

## Section 1.0

### Urine and Blood Toxicology

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#### 1.0 Enzyme-Linked Immunosorbent Assay Screening for Drugs of Abuse

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##### 1.1 BACKGROUND

ELISA is an acronym for enzyme-linked immunosorbent assay. An ELISA is an enzyme immunoassay (EIA) in which one reactant is immobilized on a solid phase and the signal generator is an enzyme. The enzyme delivers a signal to indicate to what extent a particular antigen-antibody reaction has occurred. This reaction takes place inside of a polystyrene microtiter plate well. Horseradish peroxidase is an enzyme commonly employed as a signal generator. The small size of HRP, the ease with which small conjugates can be produced through oxidation of its carbohydrate moieties to reactive aldehyde, its rapid kinetics, and reasonable price, make it popular in ELISA.

For the qualitative determination of a specific drug, or class of drugs in blood and urine this method utilized competitive micro-plate immunoassay. Each of the assays requires a predilution step for samples, controls and calibrators. This brings the analytes into an acceptable range for optimum performance of the bound microplate antibodies. Dilutions are either performed manually, with an air displacement pipette or a dilutor, or using the automated dilution capability of the instrument. Samples, calibrators or controls are added to individual wells of the microplate along with the conjugate, which is the drug or hapten labeled with the enzyme horseradish peroxidase (HRP). There is a competition between the free drug in the matrix sample (blood or urine) and drug bound to enzyme (conjugate) for antibody (sheep or rabbit) fixed on the well. The wells are washed with DI water, the substrate (3,3',5,5' tetramethylbenzidine (TMB) with peroxide ( $H_2O_2$ ) is added, and a color is produced. HRP catalyzes  $H_2O_2$  oxidation of the substrate by transferring one electron from the TMB to the peroxide to yield a blue colored product. The reaction is stopped when 2.0N sulfuric acid is added to the well. This acidic environment provides the necessary conditions for the loss of an additional electron to produce the final yellow color. The acidic environment also serves to inactivate the enzymatic activity of the HRP. The resulting absorbance at 450nm is inversely proportional to the amount of drug present in the sample or standard. Consequently, a more intense yellow color results in a greater absorbance and indicates a lower concentration of drug in the sample. The Micro-Plate EIA utilizes two matrix matched calibrators, one containing no drug (negative calibrator) and one at the concentration corresponding to the accepted cut-off for the drug (cut-off calibrator). In addition, negative and positive controls are used to assess the performance of the kit. An automated microplate analyzer is used for processing on the microplates. The analyzer automatically dispenses samples and all reagents required for ELISA testing. In addition, the analyzer allows for the programming of incubation times and wash steps.

## 1.2 SCOPE

This Micro-plate assay is applied for the qualitative screening for drugs-of-abuse in blood or urine specimens. Appropriate dilutions are made for application to the screening of blood and urine. The outcome of the assay is intended as only a preliminary analytical test result. The presence of a particular drug compound must be verified through analysis with a confirmatory instrument such as a gas chromatograph equipped with a mass selective detector.

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, only that the concentration is less than the administrative cut-off. 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. The exceptions are discussed in section 1.8.2.

<i>Assay</i>	<i>Calibrator</i>	<i>Urine Cut-off</i>	<i>Blood Cut-off</i>
Benzodiazepine	Oxazepam	300ng/mL	100ng/mL
Cannabinoid	(-) 11nor-9-carboxy- $\Delta$ -9-THC	50ng/mL	15ng/mL
Cocaine Metabolite	Benzoyllecgonine	300ng/mL	50ng/mL
Methadone	Methadone	300ng/mL	50ng/mL
Methamphetamine	S(+)-Methamphetamine	500ng/mL	50ng/mL
Opiate	Morphine	300ng/mL	50ng/mL
Amphetamine	S(+)-Amphetamine	500ng/mL	
Barbiturates	Secobarbital	100ng/mL	100ng/mL

## 1.3 EQUIPMENT

### 1.3.1 Sample Diluting Supplies

- 1.3.1.1 Air-displacement pipettes and appropriate tips.
- 1.3.1.2 Repeater Pipette and appropriate tips.
- 1.3.1.3 Automatic Dilutor equipped with appropriate syringes.
- 1.3.1.4 Screening instrument programmed for automatic dilutions.

### 1.3.2 Plasticware

- 1.3.2.1 2.0mL control vials with caps
- 1.3.2.2 25mL reagent tubes with caps
- 1.3.2.3 Disposable 13x75 polypropylene tubes
- 1.3.2.4 Disposable transfer pipettes
- 1.3.2.5 Deep-well strips/plates for automated dilution

- 1.3.3 DSX Automated ELISA Instrument
- 1.3.4 Tube Rocker
- 1.3.5 Vortex Mixer

## 1.4 REAGENTS

- 1.4.1 Forensic Specimen Diluent (Remember to check expiration date prior to use.)

### Assay Kits:

Micro-plates coated with anti-drug antibodies.

Enzyme conjugate for specific drug/drug class.

TMB Substrate reagent (universal).

2N H<sub>2</sub>SO<sub>4</sub> Stopping reagent (universal).

- 1.4.2 Processing of New Assay Supplies

- 1.4.2.1 When a new kit is opened, note the expiration date of all components listed on each assay's specification sheet.

- 1.4.2.1.1 The manufacturer's kit expiration date may be based on a component not used for the analysis of blood or urine. Only the expiration of the conjugate and plate involve the use of the assay kit since the expiration date of the substrate and stop always far exceeds the expiration date of the conjugate and the plates.

- 1.4.2.2 **Cocaine Assay Conjugate Preparation**

- 1.4.2.2.1 Using a pipette, to the vial containing *Benzoylcegonine Lypophilized Stock Enzyme Conjugate*, add 2mL *Conjugate Diluent*.

- 1.4.2.2.2 Place vial on tube rocker for a minimum of 10 minutes.

- 1.4.2.2.3 Using a pipette, add the volume of reconstituted *Stock Enzyme Conjugate* listed on the kit package insert to appropriate volume of *Conjugate Diluent*. **Prepare only necessary volume of conjugate.** (Note: The *Stock Enzyme Conjugate* is lot specific.)

- 1.4.2.2.4 Gently mix *Conjugate Diluent* bottle on tube rocker for a minimum of 1 minute.

- 1.4.2.2.5 Prior to use, allow bottle to equilibrate for a minimum of 30 minutes at room temperature or overnight under refrigeration.

## 1.5 REFERENCE MATERIAL

For both urine and blood, the following calibrators and controls must be included in each analysis run. In-house calibrators and controls should be prepared each month, at a minimum. If the method is not utilized during a month, no calibrators or controls need be prepared. If a screen fails, preparation of fresh calibrators/controls is a good troubleshooting step.

### 1.5.1 Urine

#### 1.5.1.1 **Stock Reference Material Solutions**

Obtain Amphetamine (1mg/mL), Methamphetamine (1mg/mL), Benzoyllecgonine (1mg/mL), Methadone (1mg/mL), Morphine (1mg/mL), 11-nor-9-Carboxy- $\Delta$ 9-THC (100 $\mu$ g/mL), Oxazepam (1mg/mL), and Secobarbital (1mg/mL) drug reference material from an appropriate vendor. Different vendors should be used to make up the Calibrator and Control Working Solutions whenever possible. If this is not possible, different lots from the same vendor may be used. Certificates of analysis must be stored centrally.

#### 1.5.1.2 **Working Standard Solution**

Add  $\approx$ 9mL methanol to 10mL volumetric flask. Add 50 $\mu$ L each of amphetamine, methamphetamine, and c-THC stock. Add 30 $\mu$ L each of benzoyllecgonine, methadone, morphine and oxazepam stock. Add 10 $\mu$ L of secobarbital stock. QS to 10mL with methanol. Record lot numbers of stock reference material on reagent log.

*(Solution is stable for up to 12 months when stored at 4 °C.)*

#### 1.5.1.3 **Urine Calibrators:**

May be commercially obtained or prepared in-house as described in Appendix I.A.

- Negative Urine Calibrator
- Cut-off Urine Calibrator

#### 1.5.1.4 **Platform Urine Controls**

May be commercially obtained or prepared in-house as described in Appendix I.A.

- Negative Urine Control
- Positive Urine Control

#### 1.5.1.5 **Sample Rack Urine Controls**

1.5.1.5.1 Negative Urine Control  
Negative Urine

1.5.1.5.2 Positive Urine Controls  
**25% Above Cut-off Positive Control**  
Each run must include a control at 25% above the cut-off calibrator. To prepare, add 125 $\mu$ L working standard solution to 1mL of negative urine.

**Drugs-of-Abuse Positive Control**

Each run must include a commercially obtained drugs-of-abuse urine control. The concentration of analytes may be varied.

1.5.1.5.3 Urine Cannabinoid Cut-off Control  
The last sample run on a urine cannabinoid screen will be an aliquot of the cut-off calibrator. This sample will be used for evaluating results.

1.5.2 Blood

1.5.2.1

**Stock Reference Material Solutions**

Obtain Amphetamine (1mg/mL), Methamphetamine (1mg/mL), Benzoylcegonine (1mg/mL), Methadone (1mg/mL), Morphine (1mg/mL), 11-nor-9-Carboxy- $\Delta$ 9-THC (100 $\mu$ g/mL), Oxazepam (1mg/mL), and Secobarbital (1mg/mL) drug reference material from an appropriate vendor. Different vendors should be used to make up the Calibrator and Control Working Solutions whenever possible. If this is not possible, different lots from the same vendor may be used. Certificates of analysis must be stored centrally.

1.5.2.2

**Working Standard Solution**

Add  $\approx$ 9mL methanol to 10mL volumetric flask. Add 50 $\mu$ L each of stock amphetamine, methamphetamine, benzoylcegonine, methadone and morphine. Add 100 $\mu$ L each of stock oxazepam and secobarbital. Add 150 $\mu$ L of stock c-THC. QS to 10mL with methanol. Record lot numbers of stock reference material on reagent log.

*(Solution is stable for up to 12 months when stored at 4 °C.)*

1.5.2.3

**Blood Calibrators**

May be commercially obtained or prepared in-house as described in Appendix I.B.

- Negative Blood Calibrator

- Cut-off Blood Calibrator
- 1.5.2.4 **Platform Blood Controls**  
May be commercially obtained or prepared in-house as described in Appendix I.B.

- Negative Blood
- Positive Blood Control

1.5.2.5 **Sample Rack Blood Controls**

- 1.5.2.5.1 Negative Blood  
Negative Whole Blood

- 1.5.2.5.2 Positive Whole Blood Controls  
**25% Above Cut-off Positive Control**  
Each run must include a control at 25% above the cut-off calibrator. To prepare, add 25µL working standard solution to 2mL of negative blood.

**Drugs-of-Abuse Positive Control**

Each run must include an in house or commercially obtained drugs-of-abuse blood control. The concentration of analytes may be varied.

## 1.6 PROCEDURE

1.6.1 General Rules of Operation for analyzer

- 1.6.1.1 Care should be taken to not impede the arm action, as it can be both detrimental to the instrument and dangerous to the user.
- 1.6.1.2 It is recommended, but not required, that the instrument run with the top down.

1.6.2 Initial Processing of Samples

- 1.6.2.1 Place laboratory number on each sample container.
- 1.6.2.2 Urine samples with an unusually high turbidity may be centrifuged prior to analysis.
- 1.6.2.3 Urine samples cannot contain the preservative sodium azide, as this will destroy the conjugate.
- 1.6.2.4 If particulates or clots are visible in a blood sample, it may be homogenized with tissue grinder or clarified by centrifuging.

1.6.3 Sample Dilution1.6.3.1 **Appropriate Dilution for Each Assay**1.6.3.1.1 Urine

Dilution	Assays
1 in 60	Amphetamine, Methamphetamine, Cannabinoids, Opiates, Benzodiazepines, Cocaine Metabolite, and Methadone
1 in 5	Barbiturates

1.6.3.1.2 Blood

Dilution	Assays
1 in 5	Methamphetamine, Cannabinoids, Opiates, Benzodiazepines, Cocaine Metabolite, Barbiturates and Methadone

1.6.3.2 **Suggested Volumes for Manual Dilution**1.6.3.2.1 1 in 60 parts dilution

Sample	Forensic Diluent
9 $\mu$ L / 1:5 dilution	1000 $\mu$ L
15 $\mu$ L	885 $\mu$ L

1.6.3.2.2 1 in 5 parts dilution

Sample	Forensic Diluent
160 $\mu$ L	640 $\mu$ L
200 $\mu$ L	800 $\mu$ L
250 $\mu$ L	1000 $\mu$ L

1.6.4 Preliminary Tasks

1.6.4.1 Fill wash bottles with deionized water; make sure waste container is empty.

1.6.4.2 Check pipette tip tray supply. If necessary, fill with appropriate disposable tips.

1.6.4.3 Check printer paper supply. Refill if necessary.

- 1.6.4.4 Remove samples and reagents from refrigerator for a minimum of one hour prior to starting analysis.
- 1.6.4.5 Prepare samples for analysis. Dilute as indicated under section 1.6.3.1.
- 1.6.5 Session Preparation
- 1.6.5.1 Turn on instrument, then turn on computer.
- 1.6.5.2 Double click on the Revelations icon on the desktop. Select “Connect to DSX”, then “Do it.” The instrument should perform a self-test at this time; make sure ALL TESTS PASSED before proceeding.
- 1.6.5.3 From Open Session Screen, select appropriate template from buttons. Then “Add assays using a new batch of samples” and click “OK.”
- 1.6.5.4 If there is no appropriate template, select “New,” then “Worklist.” Select “New Plate” and choose the type of assay to run.
- 1.6.5.5 Select “Open.” Note: If multiple assays are to be tested on one plate, select the first assay then hold the Ctrl button to select the others. If using the Template button, it is still possible to run multiple assays on the same plate by left-clicking the assay immediately to the right of the first plate and selecting “Combined with assay on right.” The Cannabinoid assay must be the only assay run on its plate, and the run is limited to 25 blood case samples (run in duplicate) or 52 urine case samples.
- 1.6.5.6 Click and drag under the “Test” column to select the assays for the samples. Enter ID numbers for the samples, then click “OK.” NOTE: Blood samples are to be run in duplicate. The assays are programmed to do this automatically, depending on the matrix selected.
- 1.6.5.7 Select the “PLAY” button (green triangle). The timeline will now be built and displayed. NOTE: To view the timeline, click the down (↓) arrow button; to hide the timeline, click the up (↑) arrow button. To view the *entire* timeline, click the minus (marked as a dash) button.



- 1.6.5.8 When ready to load the reagents and consumables, click the “FAST FORWARD” button (two green triangles followed by a green vertical line).
- 1.6.5.9 Follow directions on the screen to load all reagents and consumables. Note: when asked to enter “Lot Name,” enter the name of the assay (e.g. METHAMPHETAMINE, or METH), followed by the kit lot number and kit expiration date. Click green check mark when done.
- 1.6.5.10 Load all samples into sample caddy, as instructed on the screen, then click the green check mark.
- 1.6.5.11 Once plate tray is ejected, remove the plate holder, insert assay plate (with the correct number of strips in place) into the plate holder, and place the plate holder back into the tray, as instructed on the screen. Change “Plate Identifier” to the assay being run for that plate, followed by the date (e.g. AMP011212). Click green check mark to proceed to next plate. Repeat for each plate being loaded, making sure to change the plate identifier for each.
- 1.6.5.12 Load all calibrator and control fluids as instructed on the screen. Make sure to load at least the minimum volumes required for each, as the software instructs. Click green check mark.
- 1.6.5.13 Make sure the washer bottle(s) contain sufficient DI water. Click green check mark.
- 1.6.5.14 Make sure sufficient sample and reagent tips are loaded. Click green check mark.
- 1.6.5.15 Make sure there is sufficient space for waste in tip disposal and waste disposal containers. Click “OK.” Note: once you click “OK” the instrument cycle will start, so make sure all objects are out of the way of the arm.
- 1.6.5.16 Once all runs are complete, click the “STOP” button (red square). This will cause the plate tray to be ejected. Once the plate tray is ejected, click on the “EJECT” button (a square with an arrow pointing down), to fully eject the tray. Once the tray is fully ejected, the plate holders will be unlocked and easily removed.

1.6.5.17 Remove the plate holders, dispose of the strips, then return the plate holders to the tray. Push the “IN” button (a square with an arrow pointing up) to make the tray return to its “IN” position.

1.6.6 Obtaining Results - Post-Run

1.6.6.1 To display the results, click the blue “UP” arrow. Select the pop-up window displaying assay results.

1.6.7 Obtaining Archived Results

1.6.7.1 Results from past runs can be viewed by clicking “Open,” then clicking on the “Plates” folder. From here, you can select the appropriate document.

1.6.7.2 Text files of all runs are also stored. To access the text files, click “Open,” then click on the “Text” folder. From here, you can select the appropriate document. These documents can be then be opened in Excel.

1.6.8 Post-run Tasks

1.6.8.1 **General Clean-up**

1.6.8.1.1 Return conjugates, stop, and diluent reservoirs to refrigerator.

1.6.8.1.2 Dispose of used calibrators, controls, samples, and tips into appropriate biohazard waste container.

1.6.8.2 **Instrument Shut-down**

1.6.8.2.1 Close out of Revelations, then shut down computer.

1.6.8.2.2 Power off the instrument.

**1.7 RUN ACCEPTANCE CRITERIA**

1.7.1 Calibrators and Controls

1.7.1.1 The individual replicates for the absorbance of the *negative calibrator* must be less than 1.2 times the mean *negative calibrator* and greater than 0.8 times the mean *negative calibrator*.

1.7.1.2 The individual replicates of the *cut-off calibrator* must be less than 1.2 times the mean *cut-off calibrator* and greater than 0.8 times the mean *cut-off calibrator*.

- 1.7.1.3 The mean absorbance for the *negative urine or blood* is greater than the absorbance for the *negative calibrator*.
- 1.7.1.4 The absorbance for the *negative calibrator* is greater than the mean absorbance for the *cut-off calibrator*.
- 1.7.1.5 The mean absorbance for the *cut-off calibrator* is greater than the absorbance for the *positive control*.
- 1.7.1.6 The quality control equations  $((\text{KIT1}-\text{KIT2})/\text{KIT1}) * 100 > 20$  and  $((\text{NEG}-\text{CUT})/\text{NEG}) * 100 > 30$  are default settings allowing tracking of the OraSure immunoassay kits' performance through calculation of percent displacement. These criteria are not critical; they are used for tracking the performance of the specific assay. The default settings may be adjusted as appropriate.

#### 1.7.2 Urine and Blood Sample Controls

- 1.7.2.1 Matrix matched urine and blood controls, analyzed as samples, should indicate an appropriate positive or negative response
- 1.7.2.1 For purposes of this criterion, a significantly depressed absorbance qualifies as a positive result.

### 1.8 INTERPRETATION OF RESULTS

#### 1.8.1 Positive Result

A positive result for a sample is indicated by an absorbance less than or equal to the *Cut-off Calibrator*. Urine samples that have an absorbance less than or equal to the cut off control run at the end of the assay will be considered positive. Blood samples are run in duplicate; if the coefficient of variation is over 10% for a sample, the analyst will use the value with the lowest absorbance for evaluating the sample result.

#### 1.8.2 Depressed Absorbances

At the discretion of the analyst, confirmatory techniques may be applied to samples that exhibit depressed absorbances. For purposes of this exception, depressed absorbances are those which fall between the value observed for the cut-off calibrator and the negative urine or blood. 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. Blood samples are run in duplicate; if the coefficient of variation is greater than 10% for a sample, the analyst will use the value with the lowest absorbance for evaluating the sample result.

### 1.8.3 Negative Result

A negative result for a sample is indicated by an absorbance that is greater than the Cut-off Calibrator. Special considerations may apply as outlined above (see 1.8.2).

## 1.9 DISTRIBUTION OF ASSAY INFORMATION

1.9.1 Assay results are to be recorded in the LIMS system.

1.9.2 The original data printouts will be stored centrally in the laboratory where the analysis was performed.

1.9.3 A copy of assay results may be stored electronically in a central location and need not be included in individual case files. When necessary, a copy of the control and standard printouts may be prepared from the centrally stored document.

## 1.10 DSX MAINTENANCE

### 1.10.1 When-in-use Daily Maintenance/Tasks

1.10.1.1 Run an optional “Wash” cycle before doing sample runs. It is also recommended that the “Wash” cycle be run after sample runs as well. If any wash ports are clogged, perform the periodic maintenance procedure for unclogging the ports.

### 1.10.2 Periodic Maintenance

The extent of DSX use should be used as the indicator of when periodic maintenance is warranted. The following is intended as suggested maintenance frequency, and is not required if the instrument is lightly used in any given month. The schedule may be adjusted according to individual laboratory needs.

#### 1.10.2.1 **Monthly Maintenance**

1.10.2.1.1 Wash, air dry and replace the Wash Tanks water.

1.10.2.1.2 Check the wash and vacuum needles for blockages – unclog as needed.

1.10.2.1.3 Disinfect the discard tip tray by rinsing with 70% isopropanol. NOTE: Bleach fumes can detrimentally affect assays; do not use if avoidable. If bleach must be used, make sure no fumes or residue remains to disrupt assay results.

#### 1.10.2.2 **Six-Month Maintenance**

1.10.2.2.1 Replace the dispense tubing as needed.

1.10.2.2.2 Replace the aspiration tubing as needed.

1.10.2.3 **Preventive Observations**

1.10.2.3.1 During wash cycle, check the washer dispensing apparatus to ensure each port is correctly dispensing water. This may be most effectively accomplished when aided by a flashlight.

1.10.2.3.2 After wash cycle, check plate to make sure most water is removed from wells and remaining water is uniform among the wells.

**1.11 REFERENCES**

- 1.11.1 Butler, J.E. **Enzyme-Linked Immunosorbent Assay**. pp. 759-803 *In*: "Immunochemistry". Van Oss, C.J.; van Regenmortel, M.H.V., eds., Marcel Dekker, inc., New York, NY: 1994.
- 1.11.2 DSX Automated ELISA System™ User's Manual, REV.04-20-05, 2005.
- 1.11.3 OraSure Technologies DSX™ Startup Procedure and Setup of a Worklist.
- 1.11.4 OraSure Technologies Package Inserts for Serum Microplate EIA.

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## 1.12 History

### Revision # Issue Date History

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Revision	Date	History
0	04-24-00	Original Issue
1	04-24-02	Updated and made STC name change corrections
2	09-13-02	Clarification of distribution of assay information (1.7.1)
3	01-03-03	Further clarification of distribution of assay information (1.7.1)
4	08-13-04	Reformat, software/computer upgrade and assay configuration changes
5	05-07-07	Revamp, updated.
6	04-10-12	Method updated for use of DSX instrument and cut-offs updated for Methamphetamine. Required controls updated. Urine portion and amphetamines removed from method.
7	07-02-12	Amended to add urines, additional control for urine cannabinoid added and additional interpretation for positive result added for urine cannabinoids.
8	01-07-13	Added Barbiturate assay to method.
9	01-16-14	Updated 1.9 to reflect changes for new LIMS system, updated 1.6.5.5 to describe the maximum number of urine case samples that can be run in a cannabinoid run.
10	10-03-14	Updated calibrator/control preparation interval requirements. Updated maintenance section. Corrected minor formatting problems.

## **Appendix I:**

### **A. Urine Control In-House Preparation Guide**

#### I.A.1 Negative Urine Calibrator (1/2x)

##### I.A.1.1 **Direct Spiking Preparation**

Add 50 $\mu$ L working standard solution to 1mL negative urine.

##### I.A.1.2 **Serial Dilution Preparation**

Prepare 200% of cut-off solution as described in Appendix II, Table 1; then dilute as described in Appendix II, Table 2.

#### I.A.2 Cut-off Urine Calibrator (x)

##### I.A.2.1 **Direct Spiking Preparation**

Add 100 $\mu$ L working standard solution to 1mL negative urine.

##### I.A.2.2 **Serial Dilution Preparation**

Prepare 200% of cut-off solution as described in Appendix II, Table 1; then dilute as described in Appendix II, Table 2.

#### I.A.3 Negative Urine Control (NEG)

##### I.A.3.1 Negative Urine.

#### I.A.4 Positive Urine Control (2x)

##### I.A.4.1 Add 200 $\mu$ L working standard solution to 1mL negative urine.

### **B. Blood Control In-House Preparation Guide**

#### I.B.1 Negative Blood Calibrator (1/2x)

##### I.B.1.1 **Direct Spiking Preparation**

Add 5 $\mu$ L working standard solution to 1mL negative blood.

##### I.B.1.2 **Serial Dilution Preparation**

Prepare 200% of cut-off solution as described in Appendix II, Table 3; then dilute as described in Appendix II, Table 4.

#### I.B.2 Cut-off Blood Calibrator (x)

##### I.B.2.1 **Direct Spiking Preparation**

Add 10 $\mu$ L working standard solution to 1mL negative blood.

##### I.B.2.2 **Serial Dilution Preparation**

Prepare 200% of cut-off solution as described in Appendix II, Table 3; then dilute as described in Appendix II, Table 4.

#### I.B.3 Negative Blood (NEG)

##### I.B.3.1 Negative Whole Blood.

#### I.B.4 Positive Blood Control (2x)

##### I.B.4.1 Add 20 $\mu$ L working standard solution to 1mL negative blood.

**Appendix II:****Table 1:****Urine Direct Spiking:**

To 1mL of negative urine, add working standard solution as indicated below.

Control Type	% of cutoff	Working Standard Solution
Negative	50%	50µL
Positive	200%	200µL

**Table 2:****Urine Serial Dilution:**

Control Type	% of cutoff	Urine Stock	Urine Dilution
Cut-off	100%	500µL of 200%	500µL
Negative	50%	250µL of 200%	750µL

**Table 3:****Whole Blood Direct Spiking:**

To 1mL of negative blood, add working standard solution as indicated below.

Control Type	% of cutoff	Working Standard Solution
Negative	50%	5µL
Positive	200%	20µL

**Table 4:****Whole Blood Serial Dilution:**

Control Type	% of cutoff	Whole Blood Stock	Whole Blood Dilution
Negative	50%	250µL of 200%	750µL
Cut-off	100%	500µL of 200%	500µL