

SCREENING OF BIOLOGICAL SPECIMENS BY ENZYME MULTIPLIED IMMUNOASSAY TECHNIQUE (EMIT)

18.1 METHOD

This test method may be used to presumptively identify several drugs or drug metabolites and/or drug classes as being present in biological specimens.

18.2 PRINCIPLE

Immunoassays are scientific tests that use antibodies to identify and qualitatively measure amounts of a chemical substance. In forensic toxicology, these are typically used to screen biological samples for the presence of an antigen; most commonly a drug. These are competitive binding assays and are rapid methods for qualitatively detecting individual drugs or metabolites, or classes of drugs in urine or extracted blood or tissue samples.

The assay is based on competition between drug in the specimen and drug labeled with the enzyme glucose-6-phosphate dehydrogenase to antibody binding sites. Enzyme activity decreases upon binding to the antibody, so the relative drug concentration in the specimen can be compared to a known concentration. The enzyme converts NAD⁺ to NADH, resulting in a change in absorbance that is measured by a spectrophotometer.

Specificity (the degree to which the assay correctly identifies only the compound(s) of interest) is a critical component of immunoassays. Cross-reactivity to structurally similar compounds is inherent. Understanding the compounds that exhibit cross-reactivity is important to data interpretation.

18.3 SPECIMENS

Specimens include whole blood, serum, plasma, urine, vitreous humor and tissue homogenate. Standard volume for extracted specimens is 1 mL. Serum, plasma, and urine do not require any sample preparation; approximately 8 drops of each sample type is sufficient for un-extracted analysis.

NOTE: Analysis of tissue homogenate specimens requires matrix-matching of calibrators and controls.

18.4 REAGENTS, MATERIALS AND EQUIPMENT

18.4.1 REAGENTS

- Acetonitrile (ACN), reagent grade
- Certified blank blood and/or other biological matrices
- Certified blank urine
- Deionized water (DI H₂O), laboratory general-use
- Dilute aqueous *Emit*® Drug Assay Buffer

Emit® Drug Assay Buffer Concentrate (from TCA reagent kit) is diluted 1:14 with DI H₂O. (For example: Add 5 mL concentrated EMIT buffer to 70 mL of DI H₂O and mix, for total volume of 75 mL dilute aqueous EMIT buffer). This solution is used to prepare the working blood EMIT buffer.

- *Emit*® Drug Assay Buffer Concentrate
- *Emit*® *II Plus* reagents for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolite and opiates (supplied as ready-to-use liquids, kept refrigerated).
- *Emit*® *tox*TM serum tricyclic antidepressants calibrator and controls, kept refrigerated (as needed for supply of *Emit*® Drug Assay Buffer Concentrate).
- Methanol (MeOH), reagent grade
- Working blood EMIT buffer

Equal parts dilute aqueous EMIT buffer and MeOH are mixed to prepare working blood EMIT buffer. (For example: Mix 525 mL dilute EMIT buffer and 525 mL MeOH for a total volume of 1050 mL working EMIT buffer). The solution is stored in a plastic or glass bottle at room temperature and expires one year from the date of preparation.

NOTE: Adjustments to the final volume of prepared buffers are permitted, provided that the proportions are maintained.

18.4.2 MATERIALS

- Disposable extraction tubes (16 x 125 mm recommended) with closures
- Disposable 12 x 75 mm tubes with closures
- Fisherbrand conical sample cups (or equivalent)
- Disposable centrifuge tubes with closures
- Laboratory glassware (graduated cylinders, flasks)

18.4.3 EQUIPMENT

- Olympus AU400e
- Calibrated, adjustable piston pipettes and verified, adjustable repeater-pipette with disposable pipette tips
- General use equipment (centrifuge, vortex mixer, evaporator)

18.5 STANDARDS, CALIBRATORS AND CONTROLS

18.5.1 STANDARDS

- Working standard
- Working control standard
- Urine working control standard

See Appendix A for target compounds, concentrations and preparation information.

18.5.2 CALIBRATORS – All calibrations are single-point, qualitative only.

18.5.2.1 Blood, Serum, Plasma, Vitreous or Tissue Homogenate:

- a. Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 18.6 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices (e.g., tissue homogenate).
- b. Calibrations are generated from calibrator level 1 for barbiturates, cannabinoids and cocaine metabolite, and from calibrator level 2 for amphetamines, benzodiazepines and opiates. Single-point calibration levels represent the cutoff concentrations.

18.5.2.2 Urine calibration is performed as necessary, when testing of urine specimens is performed. The *Emit® II Plus* calibrators (level 1, 2, and 3) are supplied as liquids, ready to use, and kept refrigerated. The calibrators are stable until the expiration date printed on the vials.

18.5.3 CONTROLS

NOTE: Controls (positive or negative) must make up at least 10% of the extracted batch (based on number of case specimen samples), with case specimens bracketed by positive controls. Positive controls in both whole blood and/or alternate matrices may be used to bracket case specimens.

If a batch contains more than 20 case specimens, additional negative or positive controls are created for each additional 10 samples to bring the total percentage of controls to 10% of the batch. The additional controls are spaced as evenly as possible throughout the test batch. When analyzing compounds in multiple matrices, both whole blood and alternate matrix controls apply towards 10% of the batch.

18.5.3.1 Blood, Serum, Plasma, Vitreous, or Tissue Homogenate

18.5.3.1.1 Negative Controls

At least two negative whole blood controls are tested in each batch. One is to function as the matrix blank for the spectrophotometer, and the remaining functions as a negative control. All controls are prepared using certified blank blood. When analyzing tissue homogenate, two negative controls must be prepared in that matrix. If analyzing only tissue homogenate specimens, whole blood negative controls are not required.

18.5.3.1.2 Positive Controls

At least two whole blood positive controls are tested with every batch. The preparation of the positive whole blood controls is detailed in 18.6 SAMPLE PREPARATION. When analyzing tissue homogenate, at least two positive controls must be prepared in that matrix. If analyzing only tissue homogenate specimens, whole blood positive controls are not required.

18.5.3.2 Urine

18.5.3.2.1 Quality Control

The Green QC rack (see 18.7.3.1) is run once daily (as needed, on days urine testing is performed) and consists of:

- a. The negative control, made up of certified blank urine.

- b. The urine positive control; prepare by adding 30 μL of the urine EMIT working control standard (using a calibrated pipette) to 1.0 mL blank urine in a 12 x 75 mm tube. Cap the tube and vortex-mix. Transfer contents to a sample cup.

NOTE: Multiple positive control sample cups may be filled from this control. Where the green QC rack is run concurrently with the test batch, a sample of the spiked control serves as the positive control at the start of the batch, to bracket case specimens.

18.5.3.2.2 Within-batch Quality Controls

At least one negative control (certified negative urine) and one positive control are tested at the start of every batch. Note that if the green QC rack is run concurrently with the test batch, this fulfills the requirement.

18.6 SAMPLE PREPARATION (Blood, Vitreous, Tissue Homogenate, Urine)

18.6.1 Blood, Vitreous, or Tissue Homogenate

NOTE: Serum or plasma will be run with contemporary blood calibrators and controls, but do not require sample preparation. Skip to step 18.6.1.1 for un-extracted serum or plasma samples. Serum or plasma samples may be extracted, as determined by available sample volume.

- 18.6.1.1 Label a clean extraction tube for each member of the test batch. (i.e., calibrators, controls, or case samples).
- 18.6.1.2 Using a calibrated pipette, add 1 mL of certified blank whole blood into each of the two calibrator tubes (low and high), the matrix blank, and the positive and negative control(s).
- 18.6.1.3 Using a calibrated pipette and the working standard, spike the low and high calibrators as follows: Use a calibrated pipette to add 20 μL of working standard to the low calibrator tube and 40 μL to the high calibrator tube.
- 18.6.1.4 Using a calibrated pipette and the working control standard, spike the low and high positive controls as follows: Use a calibrated pipette to add 10 μL of working control standard to the low positive control tube and 20 μL to the high positive control tube.
- 18.6.1.5 Using a calibrated pipette, sample 1 mL of each case specimen into its respective tube.
- 18.6.1.6 To each tube, add 1 mL of methanol, followed immediately by 3 mL of acetonitrile. Vortex mix approximately 30 seconds.
- 18.6.1.7 Centrifuge the tubes for 5 minutes at 2000rpm (recommended for 16 x 125 mm tubes) to achieve separation.
- 18.6.1.8 Decant the supernatant into a conical centrifuge tube, and evaporate under air at 50°C to approximately 100 μL .
- 18.6.1.9 Remove the tubes from the evaporator and add 350 μL of working blood EMIT buffer and vortex mix.

18.6.1.10 Centrifuge the tubes for 5 minutes at 2000 rpm.

18.6.1.11 Transfer the clear supernatant to labeled conical sample cups for analysis on the Olympus AU400e. See section 18.7 INSTRUMENTAL PARAMETERS for guidance.

18.6.2 Urine

Urine samples require no sample preparation. A few drops (8-10) of sample are transferred into labeled conical sample cups for analysis on the Olympus AU400e (see 18.7 INSTRUMENTAL PARAMETERS).

18.7 INSTRUMENTAL PARAMETERS

18.7.1 INSTRUMENT MAINTENANCE

Daily, weekly, monthly, 3-month, and 6-month maintenance is required to keep the AU400e in working order. See the *Procedure for Instrument Maintenance and Performance Verification (PTmp12504)* and refer to the *Olympus AU400e Maintenance Manual* for instructions on routine maintenance, as well as troubleshooting any issues that may arise. Maintenance reports are filed in the instrument maintenance/QC binder.

18.7.2 INSTRUMENT CALIBRATION

18.7.2.1 From the main computer screen, under USER, select "Start Condition/New Data Index". Change the data index to reflect the current time and set the operator name.

18.7.2.2 Under USER, select "Order Calibration from Racks", select which sample type (serum or urine- select serum if using blood, serum, plasma, vitreous or tissue homogenate). Click on "Start Entry" and enter "1" or "3" under "Profile" if using a serum panel, "2" for the urine panel. Click "Entry" to make the selection, and then click on "Exit."

NOTE: Serum panel "1" includes all compound classes listed in 18.5.2.1.b. Serum panel "3" includes all compound classes listed, with the exception of cannabinoids; panel "3" is used for specimens that will be screened with a separate method for the presence of cannabinoids.

18.7.2.3 For blood (and other associated matrices), use a blue sample rack, with the supernatant from the extracted matrix blank in position 1. Fill position 1 of the red-striped yellow sample rack with the supernatant from the 20 µL low calibrator, and position 2 with the 40 µL high calibrator.

18.7.2.4 For urine calibration, use a blue sample rack with DI H₂O in position 10. Fill each labeled position in a yellow sample rack with the appropriate calibrator.

NOTE: The urine calibration is performed weekly, or as needed, with calibration data filed in the instrument maintenance/QC binder.

18.7.3 ORDERING CONTROL SAMPLES

NOTE: Ensure that step 18.7.2.1 (setting the operator name and data index) is performed before ordering any quality controls or sample tests.

18.7.3.1 The negative, low, and high controls for blood and other associated matrices are ordered as samples. See section 18.7.4 ORDERING SAMPLE TESTS.

18.7.3.2 To order urine controls, select "Order QC from Green Rack" under USER. Select urine for the sample type. Fill the labeled sections of a green sample rack with the negative and positive controls.

NOTE: The spiked urine control (as described in 18.5.3.2.1.b), ordered as a "QC" in the green rack and as a "sample" when run at the end of the batch, serve to bracket case specimens.

18.7.4 ORDERING SAMPLE TESTS

18.7.4.1 Under USER, choose "Select Report Format". Select either the urine report or blood report appropriately (choose the blood report format that corresponds to panel "1" or panel "3").

18.7.4.2 Under USER, select "Order Sample Tests". Set the sample type appropriately.

18.7.4.3 Click on "Start Entry" at the bottom of the screen, and enter the sample ID (for example "Negative Control" or an ST# associated with a case).

18.7.4.4 Click on "Profile" and select "1" or "3" for serum and "2" for urine.

18.7.4.5 Click on "Entry" at the bottom of the screen to accept the selections. Continue as above until all sample tests have been ordered.

18.7.4.6 Samples are placed in labeled conical sample cups in gray sample racks for urine, and in red-striped gray racks for blood and other associated matrices. Ensure that the position of each sample cup matches the position ordered as above in 18.7.4.3.

18.7.4.7 Once all calibration, quality control samples, and case samples have been ordered, and the racks have been placed in the rack loading area of the AU400e, the green "PLAY" arrow is pressed at the top of the screen to start the analysis.

18.8 CRITERIA FOR BATCH ACCEPTANCE

If the analysis of the batch meets the criteria listed below, the results for the specimens are accepted.

18.8.1 Controls

18.8.1.1 Negative control – blood and associated matrices

The negative control must read negative for all target compounds/classes. If the negative control is positive for any target compound/class (response of >100) all unknowns must be reanalyzed for that target compound/class.

18.8.1.2 Negative control – urine

The negative control must read negative (<25) for all target compounds/classes. If any target compound/class reads 25 or higher, all unknowns must be reanalyzed for that target compound/class.

18.8.1.3 Positive controls – blood and associated matrices

- a. The low control is prepared to illicit responses slightly above the cutoff concentration (i.e., response just over 100). In the event that one or more target compound/class does not read >100, that target compound/class response is multiplied by 0.75, and truncated to a whole number, to determine the new cutoff response. Any unknown samples reading greater than or equal to the new cutoff response shall be confirmed for that target compound/class. For example, if the opiate control reads 94, the resulting cutoff is 70. All cases with an opiate response ≥ 70 will be confirmed for opiates.
- b. The high control must read positive for all target compounds/classes (>100). In addition, the response for the high control should be greater than the response for each analyte in the low control. If a target compound/class response is negative (or less than the low control response for that target compound/class), all unknowns must be reanalyzed for that target compound/class.

18.8.1.4 Positive controls – urine

- a. Positive quality control (spiked using working control standard - see 18.5.3.2.1.b) must read >80 for target compounds/classes.
- b. If any of the above criteria are not met for a target compound/class, all unknowns must be reanalyzed for that target compound/class.

18.8.2 Case Specimens

If the target compound/class response printed on the report is >100, (or greater than or equal to the new cutoff as described in 18.8.1.3.a), it is reported as presumptive positive.

18.9 REPORTS AND DOCUMENTATION

18.9.1 Batch documentation includes instrument report printouts for all members of the test batch, including case samples, quality control, and calibration data (for blood and other associated matrices only) and the Excel worklist.

Typo in 18.9.2; Only analyst initials required on quality control and calibration printouts (applied from 7/9/19). AB 5/15/20

18.9.2 The analyst performing the test reviews the instrument reports, adds initials/date to each quality control and calibration printout, adds initials to each specimen report, and signs and dates the worklist prior to submission for peer review.

18.9.3 The peer review process includes verification that the calibration and all quality controls are acceptable (or that positive low blood control responses <100 are appropriately documented and/or new cutoff values are determined), and a report is included for all samples listed on the worklist. Batch review is documented by the reviewer adding initials/date to the quality control and calibration reports and signing/dating the worklist.

18.9.4 For blood and other associated matrices, the original sample report and copies of the calibration, negative controls, positive controls, and worklist are included in the respective case file. The original calibration data, control results, and worklist are filed in the batch file.

For urine, the original sample report and copies of the control results and the worklist are included in each respective case file. The original control results and worklist are filed in the batch file.

18.10 REPORTING

18.10.1 Any positive results reported from this assay are indicated as “presumptive positive” in the LIMS panel.

18.10.2 All presumptive positive results that are chosen to be confirmed must be confirmed by a separate method, in the same matrix or from a different matrix from the same case; if the confirmation method indicates results are not reportable, the presumptive positive EMIT result(s) is removed from the LIMS panel.

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APPENDIX A

NOTE: Refer to the *Standard Solution Preparation procedure (PQ12702)* for additional information.

Stock standards (1.0 mg/mL) are purchased from an approved reference material supplier and include the following:

- Benzoylecgonine
- Morphine
- Oxazepam
- Secobarbital
- 11-nor-9-COOH- Δ 9-THC
- d-Methamphetamine

Preparation

Adjustments to the final volume of prepared standards are permitted, provided that the proportions and final concentration is maintained.

Working standard (used for all matrices except urine)

Using a calibrated pipette, add the following volumes of each compound to a 50 mL Class A volumetric flask and bring to nominal volume with methanol.

Compound	Volume (μ L)	Final Standard Concentration (mg/L)	Cal Level 1 Concentration (mg/L)	Cal Level 2 Concentration (mg/L)
benzoylecgonine	250	5	0.10 mg/L	-
morphine	62.5	1.25	-	0.05 mg/L
oxazepam	125	2.5	-	0.10 mg/L
secobarbital	250	5	0.10 mg/L	-
(-)-11-nor-9-COOH- Δ 9-THC	25	0.5	0.01 mg/L	-
d-methamphetamine	250	5	-	0.20 mg/L

Working control standard (used for all matrices except urine)

Using a calibrated pipette, add the following volumes of each compound to a 10 mL Class A volumetric flask and bring to nominal volume with methanol.

Compound	Volume (μ L)	Final Standard Concentration (mg/L)	Low Pos Ctl Concentration (mg/L)	High Pos Ctl Concentration (mg/L)
benzoylecgonine	125	12.5	0.125 mg/L	0.25 mg/L
morphine	60	6	0.06 mg/L	0.12 mg/L
oxazepam	200	20	0.20 mg/L	0.40 mg/L
secobarbital	150	15	0.15 mg/L	0.30 mg/L
(-)-11-nor-9-COOH- Δ 9-THC	12.5	1.25	0.0125 mg/L	0.025 mg/L
d-methamphetamine	200	20	0.20 mg/L	0.40 mg/L

Urine working control standard

Using a calibrated pipette, add the following volumes of each compound to a 10 mL Class A volumetric flask and bring to nominal volume with methanol.

Compound	Volume (µL)	Final Standard Concentration (mg/L)	Pos Ctl Concentration (mg/L)
benzoylecgonine	125	12.5	0.375 mg/L
morphine	125	12.5	0.375 mg/L
oxazepam	80	8.0	0.24 mg/L
secobarbital	80	8.0	0.24 mg/L

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LIST OF CHANGES

Revision Date	Description	Page Number
5/30/13	Method approved by the State Toxicologist. See DRA dated 5/28/13. Method released for evidentiary use as of 5/30/13.	All
6/13/14	Added target concentrations to calibrator description table and added table for preparation of working control standard to spike positive controls, in lieu of prepared whole blood controls, to section 18.6.1. Changed opiates cutoff concentration from 20 ng/mL to 50 ng/mL in 18.6.1. Noted calibrator levels (cutoff concentrations) for compound classes in 18.6.2. Changed control description to reflect spiked positive controls in 18.6.3 and 18.7.1.4. Corrected reconstitution expiration for UTAK 0 to 30 days in 18.6.3. Added Keto-Diastix® to materials list in 18.5.2 and amended wording in 18.7.2. Removed 18.11.3, which described presumptive reporting.	3-8
6/1/15	Added "Technique" to title. Minor changes throughout document needed for clarification or grammatical reasons. Changes made to '18.6.3 Controls' to reflect the need for 10% controls with every batch, that controls must bracket casework, and all batches must end with a positive control.	All
6/30/15	Preparation instructions for the within-run spiked urine positive control in 18.6.3.2.2 (c) were modified for use of 0.25 mL blank urine (previously 0.5 mL), to provide an opiates concentration above the target cutoff (calibrator level).	7
10/7/15	Replaced "UTAK 0" in 18.9.1.2 with "negative" to reflect use of either the UTAK 0 negative serum control or blank urine.	10
3/16/16	Edited 18.6.1.4 to indicate use of the working controls standard to prepare positive controls for all matrices. Added wording to 18.9.1.4 to describe acceptance criteria for the within-batch (spiked) positive controls and a note to clarify that the UTAK 5, TCA and acetaminophen/salicylate positive controls (not spiked with working control standard) are positioned in the green quality control sample rack on the instrument. Other minor edits throughout. See DRA dated 1/28/16.	4, 7, 9, 10-11
11/7/16	Removed "...and Enzymatic Assay" from title, as acetaminophen/salicylates testing is no longer performed. Edited 18.2 to specify compounds/classes for urine versus other specimen types. Removed listed reagents in 18.5.1 for acetaminophen/salicylates, TCA, methadone and PCP and preparation instructions for acetaminophen/salicylates and TCA reagent kits. Removed PCP, methadone and nortriptyline from 18.6.1.2 list of stock standards and from 18.6.1.3 and 18.6.1.4 for preparation of working standard and working control standard. Updated 18.6.2.2 and 18.6.3.2 to reflect that urine calibration and QC check are only performed as needed and to remove acetaminophen/salicylates and TCA references. Removed description of ketones/glucose testing from 18.7.2 and updated 18.9.1.4 to remove acetaminophen/salicylates, TCA, methadone and PCP references and removed NOTE in 18.10.1 for acetaminophen/salicylates criteria. Removed reporting description for ketones/glucose testing in 18.11. Other minor edits throughout.	All
7/9/19	Removed policy and purpose sections and replaced with 18.1 METHOD. Added specific wording regarding matrix-matching for tissue homogenate in 18.3 SPECIMENS. Edited STANDARDS section – moved list of compounds and preparation to APPENDIX A. Updated 18.6 SAMPLE PREPARATION for use of spiked urine control. Other minor edits throughout. See DRA dated 7/2/19.	All