SCREENING OF BIOLOGICAL SPECIMENS BY ENZYME MULTIPLIED IMMUNOASSAY (EMIT) AND ENZYMATIC ASSAY

18.1 POLICY

This test method may be used to presumptively identify several drug classes as being present in biological specimens.

Any adjustments or deviations from the procedures below must be approved by the State Toxicologist, a Manager, or a Supervisor, and appropriately documented in the batch.

18.2 PURPOSE

The purpose of this standard operating procedure (SOP) is to provide technical direction for the presumptive identification of amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolite, methadone, opiates, phencyclidine (PCP), and tricyclic antidepressants (TCA), in urine, whole blood, serum, plasma, vitreous humor, or tissue homogenate, and presumptive identification of acetaminophen and salicylate in urine.

18.3 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 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 indentifies 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.

18.4 SPECIMENS

18.4.1 Specimens include whole blood, serum, plasma, urine, vitreous humor and tissue homogenate.

18.4.2 The standard specimen volume of whole blood, tissue homogenate, or vitreous humor is 1 mL.

18.4.3 Smaller volumes of specimens may be analyzed at the Forensic Scientist's discretion.

18.4.4 Analysis of larger specimen volumes must be approved and documented.

18.4.5 Given that serum, plasma, and urine do not require any sample preparation, approximately 8 drops of each sample type is sufficient to complete testing.

18.5 REAGENTS, MATERIALS AND EQUIPMENT

18.5.1 REAGENTS

18.5.1.1 Acetonitrile

18.5.1.2 Certified blank blood

18.5.1.3 Certified blank urine

18.5.1.4 Dilute aqueous *Emit*® Drug Assay Buffer

Emit® Drug Assay Buffer Concentrate (from TCA reagent kit) is diluted 1:14 with DI H_2O. (For example: Add 5 mL concentrated EMIT buffer to 70 mL of DI H_2O and mix, for total volume of 75 mL dilute aqueous EMIT buffer). Changes to the final volume are permitted, provided that the proportions are maintained. Solution is for use on date of preparation only. This solution is used to prepare the working blood EMIT buffer (18.5.1.8) and to reconstitute the TCA reagents (18.5.1.9).

18.5.1.5 *Emit*® Drug Assay Buffer Concentrate

18.5.1.6 *Emit*® *II Plus* reagents for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolite, methadone, opiates and phencyclidine (PCP) (supplied as ready-to-use liquids, kept refrigerated).

18.5.1.7 *Emit*® *tox*™ serum tricyclic antidepressants calibrator and controls, kept refrigerated.

18.5.1.8 Methanol (MeOH)

18.5.1.9 Working blood EMIT buffer

Equal parts dilute aqueous EMIT buffer (prepared in 18.5.1.3) 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). Changes to the final volume are permitted, provided that the proportions are maintained. The solution is stored in a plastic or glass bottle at room temperature and expires one year from the date of preparation.

18.5.1.10 Stanbio Laboratory Acetaminophen Liquacolor® reagents (supplied as a ready-to use liquids, kept refrigerated).

18.5.1.11 Stanbio Laboratory Salicylate Liqui-UV® reagents (supplied as liquids, kept refrigerated). Reagent A (R1) is ready to use without modification. Reagent B (R2) is diluted to a total volume of 10 mL with DI H_2O.

18.5.1.12 TCA reagents, reconstituted

TCA reagents A and B are reconstituted with 3 mL DI H_2O, using a volumetric pipette. TCA reagents R1 and R2 are prepared as follows:

•Reagent 1 (R1) is prepared by mixing one part reconstituted reagent A with 8 parts dilute aqueous EMIT buffer (prepared in 18.5.1.3). (For example, the 3 mL of reconstituted reagent A is

mixed with 24 mL dilute aqueous EMIT buffer for a total volume of 27 mL R1).

•Reagent 2 (R2) is prepared by mixing one part reconstituted reagent B with 8 parts dilute aqueous EMIT buffer.

18.5.1.13 UTAK urine toxicology controls (UTAK 0 negative control, UTAK 5 positive control), kept refrigerated.

18.5.2 MATERIALS

18.5.2.1 Disposable 16 x 125mm tubes with closures

18.5.2.2 Fisherbrand conical sample cups (or equivalent)

18.5.2.3 Disposable centrifuge tubes with closures

18.5.2.4 Disposable pipette tips

18.5.2.5 Laboratory glassware (graduated cylinders, flasks)

18.5.2.6 Volumetric glassware (flasks, pipettes)

18.5.3 EQUIPMENT

18.5.3.1 Olympus AU400e

18.5.3.2 Calibrated, adjustable air-displacement pipettes

18.5.3.3 Centrifuge

18.5.3.4 Vortex mixer

18.5.3.5 Evaporator (Caliper LS, formerly Zymark, TurboVap)

18.6 STANDARDS, CALIBRATORS AND CONTROLS

18.6.1 STANDARDS

18.6.1.1 Reference materials (referred to interchangeably in this method as stock standards) are used for the preparation of working standards which in turn are used to produce calibrators for use with all matrices except urine.

18.6.1.2 Stock standards are purchased from an approved reference material supplier and include the following:

a.	Benzoylecgonine:	1.0 mg/mL
b.	Morphine:	1.0 mg/mL
c.	Oxazepam:	1.0 mg/mL
d.	Secobarbital:	1.0 mg/mL
e.	11-nor-9-COOH-Δ9-THC	1.0 mg/mL
f.	d-Methamphetamine	1.0 mg/mL
g.	Phencyclidine	1.0 mg/mL
h.	Methadone	1.0 mg/mL
i.	Nortriptyline	1.0 mg/mL

18.6.1.3 Working standard (used for all matrices except urine)

a. Using a calibrated pipette, add the following volumes of each compound to a 50 mL Class A volumetric flask:

Compound	Volume (µL)	Final Concentration (mg/L)
benzoylecgonine	250	5
morphine	25	0.5
oxazepam	125	2.5
secobarbital	250	5
(-)-11-nor-9-COOH-Δ9-THC	25	0.5
S(+)-methamphetamine	250	5
phencyclidine	25	0.5
methadone	250	5
nortriptyline	250	5

b. Add MeOH to the flask to the designated volume.
c. Adjustments to the final volume are permitted, provided that the proportions and final concentration is maintained.
d. The final concentration of the working standard is listed in the table above for each compound. The working standard is stored in the freezer in an amber bottle and expires one year from the date of preparation.

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

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

Calibrators are prepared in certified blank blood at the time of analysis using the working standard. The preparation of the calibrators is detailed in 18.7 SAMPLE PREPARATION. If necessary, calibrators may be prepared in alternate matrices provided that the matrix has been previously determined to not contain any of the compounds tested for by this procedure.

18.6.2.2 Urine: Urine calibration is performed at least once a week (at conclusion of weekly instrument maintenance).

The following calibrators are supplied as liquids, ready to use, and kept refrigerated:

a. *Emit® II Plus* calibrators (level 1, 2, and 3). Stable until expiration date printed on vial.
b. Stanbio Laboratory acetaminophen calibrator (300 mg/L). Stable until expiration date printed on vial.
c. Stanbio Laboratory salicylate calibrator (300 mg/L). Stable until expiration date printed on vial.

The following calibrator requires reconstitution:

Emit® tox™ TCA calibrator (300 mg/L nortriptyline, reconstituted with 3 mL DI H_2O using a volumetric pipette). Stable for 12 weeks from date of reconstitution.

18.6.3 CONTROLS

18.6.3.1 Negative Controls

Blood, Serum, Plasma, Vitreous, or Tissue Homogenate

a. Two negative whole blood controls are tested with every batch. One is to function as the matrix blank for the spectrophotometer, and one as the negative control. Both controls are prepared using certified blank blood.

Urine (Run once daily)

a. The urine negative control, UTAK 0, is reconstituted with 5 mL DI H_2O using a volumetric pipette. Must be used within 25 days of reconstitution.

18.6.3.2 Positive Controls

Blood, Serum, Plasma, Vitreous, or Tissue Homogenate

a. At least two whole blood positive controls are tested with every batch.
b. Stock standards used to prepare positive controls are obtained from an approved reference material supplier.
c. Prepared whole blood positive controls, at low and high levels, are provided by QA personnel.

Urine (Run once daily)

a. The urine positive control, UTAK 5, is reconstituted with 5 mL DI H_2O using a volumetric pipette. Must be used within 25 days of reconstitution. This serves as the positive urine control for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolite, methadone, opiates and PCP.
b. The *Emit*® *tox*™ TCA positive urine control requires reconstitution with 3 mL DI H_2O using a volumetric pipette. Solution is stable for 12 weeks from date of reconstitution.
c. A salicylate/acetaminophen positive urine control is prepared in-house as follows:

•Stock solutions (2.0 mg/mL)

Add 200 mg acetaminophen to 50 mL DI H_2O in a Class A 100 mL volumetric flask. Fill to designated volume with DI H_2O and mix, adding low heat as necessary for complete dissolution.

Add 200 mg salicylic acid to 50 mL DI H_2O in a Class A 100 mL volumetric flask. Fill to designated volume with DI H_2O and mix, adding low heat as necessary for complete dissolution.

Stock solutions may be stored refrigerated in an amber bottle for up to one year from the date of preparation.

•Urine control

Add 5 mL of each stock solution to a Class A 100 mL volumetric flask. Fill to designated volume with fresh, drug-free urine. The control solution is stored in the refrigerator in an amber bottle and expires 3 months from the date of preparation.

NOTE: If preparing the acetaminophen/salicylate positive control from stock solutions that have been refrigerated, the stock solutions may need to be carefully heated and mixed (approximately one hour) to ensure complete dissolution before preparing urine control.

18.7 SAMPLE PREPARATION (Blood, Vitreous, or Tissue Homogenate)

18.7.1 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 18.7.1.11 for unextracted serum or plasma samples. Serum or plasma samples may be extracted, as determined by available sample volume.

18.7.1.1 Label a clean 16 x 125mm tube for each member of the test batch. (i.e. calibrators, controls, or case samples).

18.7.1.2 Add 1 mL of certified blank whole blood into each of the two calibrator tubes (low and high), the matrix blank, and the negative control.

18.7.1.3 Using the working standard, spike the low and high calibrators as follows: Add 20µL of working standard to the low calibrator tube and 40µL to the high calibrator tube.

18.7.1.4 Transfer 1 mL of each of the prepared whole blood control into their corresponding tubes.

18.7.1.5 Sample 1 mL of each case sample into its respective tube.

18.7.1.6 To each tube, add 1 mL of methanol, followed immediately by 3 mL of acetonitrile. Vortex mix approximately 30 seconds.

18.7.1.7 Centrifuge the tubes for 5 minutes at 2000rpm to achieve separation.

18.7.1.8 Decant the supernatant into a conical centrifuge tube, and evaporate under air at 50°C to approximately 100µL.

18.7.1.9 Remove the tubes from the evaporator and add 350µL of working blood EMIT buffer and vortex mix.

18.7.1.10 Centrifuge the tubes for 5 minutes at 2000rpm.

18.7.1.11 Transfer the clear supernatant to labeled conical sample cups for analysis on the Olympus AU400e. See section 18.8 INSTRUMENTAL PARAMETERS for guidance.

18.7.2 Urine

18.7.2.1 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 18.8 INSTRUMENTAL PARAMETERS).

NOTE: Ketones and glucose screening can be performed simultaneously using Keto-Diastix® test strips (follow manufacturer's instructions). The results are documented at the bottom of the urine report from the AU400e.

18.8 INSTRUMENTAL PARAMETERS

18.8.1 INSTRUMENT MAINTENANCE

Daily, weekly, monthly, 3-month, and 6-month maintenance is required to keep the AU400e in working order. Refer to the Olympus AU400e Maintenance Manual for instructions on routine maintenance as well as troubleshooting any issues that may arise. Maintenance records are filed in the instrument maintenance/QC binder.

18.8.2 INSTRUMENT CALIBRATION

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

18.8.2.2 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" under "profile" if using the serum panel, "2" for the urine panel. Click "Entry" to make the selection, and then click on "Exit."

18.8.2.3 For urine calibration, use a blue sample rack with DI H_2O 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.

18.8.2.4 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 20μL low calibrator, and position 2 with the 40μL high calibrator.

NOTE: Blood (and other associated matrices) calibration is performed with each batch containing those matrices.

18.8.3 ORDERING CONTROL SAMPLES

NOTE: Ensure that step 18.8.2.1 (setting the operator name and data index) is performed before ordering any quality controls or sample tests.

18.8.3.1 To order urine controls (performed once daily, with original report filed in the instrument maintenance/QC binder), select "Order QC from Green Rack" under USER. Select urine for the sample type. Press "Exit". Fill the labeled sections of a green sample rack with the appropriate quality controls.

18.8.3.2 The negative, low, and high control for blood and other associated matrices are ordered as samples. See section 18.8.4 ORDERING SAMPLE TESTS.

18.8.4 ORDERING SAMPLE TESTS

18.8.4.1 Under USER, choose “Select Report Format”. Select either the urine report or blood report appropriately.

18.8.4.2 Under USER, select “Order Sample Tests”. Set the sample type appropriately.

18.8.4.3 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).

18.8.4.4 Click on “Profile” and select “1” for serum and “2” for urine.

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

18.8.4.6 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 18.8.4.3.

18.8.4.7 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.

18.9 CRITERIA FOR BATCH ACCEPTANCE

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

18.9.1 Controls

18.9.1.1 Negative control – blood and associated matrices

The negative control must read negative for all analytes. If the negative control is positive for any analyte, (response of >100) all unknowns must be reanalyzed for that analyte.

18.9.1.2 Negative control – urine

The UTAK 0 control must read negative (<25) for all analytes. If any analyte reads 25 or higher, all unknowns must be reanalyzed for that analyte.

18.9.1.3 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 analytes does not read >100, that analyte response is multiplied by 0.75 to determine the new cutoff response. Any unknown samples reading greater than or equal to the new cutoff response shall be confirmed for that analyte.

For example, if the opiate control reads 92, the resulting cutoff is 69. All cases with an opiate response ≥69 will be confirmed for opiates.

b. The high control must read positive for all analytes (>100). In addition, the response for the high control should be greater than the response for each analyte in the low control. If an analyte's response is negative, all unknowns must be reanalyzed for that analyte.

18.9.1.4 Positive controls – urine

a. The UTAK 5 control response must read positive (>100) for amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolite, methadone, opiates and PCP.

b. The TCA positive control response must read >100.

c. The acetaminophen and salicylate control response must read between 80 and 120, inclusive.

d. If any of the above criteria are not met for an analyte, all unknowns must be reanalyzed for that analyte.

18.10 CRITERIA FOR CASE SAMPLE ACCEPTANCE

If the criteria for batch acceptance have been satisfied, the results of individual case samples are deemed suitable for reporting if the following criteria are met.

18.10.1 If the analyte response printed on the report is >100, (or greater than or equal to the new cutoff as described in 18.9.1.3.a), it may be reported as presumptive positive.

NOTE: For acetaminophen and salicylate in urine, the response must read >20 to be reported as presumptive positive.

18.10.2 The entire batch, including the Excel worklist, case samples, quality control, and calibration data (for blood and other associated matrices only), is submitted for technical peer review.

18.10.3 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.

18.10.4 For blood and other associated matrices, the original sample report and copies of the calibration, negative control, low control, high control, and worklist are included in the respective case file. The original calibration data, control results, and worklist are retained in the case file of the first sample in the batch.

For urine, the original sample report and copies of QC results (from date of analysis) and the worklist are included in the respective case file. The original control results (or reprinted if from a previous run the same day) and worklist are retained in the case file of the first sample in the batch.

18.11 REPORTING

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

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

18.11.3 In the case of death investigations, cannabinoid positive results may be left unconfirmed by a separate method, provided there is a positive EMIT result for two matrices (i.e. urine and blood), and language appears at the bottom of the report indicating that the EMIT positive results are not confirmed.

18.11.4 For reporting of positive glucose and/or ketones results from testing with Keto-Diastix® strips, the estimated result (based on test strip color comparison to ranges in color chart provided by the manufacturer) is reported in units of mg/dL.

18.12 TRACEABILITY

18.12.1 Traceability of the reference materials to SI units is provided through the certificate of analysis provided by the approved reference material supplier.

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