Section 1.0
Enzyme Immunoassay Screening for Drugs-of-Abuse in Urine and Blood

1.1 Enzyme Immunoassay Screening for Drugs-of-Abuse in Urine

1.1.1 BACKGROUND

Enzyme Multiplied Immunoassay Technique (EMIT) is a competitive binding assay based on the enzyme activity of drug-labeled Glucose-6-Phosphate Dehydrogenase (G-6-P-DH). Glucose-6-Phosphate Dehydrogenase (G-6-P-DH) catalyses the conversion of the substrate Glucose-6-Phosphate (G-6-P), this activity simultaneously results in the conversion of the co-factor Nicotinamide-Adenine Dinucleotide (NAD) to the reduced form NADH. This conversion results in a subsequent increase in absorbance at 340 nm. Both the degree of absorbance and reaction rate is monitored spectrophotometrically.[2]

Note that in this analytical method, the terms calibrator and calibration are not used in the ISO/IEC 17025:2005 sense. The manufacturer term *calibrator* refers to a urine sample with a known drug reference material concentration. This spiked urine is used for a one point *calibration* to establish a direct relationship between an amount of drug in the spiked urine and the degree/rate of absorbance as described below. The terms *calibrator* and *calibration* are used to coincide with the terminology in manufacturer manuals and package inserts.

For drugs-of-abuse applications, the G-6-P-DH is labeled with the particular drug, which the assay is designed to detect.[9,10] In the EMIT reaction, a drug in a urine specimen competes with the drug-enzyme for the binding site on a drug or drug-class specific antibody. The amount of NADH produced during the EMIT reaction is directly proportional to the amount of drug present. The EMIT reaction takes place over a finite time interval.[9,10] The rate of NADH production is what is used to provide a preliminary indication of the presence of a drug or drug class in the urine specimen. The initial set-up of the automated chemistry analyzer used for the EMIT reaction monitors the rates of production of NADH for various calibrators and controls containing known concentrations of drug. This information is then used to detect drugs in an unknown sample. The following examples outline how the EMIT reaction detects drugs-of-abuse.

Example 1: Specimen Contains Drug(s) of Interest

- An aliquot of the urine, Reagent A and Reagent B are added together.
- The drug in the urine and the drug-labeled enzyme compete with each other for available binding sites on the antibody.
- As the concentration of the drug in the urine is higher than that of the drug-labeled enzyme, a larger proportion of the free drug will bind to the antibody.
- The antibody binding sites become filled predominantly with drug from the sample.
- This results in a higher proportion of drug-labeled enzyme unbound in solution.
- This enzyme is available to breakdown G-6-P.
- When G-6-P is broken down, along with the product, a single Hydrogen ion is released.
- The NAD reacts with the H^+ forming NADH.

- The amount of NADH formed is directly proportional to the amount of free drug in the urine. Thus more NADH indicates more drug(s) is present in the urine specimen.
- NADH has an absorbance wavelength of 340nm (UV).

Example 2: Specimen Contains No Drug(s) of Interest

- An aliquot of the urine, Reagent A and Reagent B are added together.
- The drug in the urine and the drug-labeled enzyme compete with each other for available binding sites on the antibody.
- Due to the absence of competing drug, much of the drug-labeled enzyme will bind to the antibody.
- Due to the effect of steric hindrance - the active site on the enzyme is blocked by the sheer size of the antibody.
- Bound enzyme therefore cannot breakdown G-6-P.
  - Note - In the absence of drug, some enzyme-labeled drug does remain free and some NADH is formed. Thus a negative does have some measurable absorbance. This absorbance is clearly differentiated from the absorbance of the cut-off calibrator by a defined level of separation. In addition to the absorbance change, the rate of conversion from NAD to NADH is monitored and must occur within established time limits.
- With less hydrogen ions liberated, significantly less NADH is formed.
- A low reading at 340nm indicates the absence of drug.

1.1.2 SCOPE

This analytical method employs EMIT for the qualitative screening for drugs-of-abuse in urine specimens. EMIT is commonly used for the detection of drugs-of- abuse in urine. The EMIT assays are run on a microprocessor-controlled automatic chemistry analyzer. The assay results are intended as only a preliminary analytical test result. Confirmatory analysis is performed with an instrument such as a gas chromatograph or liquid chromatograph equipped with a mass selective detector. If EMIT results are reported out, the report must clearly state that the results are from initial screening and confirmatory testing may be requested.

As indicated in the table below, each assay in use has an established administrative threshold or cut-off. For this reason, a negative result does not indicate that no drug is present; the concentration of the drug may be less than the administrative cut-off, or a drug may have poor cross-reactivity to the assay. For this reason there may be situations where confirmation of an analyte may be pursued even if a negative result is indicated for the compound or a class of compounds in question.

Assay	***Calibrator***	***Urine Cut-off***
Amphetamines	d-Methamphetamine	500ng/mL
Benzodiazepines	Lormetazepam	300ng/mL
Cannabinoids	11-Nor-9-Carboxy-THC	50ng/mL
Cocaine Metabolite/-M	Benzoylecgonine	300ng/mL
Methadone	Methadone	300ng/mL
Opiates	Morphine	300ng/mL

1.1.3 EQUIPMENT

1.1.3.1 Viva-Junior™ Analyzer
1.1.3.2 Disposable polyethylene pipettes
1.1.3.7 Disposable 1 mL plastic specimen cups
1.1.3.8 Disposable 13X75 polypropylene tubes
1.1.3.9 15mL HDPE Bottle
1.1.3.10 30mL HDPE Bottle

1.1.4 REAGENTS

1.1.4.1 DI water
1.1.4.2 Syva EMIT Assay Kits

Antibody/Substrate Reagent 1:
Antibodies to drug(s) of interest, bovine serum albumin, Glucose-6-Phosphate (G-6-P), Nicotinamide Adenine Dinucleotide (NAD^+), preservatives, and stabilizers.

Enzyme Reagent 2:
Drug(s) of interest labeled with bacterial Glucose-6-Phosphate Dehydrogenase (G-6-P-DH), Tris buffer, bovine serum albumin, preservatives, and stabilizers.

1.1.4.3 Manufacturer Provided Assay Reagents
- 0.1N Hydrochloric Acid (Cleaning Solution A)
- 0.1N Sodium Hydroxide (Cleaning Solution B)
- System Solution (Added to DI water for rinsing)
- Sodium Hypochlorite (Needle Rinse)

1.1.5 REFERENCE MATERIAL

1.1.5.1 **EMIT® Cut-off Calibrators**

The following table indicates which level of EMIT urine calibrator contains the selected cut-off concentration.

Assay/ Selected Cutoff (ng/mL)	Level 1	Level 2	Level 3	Level 4
Amphetamine/500		☠		
Benzodiazepine/300				☠
Carboxy-THC/50			☠	
Cocaine-M/300			☠	
Methadone/300			☠	
Opiate/300	☠			

1.1.5.2 **EMIT Urine Controls**

1.1.5.2.1 EMIT® Urine Controls
- EMIT® Level 0/Negative Control
- EMIT® Level 5/High Positive Control

Assay/ Cutoff (ng/mL)	Level 0	Level 5
Amphetamine/500	0	2000
Benzodiazepine/300	0	1000
Carboxy-THC/50	0	200
Cocaine-M/300	0	1000
Methadone/300	0	1000
Opiate/300	0	4000

1.1.5.3 **Commercially Obtained Enzyme Immunoassay Positive Urine Controls**
Obtain positive urine controls with concentrations which challenge each EMIT® assay at below, just above or well above the cut-off for each assay. Ideally the control should contain the analytes that are present in calibrators. Positive control can be obtained through BIORAD, UTAK or other suitable vendor.

1.1.5.5 **Negative Control**
Drug-free urine. Negative control can be provided in-house or obtained through BIORAD, UTAK or other suitable vendor.

1.1.6 PROCEDURE

1.1.6.1 Analyzer Calibration and Pre-run Controls

1.1.6.1.1 EMIT calibrators are used to set-up the analyzer for each assay at the selected assay cut-off. These cut-offs will be programmed during analyzer installation.

1.1.6.1.2 To confirm that the analyzer is properly calibrated for each assay, controls are analyzed and evaluated. Prior to each casework run, a minimum of an EMIT Level 0/negative and an EMIT Level 5/High positive control must be run. Two additional urine controls, commercially obtained (see section 1.1.5.3) should be included.

1.1.6.1.3 For the Viva-Junior™ Analyzer, the calibration for urine assays is valid as long as analyzer provides appropriate responses for controls.

1.1.6.1.4 Validity of calibration is verified by:

- Comparing calibrator reaction rates against those of last calibration.
- Controls responding appropriately as outlined in 1.1.6.1.6.

1.1.6.1.5 Appropriate control responses are:

- Level 0/Negative Control indicating negative response.
- Level 5/High Control indicating positive response.
- Commercially obtained controls responding appropriately.
- Comparison of control reaction rates against those of previous controls, analyzed with current calibration, indicates no significant change.
- Evaluation of assay/level specific manufacturer provided rate separations indicates suitable separations between the following:
  - Negative control/Level 0 and cut-off.
  - Cut-off and High control/Level 5.

1.1.6.1.6 There are no absolute evaluation criteria due to variation between analyzers and assays. However, at the discretion of the analyst, any significant departure from previous values should warrant recalibration of the analyzer.

1.1.6.1.7 If controls fail, the instrument calibrators must be run followed by analysis of additional urine controls.

1.1.6.2 <u>In-run Controls</u>

1.1.6.2.1 In each casework analysis run, a minimum of one negative and one positive urine control must be included in rotor sample positions. Refer to section 1.1.5.2 for urine control options.

1.1.6.2.2 Appropriate control responses are:

- Negative urine control indicating negative response consistent with that observed for EMIT Level 0/Negative control.
- Positive urine controls indicating appropriate positive response relative to cut-off.
- No significant change is noted when control reaction rates are compared to those of previous controls analyzed with current calibration.

1.1.6.3 <u>Sample Run Preparation</u>

1.1.6.3.1 Program instrument with laboratory numbers and urine control information.

1.1.6.3.2 **1mL Plastic Cups (Pediatric)**

Dispense urine unknowns, negative and positive urine controls into EMIT immunoassay cup. Do not overfill cup.

- Based on sample volumes and dead volume, minimum sample to run our selected assays is ≅171µL.

Place cup into pediatric adapter and load in designated position on sample rotor.

1.1.6.3.3 **13 x 75 mm Tubes**

Dispense urine unknowns, negative and positive urine controls into tube. Do not overfill tube.

- Based on sample volumes and dead volume, minimum sample to run our selected assays is ≅421µL.

Place tube into designated position of sample rotor.

1.1.6.4 Viva Junior™ Operation and Maintenance[5,6]

Refer to current Viva Junior™ Operation Guide and manual.

1.1.7 DETECTION CRITERIA

1.1.7.1 Positive Case Sample Result

Provided that calibration and control evaluation indicate that analyzer has quality assurance conditions suitable for use, a positive result for a sample is indicated by a change in absorbance at a rate value (dABS/m) of equal to or greater than the *Cut-off Calibrator*.

1.1.7.2 Elevated Absorbance

At the discretion of an analyst, confirmatory techniques may be applied to samples that exhibit an elevated absorbance rate. An elevated absorbance rate is that greater than that of the negative control/Level 0 but less than the cut-off calibrator. If data for confirmatory techniques supports the presence of an analyte, the analyte may be reported as present. In addition, samples with compounds that have low cross reactivity may be confirmed and reported with a negative screen result.

1.1.7.3 Negative Result

A negative result for a sample is indicated by a change in absorbance at a rate that is less than the Cut-off Calibrator. Special considerations may apply as outlined above (1.1.7.2).

1.1.8 DISTRIBUTION OF ASSAY INFORMATION

1.1.8.1 Electronic copy of EIA analysis report must be attached to the case in LIMS. Case results are also to be recorded in the LIMS system.

1.1.8.2 A copy of data for calibrators and controls may be stored electronically in a central location.

1.1.8.3 Original data for calibration and controls for each analysis must be stored centrally in the laboratory where the analysis was performed, until archiving or destruction.

1.1.9 QUALITY ASSURANCE REQUIREMENTS

1.1.9.1 Refer to method 5.8 for storage requirements.

1.1.9.2 Refer to toxicology manual section 5.10 for authentication of reference material requirements.

1.1.10 REFERENCES

1.1.10.1 Thompson, S.G., *Principles for Competitive-Binding Assays. in:* Clinical Chemistry: Theory, Analysis, Correlation, edited by Kaplan, L.A., Pesce, A.J. and Kazmierczak, S.C., pp. 246-260, Mosby, 2003.

1.1.10.2 Hand, C. and Baldwin, D., *Immunoassays in:* Clarke's Analytical Forensic Toxicology, edited by Jickells, S. and Negrusz, A., pp. 375-391, Pharmaceutical Press, 2008.

1.1.10.3 E.M.I.T. Urine Screening Procedure, Montana Department of Justice Forensic Sciences Division, Courtesy of Jim Hutchison, May 2008.

1.1.10.4 Enzyme Multiplied Immunoassay (EMIT) Enzymatic Assays for Drug Screening in Urine, Whole Blood Extracts and Other Biological Fluids, Washington State Toxicology Laboratory, Courtesy of Melissa Pemberton, August 2008.

1.1.10.5 Viva-Jr™ Operator's Manual, Article No.: 6002-940-410, Version number: 01/04-06.

1.1.10.6 Viva-Jr™ System Operations Guide, T268, 6/25/07, D01373.

1.1.10.7 Viva-E™ Operator's Manual, Article No.: 6002-380-410-01, Version number: 1.0/08-04

1.1.10.8 Viva-E™ System Operations Guide, T216, 6/4/07, D01320.

1.1.10.9 Leedam, D.C., EMIT Basic Power Point Presentation, February 1997 (Provided by Siemens during training October 16, 2008.)

1.1.10.10 Syva Package Inserts for Emit II Plus Assays

Amphetamines:	9C122UL.4DS_A
Benzodiazepine:	9F022UL.10DS_B
Cannabinoid:	9N022UL.9DS_A
Cocaine:	9H522UL.4DS_A
Methadone:	9E022UL.9DS_A
Opiate:	9B322UL.10DS_A

Method History

Section 1.0
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Revision No.	Issue Date	History
0	01-15-2009	Original Issue
1	11-28-2012	Removed references to Viva E, removed option, equipment and information for preparing positive controls in house. Clarified detection criteria. Cleaned up quality assurance requirements by removing the ones that did not apply and referring to the central quality assurance methods.
2	1/16/2014	Amendment to 1.1.8 in accordance with new LIMS system. Minor formatting changes
3	04/02/2015	Minor grammatical corrections or clarifications. Clarification of how results are to be recorded in LIMS.