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1.0 Purpose and Scope

1.1 This manual describes the analytical methods for the confirmation and quantitation of drugs in whole blood samples.

1.2 Samples to be analyzed may include calibration and control standards and casework samples.

1.3 The scope of this manual includes quality assurance protocols for the equipment and instrumentation used in confirmation analysis, sample workflow from preliminary screening to confirmatory analysis, data review and release of reports, documentation, and quality control criteria.

1.4 This manual does not include specific protocols for the preparation and analysis of casework samples for particular analytes of interest. These protocols are included in the method-specific standard operating procedures for each panel (TOX_P70X).

2.0 Responsibility

2.1 All analysts who perform sample preparation, extraction, or analysis by confirmatory methods as part of their prescribed job duties are responsible for following these procedures as written.

2.2 These procedures are reviewed periodically by the Toxicology Section staff. Revisions are made at that time or when there is an identified need to change this written manual to be compatible with changing needs in the analytical process. In the event that there are changes to be made to this manual, the analyst must report those changes in detail to the Toxicology Section Supervisor in a timely manner.

2.3 All analysts performing these procedures and reporting analytical results for forensic purposes must be fully trained and authorized in the use of these procedures in accordance with the Toxicology Training Manual (TOX_P301). All analysts must demonstrate initial competency in the methods and must show ongoing proficiency by successfully analyzing at least one internal or external proficiency test annually.

2.3.1 Proficiency samples will be treated as casework. Results will be unknown to the analyst prior to testing. Samples that screen positive will be confirmed using currently validated confirmatory methods.

2.3.2 Analysts trained and authorized to perform both toxicological screening and confirmation procedures must perform analysis using both procedures on at least one proficiency sample per year regardless of screening results.

2.3.3 Proficiency test results for confirmation analysis must fall within ± 3

standard deviations of the grand mean reported by the test provider for each analyte reported.

2.4 Analysts will ensure that an adequate amount of sample processing supplies are on hand at all times. Orders should be placed when supplies are low to ensure that new stock arrives and can be performance checked before supplies are completely empty.

3.0 Emergency or High Priority Situations

3.1 The Commissioner of Public Safety, Laboratory Director or Toxicology Section Supervisor can designate samples as high priority.

3.2 High priority samples are analyzed as soon as possible.

3.3 Priority sample results are reviewed and released as soon as they are available and once they pass the quality assurance criteria.

4.0 Quality Assurance

4.1 It is expected that the analyst will report any unacceptable or anomalous behavior of any analytical system immediately to the Toxicology Section Supervisor. It is further expected that appropriate actions will follow as soon as possible and be properly documented.

4.2 **Equipment**

4.2.1 Pipettes

4.2.1.1 Measurements made by the Toxicology Section using pipettes are critical.

4.2.1.2 Pipettes used by the Toxicology Section, including fixed and variable volume pipettes, have their calibration evaluated and certified by an approved vendor. The resulting documentation will be maintained.

4.2.1.3 If a pipette is sent out for service, an inspection of the package/pipette will be performed to check for any shipping and handling concerns prior to being returned for use. The calibration certificate will be reviewed in accordance with QA_P100_6.4 Equipment QC.

4.2.1.4 If a question arises regarding the proper functioning of a pipette, a performance check or calibration service by an approved vendor may be initiated.

4.2.1.4.1 Performance checks will be performed in accordance with QA_P100_6.4_Equipment QC.

4.2.1.4.2 Performance checks will be reviewed and filed in the VFL Pipettes Equipment QA/QC binder.

4.2.2 Balance(s)

4.2.2.1 The analytical balance will be checked monthly with NIST traceable weights. This check will be recorded in the corresponding VFL Balances Equipment QA/QC Binder.

4.2.2.2 The analytical balance will have its calibration evaluated and certified by an approved vendor. The resulting documentation will be maintained.

4.2.2.3 Any balance maintained to the same standard may be used as a back-up if the primary balance is unavailable.

4.2.3 Glassware

4.2.3.1 Class A volumetric glassware shall be used for the preparation of calibrators and shall be calibrated by an accredited calibration service supplier prior to use. After initial calibration, scheduled recalibration shall recur at least once every ten years by an appropriately accredited calibration service supplier.

4.2.3.2 Class A volumetric glassware used in the preparation of calibrators shall be dedicated for this purpose and maintained and stored as to protect its integrity.

4.2.3.3 Glassware to be used on the UPLC should only be cleaned with HPLC compatible solvents.

4.2.4 Centrifuge

4.2.4.1 Centrifugation times and velocities are not considered critical to analysis. Samples must be centrifuged for sufficient duration to separate a pellet of precipitated protein and cellular debris from the supernatant fluid.

4.2.4.2 The centrifuge is checked annually.

4.2.5 Positive Pressure Manifold

4.2.5.1 Before use, ensure the nitrogen tank and regulator valves are open, and pressure reaching the manifold is at least 50 PSI.

4.3 **LC-MS/MS Instrumentation**

4.3.1 All maintenance performed, including routine and preventative maintenance as well as troubleshooting activities, should be recorded in the Instrument Maintenance Log.

4.3.2 Day of use

4.3.2.1 Check nitrogen and argon supply pressures.

4.3.2.2 Check solvent waste container and source exhaust trap; empty as needed.

4.3.2.3 Prime mobile phase lines and plungers.

4.3.2.4 Run an Instrument Performance Evaluation (IPE) sample prior to running casework samples; retain electronic copy on instrument computer.

4.3.3 As Needed

4.3.3.1 If mobile phases or wash solutions have been changed, prime all solvents before running the instrument.

4.3.3.2 Regularly (typically once per week) check roughing pump oil level and gas ballast roughing pump.

4.3.3.3 Clean source housing, sample cone, and cone gas nozzle.

4.3.3.4 Replace filters, valves, seals, column, and capillary according to manufacturer's recommendations.

4.3.3.5 Clean flow-through needle injection port; replace needle and seal assembly.

4.3.4 Annual

4.3.4.1 Schedule on-site preventative maintenance with service engineer.

4.3.5 Refer to operator manual and Instrument Maintenance Log for additional maintenance and troubleshooting procedures.

4.3.6 The Instrument Maintenance Log and the listed resources are kept near the instrument.

4.4 Reagent preparation

4.4.1 Materials

4.4.1.1 Acetonitrile (HPLC grade)

4.4.1.2 Methanol (HPLC grade)

4.4.1.3 Isopropanol (HPLC grade)

4.4.1.4 Formic acid (HPLC grade)

4.4.1.5 Deionized water (diH_2O)

4.4.2 Equipment

4.4.2.1 Graduated cylinders

4.4.2.2 HPLC grade glass bottles

4.4.2.3 Pipettes

4.4.3 Mobile phase A – 0.1% Formic acid in diH_2O

4.4.3.1 Add 4 L diH_2O to clean glass bottle.

4.4.3.2 Add 4 mL formic acid.

4.4.3.3 Assigned lot number is MPA-MMDDYYYY.

4.4.3.4 Solution expires one year from date of preparation when stored in refrigerator.

4.4.3.5 Aliquots of this solution expire after one month when stored at room temperature.

4.4.4 Mobile phase B – 0.1% Formic acid in Acetonitrile

4.4.4.1 Add 4 mL formic acid to 4L bottle of HPLC grade Acetonitrile.

4.4.4.2 Assigned lot number is MPB-MMDDYYYY.

4.4.4.3 If needed for use as extraction solvent, store an aliquot in freezer.

4.4.5 Mobile phases used in the analysis of casework samples must be checked prior to being placed into service.

4.4.5.1 A solvent blank will be run on the LC-MS/MS system using the validated methods to demonstrate that the mobile phase(s) are free of analytes of interest and other interfering compounds.

4.4.6 Seal Wash – 9:1 diH_2O: Acetonitrile

4.4.6.1 Add 900 mL diH_2O to clean HPLC bottle.

4.4.6.2 Add 100 mL HPLC grade acetonitrile; mix well.

4.4.6.3 Assigned lot number is SW-MMDDYYYY.

4.4.7 MS Wash – 7:3 Methanol: diH_2O

4.4.7.1 Add 70 mL HPLC grade methanol to clean HPLC bottle.

4.4.7.2 Add 30 mL diH_2O; mix well.

4.4.7.3 Assigned lot number is MSW-MMDDYYYY.

4.4.8 Wash solvent – 1:1:1:1 methanol:isopropanol:acetonitrile:diH2O

4.4.8.1 Add 250 mL HPLC grade methanol, isopropanol, acetonitrile and diH2O to clean HPLC bottle; mix well

4.4.8.2 Assigned lot number is WS-MMDDYYYY.

4.4.9 Purge solvent – 1:1 methanol:diH2O

4.4.9.1 Add 500 mL HPLC grade methanol to clean HLPC bottle.

4.4.9.2 Add 500 mL diH2O; mix well.

4.4.9.3 Assigned lot number is PS-MMDDYYYY.

4.4.10 Solvent Blank

4.4.10.1 Add approximately 1 mL diH_2O to a clean glass vial.

4.4.10.2 Solvent blanks do not require a lot number or entry in the Reagent Preparation Log and may be stored in the instrument autosampler or refrigerator until use.

4.4.11 IPE preparation

4.4.11.1 Add 450 µL diH_2O to a clean glass vial.

4.4.11.2 Add 25 µL of the lowest concentration working stock dilution from the prepared calibration curve standards.

4.4.11.3 Add 25 µL internal standard; mix well.

4.4.11.4 IPE samples do not require a lot number or entry in the Reagent Preparation Log and may be stored in the instrument autosampler or refrigerator until use.

4.4.12 Prepared reagents are stored at room temperature unless otherwise noted.

4.4.13 Reagents expire one year from date of preparation unless otherwise noted.

4.4.14 Reagent preparation, analysis, and review (as necessary) will be recorded in the Reagent Preparation Log. Analytical results will be kept on file with the Toxicology Section. Containers will be labeled with lot number, preparation date, expiration date, and preparer initials, unless otherwise noted.

4.4.15 Volumes specified in each preparation can be changed according to use, provided the final concentration or ratio of components remains constant.

4.4.16 If the analytical method requires solvents not listed above, refer to method-specific standard operating procedure documents for information regarding preparation and performance criteria.

4.5 Reference Materials

4.5.1 Purchased Standards

4.5.1.1 Where available, NIST traceable standards are used for calibration and quality control for each analytical method. These standards are purchased from an ISO 17025 and/or ISO 17034 certified supplier. The certificates of analysis for all standards are kept on file with the Toxicology Section. Calibrator and quality control stocks prepared from NIST traceable standards shall be performance checked prior to use.

4.5.1.2 A different manufacturer of a given standard should be used in the calibration and quality control of each assay. In instances where material from a second manufacturer is not readily available, different lot numbers of the standard will be used as calibrators and controls for the assay.

4.5.1.3 Standards will be stored as specified by the manufacturer.

4.5.2 In-House Preparations

4.5.2.1 Negative Control Stock (NEG)

4.5.2.1.1 Refer to Toxicology Screening Manual (TOX_P600) for Negative Control Stock preparation.

4.5.2.1.2 Before use in casework, each lot number will be analyzed using the validated methods to verify that it is free of analytes of interest and other interfering compounds.

4.5.2.1.3 NEG may be used for each method for which it has been approved for use.

4.5.2.1.4 NEG preparation, analysis, and review will be recorded in the Reagent Preparation Log. Analytical results will be kept on file with the Toxicology Section.

4.5.2.1.5 NEG is stored in the refrigerator and is approved for use until consumed or contamination is suspected.

4.5.2.2 Refer to method-specific standard operating procedure documents for information regarding preparation and performance check criteria for additional in-house preparations.

5.0 Evidence Handling and Sample Workflow

5.1 See the Toxicology Screening Manual (TOX_P600) for details regarding evidence handling and screening procedures.

5.2 All samples determined to need confirmation testing will be confirmed using the procedures outlined in this manual and the panel-specific standard operating procedures.

5.3 Where possible, the same blood tube used for screening analysis will be used for confirmation. If sample volume is insufficient for analysis, a second tube may be opened with approval from the Toxicology Section Supervisor, or their designee.

6.0 Quantitative Confirmation Analysis

6.1 Principle

6.1.1 Prior to analysis, samples must be prepared in order to extract the analytes of interest from the potentially interfering compounds present in the sample matrix. Sample preparation is typically performed using cell lysis/ protein precipitation, centrifugation, and/or solid phase extraction.

6.1.2 The product of these steps is injected into the liquid chromatography system. The analytes of interest flow through the chromatographic column, transported by a liquid mobile phase comprised of a mixture of aqueous and organic solvents. The analyte of interest's differential affinity for the solid phase of the column relative to the strength of solvent in the mobile phase dictates the speed with which the analyte travels through the column, thereby separating different compounds of interest by retention time.

6.1.3 The mass spectrometry detector uses ionization and fragmentation of compounds to uniquely identify the analyte of interest in the flow from the LC column. As molecules are ionized, they flow through a series of quadrupoles which filter ions based on mass-to-charge ratio (m/z). Filtered ions are bombarded by charged gas molecules in the collision cell, which fragments the molecular structure of the compound. The technique of filtration and fragmentation allows the instrument to monitor ion transitions, or combinations of parent and product ions which are unique to a particular compound.

6.1.4 By correlating the results of liquid chromatography and mass spectrometry, a highly specific quantitative assay for various analytes of interest can be performed.

6.2 **Procedure**

6.2.1 Refer to method-specific standard operating procedures for information regarding quantitative analysis for particular analytes of interest.

7.0 Quality Control and Corrective Action

7.1 All analytical sequences must contain:

7.1.1 At least one solvent blank at the beginning and end of the analytical batch to demonstrate lack of contamination and instrument performance.

7.1.1.1 Solvent blanks must not contain any identifiable peaks above 50% of the lower limit of quantitation (LLOQ) for each analyte of interest.

7.1.2 Calibration standards spanning the analytical range for each analyte.

7.1.2.1 The coefficient of determination (R^2) of the calibration line must be 0.99 or greater.

7.1.2.2 Calibration results must be within ± 20% of their expected concentration.

7.1.2.3 Calibration points within ± 20% but outside ± 10% of their expected concentration may be dropped at the analyst's discretion if the point appears inconsistent with other calibration results.

7.1.2.4 Calibration curves must include no fewer than five calibration points for each analyte, including the upper and lower limits of quantitation.

7.1.3 A matrix-matched negative control sample.

7.1.3.1 The negative control must not contain any identifiable peaks above 50% of the LLOQ for each analyte of interest.

7.1.4 Matrix-matched positive quality control samples.

7.1.4.1 Quality control samples must span the analytical range of the assay for each analyte. Quality control samples will be prepared in the following concentrations:

7.1.4.1.1 Low (approximately 3 times the LLOQ).

7.1.4.1.2 Mid (approximately 50% of the highest calibrator).

7.1.4.1.3 High (approximately 80% of the highest calibrator).

7.1.4.2 Each concentration of QC sample must be present in the batch; the last QC sample in the batch must be Low concentration.

7.1.4.3 Results must be within ± 20% of their expected concentration for each analyte at each level.

7.1.4.4 Quality control results will be entered into a control chart for each concentration.

7.1.5 Casework samples.

7.1.5.1 No more than 10 casework samples may be bracketed between passing QC samples.

7.2 All quantitative samples must meet the following acceptance criteria:

7.2.1 Qualifier ion ratios for each analyte must be within ± 20% of the average qualifier ion ratio from the calibration samples in the batch.

7.2.1.1 Ion ratios may be concentration-dependent; comparison to a calibrator of similar concentration to the sample in question is acceptable if the ion ratio does not fall within ± 20% of the average ratio from all calibration samples.

7.2.2 Relative retention time (RRT), measured as a ratio between the retention times of the analyte of interest and its internal standard, must be within ± 2%.

7.2.3 Analyte peaks must exhibit acceptable shape and be free from splitting, broadening, or excessive asymmetry as compared to typical peak shapes in the calibration samples for each analyte.

7.3 Samples that do not meet the acceptability criteria for an analyte(s) may be reanalyzed for the analytes in question.

7.3.1 Casework samples bracketed by a quality control sample requiring reanalysis must also be reanalyzed unless:

7.3.1.1 Controls fail high, i.e. > 20% above the target value; all negative results may be reported. If all samples are negative, control samples do not require reanalysis.

7.3.1.2 Standard or control qualifier ion ratios fail; only those samples which do not meet the criteria must be re-injected. If re-injection does not meet quality control criteria, all casework samples must be re-extracted with quality control samples.

7.3.2 Samples may be re-injected and evaluated against the batch calibration curve in accordance with method-specific stability and storage requirements.

7.3.3 Casework samples being re-injected must be followed by a re-injected Low concentration quality control sample.

7.3.4 Re-injected or re-extracted calibration curves may be used to process previously injected samples, provided the calibration(s) are run within the method stability timeframe.

7.3.5 Samples that fail re-injection may be re-extracted and analyzed in a subsequent batch for the analyte(s) in question. The first acceptable result will be reported.

7.4 If a sample's concentration exceeds the upper level of quantitation, the subsequent sample in the run may be reanalyzed if carryover is suspected. If results from the reinjected sample are within 20% of the original injection, the original results will be reported.

7.5 Exceptions may be made on a case by case basis with the approval of the Toxicology Section Supervisor, or their designee.

7.6 Samples not meeting acceptance criteria may be reported as not meeting quality control criteria with the approval of the Toxicology Section Supervisor, or their designee.

7.7 Documentation of all analyses performed must be retained in the casework packet.

8.0 Estimation of Uncertainty of Measurement

8.1 All validated instruments for each panel are included in the estimation of measurement uncertainty.

8.2 The estimation of measurement uncertainty is performed using the Simplified GUM Approach as defined in ASCLD/LAB Guidance on the Estimation of Measurement Uncertainty.

8.2.1 A 95.45% level of confidence will be used to determine the expanded uncertainty.

8.2.2 The expanded uncertainty will be rounded up to one decimal place.

8.3 Calculate the interval for each result by multiplying the measured result by the expanded uncertainty.

8.3.1 This value will be reported along with the measured result.

8.3.2 The reported estimated measurement uncertainty interval will be rounded to the same number of decimal places as the reported result.

8.4 The estimated uncertainty of measurement will be updated annually or if any significant change in the expanded uncertainty is detected.

8.5 The reported result is the result at the time of analysis, and does not account for changes in sample composition which may occur subsequent to or before examination.

9.0 Result Reporting and Review

9.1 Reporting Guidelines

9.1.1 Drug concentrations will be reported in ng/mL, rounded to two significant figures for concentrations <10 ng/mL, and the nearest whole number for concentrations ≥10 ng/mL.

9.1.2 All positive quantitative results will be reported as a list of analytes and their associated concentrations in the format:

Item…contains:

Analyte 1: [Conc.] ± (MU) ng/mL

Analyte 2: [Conc.] ± (MU) ng/mL

Etc.

9.1.3 Analyte concentration results below the lower limit of quantitation will not be reported.

9.1.4 Samples with all results below the lower limit of quantitation will be reported as having results below the laboratory's reporting limit for all blood drug analytes.

9.1.5 Analyte concentration results greater than the upper limit of quantitation will be reported as having results greater than the laboratory's reporting limit with no associated uncertainty.

9.1.6 If results do not meet quality control criteria, quantitative results will not be reported for that analyte(s). A statement that the analytes did not meet quality control criteria will be reported.

9.1.7 Result reports will state all analytes for which a casework sample was tested, the method by which they were tested, the range of quantitation, and the sample matrix from which they were extracted.

9.2 Procedure

9.2.1 Generate sequence list, calibration summary, compound result summary, and chromatograms on the TargetLynx workstation.

9.2.2 Upon successful completion of analysis, the analyst must perform a primary data review of the confirmation data packages.

9.2.3 The completed Case File includes:

9.2.3.1 Toxicology Case Specific Review Checklist (QA_F100_7.7_17).

9.2.3.2 VFL lab report and FA worksheet.

9.2.3.3 Case specific chromatograms.

9.2.3.4 A request for analysis form (EH_F100_2).

9.2.4 The completed Batch File includes:

9.2.4.1 Blood Drug Confirmation Batch File Review Checklist (QA_F100_7.7_20).

9.2.4.2 Sample preparation worksheet.

9.2.4.3 Sequence list.

9.2.4.4 Compound results summary for each analyte.

9.2.4.5 Calibration graphs for each analyte.

9.2.4.6 All non-case specific chromatograms generated during the analytical process.

9.2.5 Analyst Review:

9.2.5.1 Ensure the criteria defined in Section 7.0 are met.

9.2.5.2 Enter quality control data into the appropriate control chart for each analyte.

9.2.5.3 Enter results and associated uncertainty values in FA. Ensure that all transcription is correct and results are reported appropriately.

9.2.5.4 Verify the sample preparation worksheet is completely filled out and all reagents were used within their expiration dates.

9.2.6 Technical Review:

9.2.6.1 A qualified analyst must perform a technical review of the complete Batch and Case Specific data packages.

9.2.6.2 Ensure that the data packages are complete and all forms are complete and accurate.

9.2.6.3 Confirm all calculations that are not part of a validated worksheet.

9.2.6.4 If data quality issues have been identified during review, the reviewer must attempt resolution through discussion with the analyst and/or Toxicology Section Supervisor. If issues cannot be resolved, it may be necessary to prepare and analyze new aliquots of the submitted sample.

9.2.7 Administrative and Director Review:

9.2.7.1 Upon completion of the technical review, an administrative and director review of the Batch and Case Specific data packages will be completed.

9.2.8 All review criteria must be met before the final result report can be released.

10.0 Backup Procedures

10.1 Any secure storage refrigerator in rooms 155A, 266, or 265B may be used to store samples.

11.0 References

11.1 Toxicology Training Manual (TOX_P301)

11.2 VFL Balances Equipment QA/QC Binder

11.3 Instrument Maintenance Log

11.4 Reagent Preparation Log

11.5 Toxicology Screening Manual (TOX_P600)

11.6 Class/analyte specific confirmation procedures (TOX_P70X)

11.7 Quality Assurance Manual (QA_P100)

11.8 Blood Drug Confirmation Batch File Review Checklist (QA_F100_7.7_20)

11.9 Toxicology Case Specific Review Checklist (QA_F100_7.7_17)

11.10 ASCLD/LAB Guidance on the Estimation of Measurement Uncertainty – Annex A; Details on the NIST 8-Step Process. ASCLD/LAB – International.

11.11 Measurement Uncertainty Binder

11.12 Waters ACQUITY UPLC H-Class Maintenance Guides

11.13 Waters Xevo TQ-S micro Overview and Maintenance Guide

DOCUMENT HISTORY			
DATE	**VERSION**	**APPROVED BY**	**ACTIVITY OR REVISION**
2/11/2019	1	Lab Director	First edition
11/9/2020	2	Lab Director	Addition of reagents; quality control criteria improved; general edits for clarity
6/14/2021	3	Lab Director	Added 8.1 Instrumentation included in MU; minor formatting changes throughout
8/10/2022	4	Lab Director	Updated section 7 to clarify QC requirements; updated section 9 for batch and case file parameters; changed pipette calibration interval removed section 10.2; updated measurement uncertainty rounding; minor formatting changes throughout