cutoff*	1	151	+0.7%	SD: 2.4 ng/mL CV: 1.6%
	2	153	+2.0%	
	3	148	−1.3%	
	4	150	±0.0%	
	5	147	−2.0%	

Carryover Studies

The laboratory must characterize the potential for one sample to carryover to another during testing. The laboratory should perform these carryover studies by analyzing highly concentrated samples followed by negative samples (i.e., without the measurand of interest) and evaluating the negative samples for carryover. The measurand concentrations in the high samples should be realistic (i.e., high concentrations that may be found in the testing population) and at least as high as the established ULOL. The ULOL is the highest value at which carryover may be assigned.

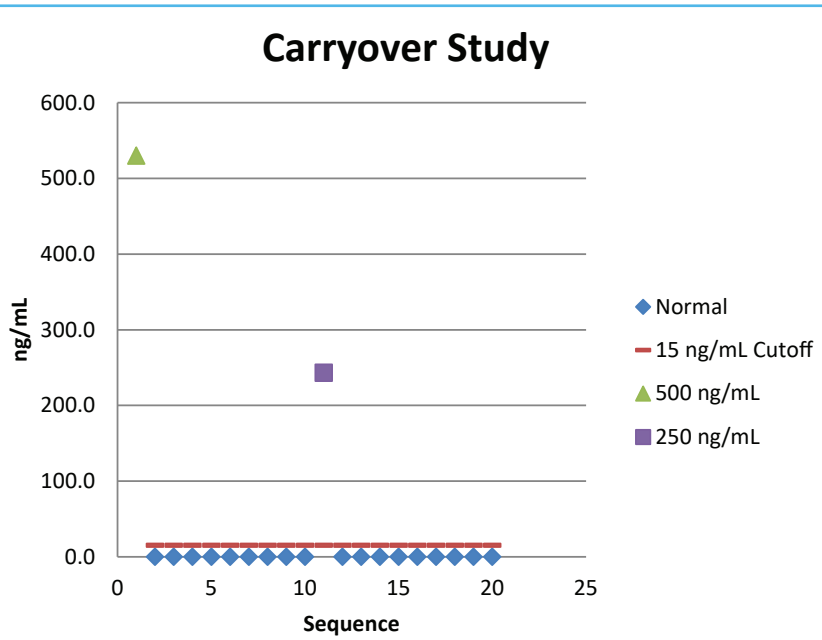
In some cases, carryover may not be demonstrated until the concentration exceeds the assay ULOL. When this occurs, the observed concentration is no longer an accurate quantitative value and is not an appropriate value to use as a prompt for corrective action. In such cases, *the laboratory must assign the carryover limit to be the same value as the ULOL*.

If the laboratory plans to use multi-well plates, the SOP must describe measures to prevent carryover in the well plate during pipetting. If evaporators are used with multi-well plates, the laboratory must perform a carryover study to ensure no cross-contamination occurs (e.g., fluorescence test).

Notes:

- *If solvent blanks are routinely injected between donor samples and the laboratory has objective criteria for reviewing the blank data, a carryover study is not required. If this approach is used, it must be described in the SOP.*
- *The laboratory must establish criteria (i.e., allowable response or concentration) for evaluating the negative sample tested after a high sample in carryover studies. These must be described in the validation study summary and the SOP.*
- *For use of multi-well plates, it is not acceptable to remove plate caps or plate mats*.

Exhibit 3. Example of GC-MS Carryover Study



Carryover Check Samples		ng/mL	
		Sample Result	Cutoff Calibrator
1	500 ng/mL THCA	530.0	15.0
2	Normal Urine	0.0	15.0
3	Normal Urine	0.0	15.0
4	Normal Urine	0.0	15.0
5	Normal Urine	0.0	15.0
6	Normal Urine	0.0	15.0
7	Normal Urine	0.0	15.0
8	Normal Urine	0.0	15.0
9	Normal Urine	0.0	15.0
10	Normal Urine	0.0	15.0
11	250 ng/mL THCA	243.0	15.0
12	Normal Urine	0.0	15.0
13	Normal Urine	0.0	15.0
14	Normal Urine	0.0	15.0
15	Normal Urine	0.0	15.0
16	Normal Urine	0.0	15.0
17	Normal Urine	0.0	15.0
18	Normal Urine	0.0	15.0
19	Normal Urine	0.0	15.0
20	Normal Urine	0.0	15.0

Specificity and Interference Studies

The laboratory must characterize the assay performance when challenged with compounds commonly encountered in the testing population. Whether the presence of a compound would reduce or increase the concentration of the measurand is of concern. Assays should include appropriate ions that prevent measurand misidentification and chromatographic column selection and temperature programming that prevents interference from co-eluting compounds. It may be necessary to validate an alternate method for specimens exhibiting interference that cannot be resolved using the primary method. In addition to selecting appropriate ions and transitions to prevent analyte misidentification, assay modifications such as temperature program changes may be needed to prevent interference from co-eluting compounds.

Compounds to be evaluated include illicit drugs and over-the-counter and prescription drugs at concentrations exceeding those encountered with therapeutic doses. Interference studies must be performed by analyzing samples containing interferents in the presence of the measurands at 40%* of the cutoff and without the measurand (see below for separate urine and oral fluid criteria).

**Note: Enantiomer (amphetamine and methamphetamine) interference studies must be performed by analyzing samples containing interferents in the presence of the measurands (d-methamphetamine, l-methamphetamine, d-amphetamine, and l-amphetamine) at a defined ng/mL and without the measurand (see below for separate urine and oral fluid criteria).*

For alternate technology initial drug tests with no hydrolysis, the laboratory must monitor the free drug and the drug as the glucuronide in the interference study for analytes that may be in the conjugated form (i.e., Δ-9-tetrahydrocannabinol-9-carboxylic acid (THCA), codeine, morphine, oxymorphone, and hydromorphone).

The **minimum** requirements for multi-analyte assay interference studies include the following concentrations and drugs to test:

Urine

- 6-acetylmorphine assays
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: hydrocodone, hydromorphone, oxycodone, oxymorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Amphetamine (amphetamine, methamphetamine, MDMA, and MDA) assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - Structurally similar compounds such as substituted phenethylamines in amphetamine assay interference studies to evaluate and document the effects of such compounds on amphetamine analysis
- Amphetamine and methamphetamine assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine

  - 5,000 ng/mL: MDA, MDMA
  - Structurally similar compounds such as substituted phenethylamines in amphetamine assay interference studies to evaluate and document the effects of such compounds on analysis for the analytes of interest.
- Enantiomer (amphetamine, methamphetamine) assays
  - Samples with 50 ng/mL: d-methamphetamine, l-methamphetamine, d-amphetamine, l-amphetamine, and samples without amphetamine or methamphetamine
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - 5,000 ng/mL: MDA, MDMA
  - Structurally similar compounds such as substituted phenethylamines are not required for enantiomer assays.
- MDA or MDMA assays
  - 50,000 ng/mL: phentermine
  - 1 mg/mL: phenylpropanolamine, ephedrine, pseudoephedrine
  - 5,000 ng/mL: amphetamine, methamphetamine
  - Structurally similar compounds such as substituted phenethylamines in amphetamine assay interference studies to evaluate and document the effects of such compounds on analysis for the analytes of interest.
- Codeine and morphine assays
  - 200 ng/mL: 6-acetylmorphine
  - 5,000 ng/mL: hydrocodone, hydromorphone, oxycodone, oxymorphone, norcodeine
- Opioid (codeine, morphine, oxycodone, oxymorphone, hydrocodone, hydromorphone, and 6-acetylmorphine) assays
  - 5,000 ng/mL: norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Oxycodone, oxymorphone, hydrocodone, and hydromorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Oxycodone and oxymorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine

  - 5,000 ng/mL: hydrocodone, hydromorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone
- Hydrocodone and hydromorphone assays
  - 200 ng/mL: 6-acetylmorphine
  - 40,000 ng/mL: free morphine, codeine
  - 5,000 ng/mL: oxycodone, oxymorphone, norcodeine, norhydrocodone
  - 1,000 ng/mL: noroxycodone, noroxymorphone

Oral Fluid

- Amphetamines (amphetamine, methamphetamine, MDMA, and MDA) assays
  - 10,000 ng/mL: ephedrine, pseudoephedrine, phentermine, 4-fluoromethamphetamine
  - Structurally similar compounds such as substituted phenethylamines in amphetamine assay interference studies to evaluate and document the effects of such compounds on amphetamine analysis
- Enantiomer (amphetamine, methamphetamine) assays
  - Samples with 5 ng/mL: d-methamphetamine, l-methamphetamine, d-amphetamine, l-amphetamine, and samples without amphetamine or methamphetamine
  - 10,000 ng/mL: phentermine, phenylpropanolamine, ephedrine, pseudoephedrine, MDA, MDMA
- Opioid (codeine, morphine, and 6-acetylmorphine) assays
  - 45 ng/mL: norcodeine, normorphine
  - 100 ng/mL: dextromethorphan, dextrorphan
- Oxycodone, oxymorphone, hydrocodone, and hydromorphone assays
  - 300 ng/mL: codeine, morphine, norcodeine, normorphine, norhydrocodone, norhydromorphone, noroxycodone, noroxymorphone
- Tetrahydrocannabinol (THC) assays
  - 2,000 ng/mL: cannabidiol
  - 100 ng/mL: delta 8-tetrahydrocannabinol (delta 8-THC)

Positive/Negative Differentiation Studies for Alternate Technology Initial Tests

The laboratory must characterize the ability of the assay to differentiate positive and negative samples using **at least two replicates of at least 10 positive** samples at different concentrations for each initial drug test measurand and **at least two replicates of at least 10 negative** samples (i.e., 40 results total for each analyte). The laboratory may analyze negative donor specimens or controls, negative specimens fortified with known amounts of the assayed measurands, non-NLCP proficiency testing samples, or discarded positive donor specimens.

Note: A positive/negative differentiation study is not necessary if the laboratory uses the same method for its initial and confirmatory drug tests (i.e., all parameters are the same).

Parallel Studies

Note: Parallel studies are not necessary if the laboratory does not have an existing assay for the analyte(s) in regulated specimens.

When the current and new methods use different technologies, the laboratory must conduct parallel studies that compare results of the new method to results from the existing procedure. The laboratory analyzes **at least 10** human specimens positive for the assay and the ***specimens from two PT cycles***. When donor samples are used, previously obtained values are used for the comparison (note that the laboratory may reanalyze positive donor specimens by the existing method if discrepant values could be caused by drug analyte stability issues). Results obtained using the new method should be within ±20% of the results using the existing method. Any discrepancies should be investigated and explained.

Human (donor specimen) study samples include:

- 5 specimens with concentrations between the cutoff and two times the cutoff, and
- 5 specimens with concentrations greater than two times the cutoff.

Additional notes:

- Use positive donor specimens for THC, marijuana metabolite (THCA), codeine, morphine, hydromorphone, and oxymorphone.
- Use samples spiked with non-conjugated measurands if positive donor specimens are not available for amphetamines, cocaine, cocaine metabolite, phencyclidine, 6-acetylmorphine, hydrocodone, and oxycodone. Spiked study samples may include:
  - 5 replicate samples prepared at one concentration that is between the cutoff and two times the cutoff, and
  - 5 replicate samples prepared at one concentration that is greater than two times the cutoff.

PT study samples include samples from the two most recent NLCP PT cycles (excluding those categorized as invalid, substituted, or adulterated).

Re-analyzing NLCP PT samples requires that the laboratory request permission to use the specimens to validate the new method. *If additional NLCP PT material is needed, submit another request to the NLCP.*

Method Parameters

Laboratories must perform a systematic evaluation of instrument parameters (e.g., mass analyzer settings, ion optics settings, chromatographic conditions, ion source settings) that would impact the analysis. The laboratory must document the evaluation and selection of ions to be monitored for drug measurands and internal standards.

Note: Staff responsible for method development and oversight must know the chemical structure of derivatives and select ions that represent the structural components that make the measurand a unique molecule.

The laboratory must use equivalent ions and transitions for the deuterated internal standard and non-deuterated measurand whenever possible. It is not appropriate to use trivial loss fragments, adduct ions (e.g., dimers), or derivatizing agent derived fragments. A stable isotope internal standard must be used for each analyte. It is not acceptable to use the same internal standard for multiple analytes.

Consider the following example: when using 4-CB (4-carbethoxyhexafluorobutyryl chloride) as the amphetamines' derivatizing reagent, laboratories should monitor the 91 or 118 ion for amphetamine because the higher *m/z* ions represent only the derivative fragment. Monitoring isotopes of a fragment is only acceptable when other suitable ions are not available.

Instrument Parameter Optimization

The laboratory must objectively evaluate instrument parameters to determine the optimal values for the particular configuration and manufacturer.

If a laboratory uses a tandem mass spectrometric method for both the initial and confirmatory drug tests, the laboratory should obtain enough information during method validation to develop appropriate acceptance criteria for each test (e.g., can use less stringent ratio criteria for the initial test). The laboratory must use at least one transition for the initial test. For confirmatory tests, the laboratory must use at least two transitions (i.e., at least one quantifier and one qualifier transition for the analyte) and evaluate the ratio of the abundance of these transitions for the target analyte. The same requirements apply to the internal standard. For single MS methods, the laboratory must use at least three ions for the analytes and two ions for the internal standard. These transitions/ions should be free of interferences and matrix effects and should be specific to the target analyte (e.g., should be a transition from the target analyte or minimally be a justifiable structure relative to the target analyte). The laboratory must provide a structural justification of the selected transitions.

Dilution Integrity

If the laboratory pre-dilutes all sample preparations for confirmatory testing method, a dilution integrity study must be performed to document that the dilution does not affect assay performance. These studies consist of the precision/accuracy studies using samples at the dilution specified in the SOP.

Note: The Dilution Integrity study must use at least five dilutions including the highest dilution used routinely by the laboratory.

Hydrolysis Studies

If the laboratory performs hydrolysis for alternate technology initial tests, a hydrolysis study must be performed. For confirmatory drug tests, hydrolysis is required for THCA and opioid confirmatory drug tests; therefore, the hydrolysis study is required.

Laboratories must demonstrate and document acceptable hydrolysis (i.e., at least 80% recovery). For THCA hydrolysis studies, the laboratory must analyze positive THCA donor specimens with and without hydrolysis. For codeine and morphine hydrolysis studies, the laboratory must accurately quantify at least 15,000 ng/mL of codeine as the glucuronide and morphine as the glucuronide (i.e., each with at least 15,000 ng/mL of the free drug). For oxymorphone and hydromorphone hydrolysis studies, the laboratories must accurately quantify at least 1,000 ng/mL of oxymorphone as the glucuronide and 1,000 ng/mL of hydromorphone as the glucuronide (i.e., each with at least 1,000 ng/mL of the free drug).

Note: Laboratories must document acceptable hydrolysis performance at the same concentrations in re-verification studies, unless the laboratory's hydrolysis controls included in each batch are at or above the specified concentrations.

Special Considerations Specific to Alternate Technology Initial Test Validation

- Determining an LOD is not required; however, laboratories must be able to quantify drug analytes at or below 40% of the cutoff. To meet this program requirement, the laboratory must establish an LOQ below 40% of the cutoff.
- Positive/Negative Differentiation Studies. The laboratory must analyze positive and negative samples that have been verified by the confirmatory drug test method to assess the ability of the assay to differentiate positive and negative samples. The laboratory may analyze a combination of negative donor specimens or controls, negative samples fortified with known amounts of the assayed drugs, non-NLCP proficiency testing samples, or discarded positive donor specimens. The laboratory should analyze a minimum of 10 positive samples at differing concentrations for each initial drug test analyte and 10 negative samples, in duplicate (i.e., 40 results for each analyte).

  Note: A positive/negative differentiation study is not necessary if the laboratory uses the same method for its initial and confirmatory drug tests (i.e., all parameters are the same).

Special Considerations Specific to Confirmatory Drug Test Validation

- Laboratories must be able to identify and quantify drug analytes at or below 40% of the cutoff. To meet this program requirement, the laboratory must establish an LOD and LOQ below 40% of the cutoff. NLCP PT samples challenge laboratories with drug analytes at approximately 40% of their confirmatory test cutoffs.
- For confirmatory drug test methods, laboratories are required to identify and accurately quantify at or below 40% of the cutoff. Therefore, laboratories must document the precision/accuracy around 40% of the cutoff and document the lack of interference of the assay in the presence of analyte at 40% of the cutoff.
- The required amphetamine/methamphetamine enantiomer validation study depends on whether the test is quantitative (i.e., determining quantitative values for methamphetamine enantiomers and, if analyzed, amphetamine enantiomers) or semi-quantitative (using area to determine the relative percentages of methamphetamine enantiomers and, if analyzed, amphetamine enantiomers).

Quantitative Enantiomer Tests

If the enantiomer test is quantitative, the laboratory must perform the following studies for assay or full instrument validation:

- Determine the LOD, LOQ, and ULOL
- Precision/accuracy
  - For urine: around 250 ng/mL (total analyte), around 100 ng/mL (total analyte), and around 50 ng/mL (each enantiomer)
  - For oral fluid: around 25 ng/mL (total analyte), around 10 ng/mL (total analyte), and around 5 ng/mL (each enantiomer)

- Carryover
- Interference
- For an assay validation: method parameters including appropriate ion or MRM transition selection
- For a full instrument validation: instrument parameter optimization

Abbreviated instrument validation studies for quantitative enantiomer assays must include the following:

- Determination of the LOD, LOQ, and ULOL
- Carryover
- Interference
- Parameter optimization

Semi-Quantitative Enantiomer Tests

If the enantiomer test is semi-quantitative, the laboratory must perform the following studies for assay validation, full instrument validation, or abbreviated instrument validation:

- Carryover
- Interference
- For an assay validation: method parameters including appropriate ion or MRM transition selection
- For a full instrument or abbreviated instrument validation: instrument parameter optimization
- Precision/accuracy of enantiomer ratios

- A laboratory may perform additional tests (i.e., other than the drug and specimen validity tests specified by the HHS Guidelines) at the request of a Medical Review Officer or at the direction of a federal agency. The validation and re-verification study requirements for these additional tests depend on the measurand.
  - Urine validation requirements for tetrahydrocannabivarin (THCV) and semi-synthetic opioid metabolites
    - determination of the LOQ
    - full linearity study (LOQ, ULOL) is required if the laboratory reports quantitative results rather than "≥ [established LOQ]"
    - interference
      - THCV must be able to accurately resolve structurally similar compounds (e.g., THCA)
      - validation for opioid metabolites has the same requirements as for opioid confirmatory drug tests (described above)

- carryover study and procedures to address carryover (as an alternative to a carryover study, the laboratory may choose to inject a negative control or solvent blank between specimens, and have criteria for evaluating carryover)
- method parameters including appropriate ion or MRM transition selection
- instrument parameter optimization

– Re-verification requirements for THCV and semi-synthetic opioid metabolites
- Determine LOQ
- ULOL (if the laboratory reports quantitative results rather than "≥ [established LOQ]")
- Interference
- Carryover

Minimum Requirements for Periodic Re-verification Studies

Note: All confirmatory drug test methods (primary and alternate) must be re-verified on at least an annual basis.

Periodic re-verification studies are required for alternate technology initial tests and confirmatory tests to verify that existing limits are still valid. These studies may not be as extensive as those performed for implementing a new method. The following are minimum requirements:

- Determining the LOQ and ULOL (LOD must be determined for confirmatory tests)
- Carryover
- Specificity/interference (for confirmatory tests)
- Hydrolysis (if performed)

LOD, LOQ, and ULOL Re-verification: The laboratory must analyze a minimum of three replicates targeted at the existing limit concentration. If the limit is not re-verified, the limit must be re-established using the validation protocol.

Notes:

- *If a control with a target concentration at the stated LOQ or ULOL is analyzed in each test batch, the laboratory may not need to perform a periodic formal study to re-verify the existing limit (LOQ or ULOL). The laboratory will have data to demonstrate the validity of the existing limit(s). The SOP would need to describe this practice and the results obtained from batch quality control records summarized for review.*
- *LOD studies are not required for confirmatory testing if the LOD is assigned the same value as the LOQ.*
- *LOD studies are not required for alternate technology initial drug tests.*

Carryover Re-verification: The laboratory must perform carryover studies unless blanks are injected between samples in each batch. Using blanks, formal studies may not be required, because the laboratory will be evaluating the potential for carryover in each batch. If this approach is used, the laboratory must have objective criteria for evaluating the blanks, and have procedures to ensure that possible carryover would be identified and appropriate corrective actions taken.

Note: For an extraction method with elution into multi-well plates, the laboratory is not required to perform a periodic formal cross-contamination study if the laboratory includes at least one contamination check sample in each batch that demonstrates the lack of cross-contamination.

Interference Re-verification: The laboratory must perform re-verification of potential interferences for all confirmatory drug tests (and for amphetamine enantiomers) unless controls with high concentrations of common interferents are analyzed in test batches. These controls may be used to replace formal periodic interference studies only if the controls are equivalent to the samples required by the NLCP for a formal interference study (i.e., controls with and without the compounds of interest, with the interfering substances at the concentrations specified by the program). If this approach is used, the laboratory must have data to support its continued ability to accurately resolve the analyte from structurally similar compounds. The SOP must describe this practice, and the results obtained from batch quality control records must be summarized for review.

Amphetamine/Methamphetamine Enantiomer Test Re-verification: Re-verification studies depend on whether the test is quantitative or semi-quantitative.

For quantitative tests (i.e., quantitative values for methamphetamine enantiomers and, if analyzed, amphetamine enantiomers), the laboratory must perform the following studies to evaluate each analyte:

- Determination of the LOD, LOQ, and ULOL
- Carryover
- Interference

For semi-quantitative enantiomer tests (i.e., relative percentages of methamphetamine enantiomers and, if analyzed, amphetamine enantiomers), the laboratory must perform the following studies:

- Carryover
- Interference

Hydrolysis Re-verification: The laboratory must perform hydrolysis studies as performed for the original method validation (see ***Hydrolysis Studies*** section).

Note: Hydrolysis studies are not needed if the laboratory includes hydrolysis controls at or above the specified concentration in each batch.

References

1. Hedenus M. Eugen Goldenstein and his laboratory work at Berlin Observatory. Astronomical Notes (Astronomische Nachrichten). 2002;323(6):567-9.
2. Nobel Media. Wilhelm Wien-Biographical [Internet]. 2019 [Available from: http://www.nobelprize.org/nobel_prizes/physics/laureates/1911/wien-bio.html.
3. Rainey PM, Baird GS. Analytical methodologies for the toxicology laboratory, mass spectrometry. In: B. Magnani, M. G. Bissell, T. C. Kwong, A. H. B. Wu, editors. Clinical toxicology testing: A guide for laboratory professionals. Northfield, IL: CAP Press; 2012. p. 90-3.
4. Gohlke RS, McLafferty FW. Early gas chromatography/mass spectrometry. Journal of the American Society for Mass Spectrometry. 1993;4(5):367-71.
5. Mandatory Guidelines for Federal Workplace Drug Testing Programs using Urine (effective October 1, 2017), 82 FR. Sect. 7920-7970 (2017).
6. Mandatory Guidelines for Federal Workplace Drug Testing Programs using Oral Fluid (effective January 1, 2020), 84 FR. Sect. 57554-57600 (2019).
7. RTI International, Center for Forensic Sciences. Manual for Urine Laboratories, National Laboratory Certification Program (NLCP). Research Triangle Park, NC: RTI International, Center for Forensic Sciences,; 2017. Contract No.: Rev. 0222.
8. RTI International, Center for Forensic Sciences Manual for Oral Fluid Laboratories, National Laboratory Certification Program (NLCP). Research Triangle Park, NC: RTI International, Center for Forensic Sciences; 2020. Contract No.: Rev. 0222.
9. ANSI/ASB Standard 036. Standard Practices for Method Validation in Forensic Toxicology. 2019. Available from: https://www.aafs.org/sites/default/files/media/documents/036_Std_e1.pdf
10. Clinical Laboratory Improvement Amendments (CLIA), Standards and Certification, Laboratory Requirements, Subpart K, Standard: Establishment and verification of performance specifications, 42 CFR. Sect. Part 493.1253.

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