ELISA Drug Screen

1.0 Purpose - This procedure specifies the required elements for the calibration and use of the Tecan Freedom EVO 75 Workstation for an ELISA Drug Screen.

2.0 Scope – This procedure applies to the Toxicology Units of the State Crime Laboratory.

3.0 Definitions

- **Performance verification –** The initial confirmation of the reliability of a previously or externally validated method or instrument.
- **Quality control (QC) check –** Periodic confirmation of the reliability of equipment, instrumentation, and/or reagents.

4.0 Equipment, Materials and Reagents

4.1 Equipment

- Tecan/Immunalysis Freedom EVO ELISA Analyzer
- Mechanical pipettes
- Liquid handling diluter/pipettor system
- Volumetric flasks, Class A, 10 mL

4.2 Materials

- Stoppers or caps
- Vortex Mixer
- Test tubes

4.3 Reagents

- Deionized water

4.4 Commercial Reagents

- Methanol, ACS and spectrophotometric grade
- Immunalysis TMB Substrate Reagent
- Immunalysis TMBZ Substrate Reagent
- Immunalysis Stop Reagent
- Immunalysis ELISA Buffer

4.5 Primary Reference Materials

- d-Amphetamine, 1 mg/mL
- Benzoylecgonine, 1 mg/mL
- Carisoprodol, 1 mg/mL
- (-)-delta-9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC-COOH), 1 mg/mL
- (±)-Methadone, 1 mg/mL
- d-Methamphetamine, 1mg/mL
- Morphine, 1 mg/mL

- Oxazepam, 1 mg/mL
- Nordiazepam, 1 mg/mL
- Oxycodone, 1 mg/ml
- Phenobarbital, 1 mg/mL
- *cis*-Tramadol HCl, 1 mg/mL
- Zolpidem, 1 mg/mL

4.6 Critical Reagents

- Two purchased blood verification standards: Verifier I contains 600 ng/mL phenobarbital, 100 ng/mL nordiazepam, 100 ng/mL morphine, 100 ng/mL benzoylecgonine, 50 ng/mL methadone, and 40 ng/mL d-amphetamine. Verifier II contains 50 ng/mL THC-COOH, 100 ng/mL cis-tramadol HCl, 40 ng/mL d-methamphetamine, 1000 ng/mL carisoprodol, 40 ng/mL zolpidem, and 50 ng/mL oxycodone.
- Negative blood/urine
- Immunoassay Blood/Urine Calibration Solutions
- Immunoassay Blood/Urine Verification Solutions
- Immunalysis 96 well coated Micro-plates for barbiturates, benzodiazepines, carisoprodol, cocaine metabolite, metabolites of delta-9-THC, methamphetamine/MDMA, methadone, opiates, tramadol, zolpidem, amphetamine/MDA, and oxycodone / oxymorphone and each respective Enzyme Conjugate

4.7 Prepared Reagents - reagents may be prepared in any amount provided that the component ratios are kept constant. The Verification Stock solutions shall be prepared using standards from manufacturers or lot numbers that differ from the ones used to prepare the calibration solutions.

4.7.1 Immunoassay Blood Calibration Solution I

4.7.1.1 To a 10 mL volumetric flask, add the following Primary Reference Materials:

- 150 µL of 1 mg/mL phenobarbital
- 25.0 µL of 1 mg/mL oxazepam
- 25.0 µL of 1 mg/mL morphine
- 25.0 µL of 1 mg/mL benzoylecgonine
- 12.5 µL of 1 mg/mL (±)-methadone
- 10.0 µL of 1 mg/mL d-amphetamine

4.7.1.2 Dilute the flask to volume with methanol.

4.7.1.3 Lot number: Eight digit format year/month/day

4.7.1.3.1 Example: 20101231

4.7.1.4 Expiration: One year.

4.7.1.5 Store in freezer.

4.7.1.6 QC check: Successful QC checks (see **5.5**).

4.7.2 Immunoassay Blood Calibration Solution II

4.7.2.1 To a 10 mL volumetric flask, add the following Primary Reference Materials:

- 12.5 µL of 1 mg/mL (-)-THC-COOH
- 250 µL of 1 mg/mL carisoprodol
- 10.0 µL of 1 mg/mL d-methamphetamine
- 25.0 µL of 1 mg/mL cis-tramadol HCl
- 10.0 µL of 1 mg/mL zolpidem
- 12.5 µL of 1 mg/mL oxycodone

4.7.2.2 Dilute to volume with methanol.

4.7.2.3 Lot number: Eight digit format year/month/day

4.7.2.3.1 Example: 20101231

4.7.2.4 Expiration: One year.

4.7.2.5 Store in freezer.

4.7.2.6 QC check: Successful QC checks (see **5.5**).

4.7.3 Immunoassay Blood Verification Solution I

4.7.3.1 To a 10 mL volumetric flask, add the following primary reference materials:

- 300 µL of 1 mg/mL phenobarbital
- 50.0 µL of 1 mg/mL nordiazepam
- 50.0 µL of 1 mg/mL morphine
- 50.0 µL mL of 1 mg/mL benzoylecgonine
- 25.0 µL of 1 mg/mL (±)-methadone
- 20.0 µL of 1 mg/mL d-amphetamine

4.7.3.2 Dilute to volume with methanol.

4.7.3.3 Lot number: Eight digit format year/month/day

4.7.3.3.1 Example: 20101231

4.7.3.4 Expiration: One year.

4.7.3.5 Store in freezer.

4.7.3.6 QC check: Successful QC checks (see **5.5**).

4.7.4 Immunoassay Blood Verification Solution II

4.7.4.1 To a 10 mL volumetric flask, add the following primary reference materials:

- 25.0 µL of 1 mg/mL (-)-THC-COOH

- 500 µL of 1 mg/mL carisoprodol
- 20.0 µL of 1 mg/mL d-methamphetamine
- 50.0 µL of 1 mg/mL cis-tramadol HCl
- 20.0 µL of 1 mg/mL zolpidem
- 25.0 µL of 1 mg/mL oxycodone

4.7.4.2 Dilute to volume with methanol.

4.7.4.3 Lot number: Eight digit format year/month/day

4.7.4.3.1 Example: 20101231

4.7.4.4 Expiration: One year.

4.7.4.5 Store in freezer.

4.7.4.6 QC check: Successful QC checks (see **5.5**).

4.7.5 Immunoassay Urine Calibration Solution

4.7.5.1 To a 10 mL volumetric flask, add the following Primary Reference Materials:

- 150 µL of 1 mg/mL phenobarbital
- 150 µL of 1 mg/mL morphine
- 150 µL of 1 mg/mL d-methamphetamine
- 75.0 µL of 1 mg/mL methadone
- 75.0 µL of 1 mg/mL benzoylecgonine
- 50.0 µL of 1 mg/mL oxazepam

4.7.5.2 Dilute the flask to volume with methanol.

4.7.5.3 Lot number: Eight digit format year/month/day

4.7.5.3.1 Example: 20101231

4.7.5.4 Expiration: One year.

4.7.5.5 Store in freezer.

4.7.5.6 QC check: Successful QC checks (see **5.5**).

4.7.6 Immunoassay Urine Verification Solution

4.7.6.1 To a 10 mL volumetric flask, add the following Primary Reference Materials:

- 300 µL of 1 mg/mL phenobarbital
- 300 µL of 1 mg/mL morphine
- 300 µL of 1 mg/mL d-methamphetamine
- 150 µL of 1 mg/mL methadone

- 150 µL of 1 mg/mL benzoylecgonine
- 100 µL of 1 mg/mL nordiazepam

4.7.6.2 Dilute the flask to volume with methanol.

4.7.6.3 Lot number: Eight digit format year/month/day

4.7.6.3.1 Example: 20101231

4.7.6.4 Expiration: One year.

4.7.6.5 Store in freezer.

4.7.6.6 QC check: Successful QC checks (see **5.5**).

5.0 Procedure

5.1 Instrument Performance Verification for New Instrumentation

5.1.1 New Tecan work stations shall be installed by a manufacturer representative and shown to meet any manufacturer's requirements.

5.1.2 The ELISA Key Operator shall complete performance verification on new Tecan work stations prior to use for casework.

5.1.3 The performance verification shall include analysis of a minimum of fifteen blood and urine samples with known results. All quality control requirements shall be met.

5.1.3.1 The known blood and urine samples may be prepared or purchased.

5.1.3.2 The results of the known samples shall be substantially comparable to their known results.

5.1.4 The data shall be filed and maintained by ELISA Key Operator to document the new instrument set up.

5.1.5 A new entry for the instrument shall be made in the Resource Manager section of FA prior to use in casework. The new entry shall include the following:

- Manufacturer's serial number.
- Unique Section identifier for the new instrument.
- Notation under "Verification Date" to reflect the date the performance verification was completed.

5.2 Maintenance

5.2.1 Record all maintenance in the instrument log at the time it is performed.

5.2.2 Notify the ELISA Key Operator or designee of instrument problems. The ELISA Key Operator or designee shall evaluate the instrument and determine if maintenance or service is needed. If the problem prevents the instrument from properly functioning, the ELISA Key Operator or designee shall note in the instrument log that the instrument

is "Out of Service." The instrument shall not be used for casework until the problem is corrected and a successful calibration is performed on one assay in triplicate. (see **5.4**). Upon correction of the problem and a successful calibration the ELISA Key Operator or designee shall note in the instrument log that the instrument is "In Service."

5.2.3 Suggested Routine Maintenance Schedule

5.2.3.1 This is a suggested maintenance schedule. Instrument use may alter the need for maintenance. The maintenance schedule shall be determined by the ELISA Key Operator or designee based upon instrument use.

5.2.3.2 Daily post-run maintenance performed by instrument operator

5.2.3.2.1 If more than one analytical run is performed on the same day, rinse washer by performing a "day rinse" prompted directly from the washer.

5.2.3.2.2 Upon completion of instrument use each day perform a "night rinse" prompted directly from the washer and turn the system off.

5.2.3.2.3 Wipe instrument with isopropyl alcohol.

5.2.3.2.4 Remove racks from the instrument surface and carefully clean using isopropyl alcohol or a mild detergent.

5.2.3.2.5 Clean the Teflon sample tip by gently wiping it with a lint-free tissue containing isopropyl alcohol.

5.2.3.2.6 Empty the waste container and clean with dilute bleach.

5.2.3.3 Weekly maintenance performed each week the instrument is in use (notify the ELISA Key Operator if any items listed between **5.2.3.3.1** thru **5.2.3.3.4** are encountered)

5.2.3.3.1 Check syringes for leaks, bubbles or visual contamination. If required, clean the syringes taking care in removing syringes. If the syringes are leaking, replace the caps on the syringe plungers.

5.2.3.3.2 Check the valve and surrounding area for signs of moisture.

5.2.3.3.3 Check the green Teflon coating of the stainless steel pipette tip for damage.

5.2.3.3.4 Check that there are no air bubbles or contamination in the pipetting tubing. Tighten the tubing connections or replace the tubing as required.

5.2.3.4 Six month maintenance

5.2.3.4.1 With a mild soap, clean liquid system and liquid system container. Thoroughly rinse container and fill with clean DI water. Flush with a bleach solution. Thoroughly rinse container and fill with clean DI water

5.2.4 Shutdown

5.2.4.1 Successful calibration shall be performed following any instrument shutdown (see **5.4**).

5.3 Sampling

5.3.1 Allow all solutions and samples to equilibrate to room temperature.

5.3.2 Ensure that all body fluids are homogenous by shaking and/or vortexing.

5.3.2.1 If a homogenous sample cannot be obtained, make a notation in the FA worksheet detailing the condition of the sample and its handling.

5.4 Calibration and Verification

5.4.1 Calibration and verification samples shall be analyzed with every run.

5.4.2 Calibration/Verification Sample Prep

5.4.2.1 Negative Calibration Standard

5.4.2.1.1 Prepare a negative standard as described in **5.7** using the appropriate negative matrix.

5.4.2.2 Blood/Urine Calibration Standards

5.4.2.2.1 Add 0.100 mL of the appropriate Immunoassay Calibration Solution to a test tube.

5.4.2.2.2 Add 4.9 mL of the appropriate negative matrix to the test tube.

5.4.2.2.2.1 The final concentration of the **Immunoassay Blood Calibration Standard I** is 300 ng/mL phenobarbital, 50 ng/mL oxazepam, 50 ng/mL morphine, 50 ng/mL benzoylecgonine, 25 ng/mL methadone, and 20 ng/mL d-amphetamine.

5.4.2.2.2.2 The final concentration of the **Immunoassay Blood Calibration Standard II** is 25 ng/mL THC-COOH, 50 ng/mL cis-tramadol HCl, 20 ng/mL d-methamphetamine, 500 ng/mL carisoprodol, 20 ng/mL zolpidem, and 25 ng/mL oxycodone.

5.4.2.2.2.3 The final concentration of the **Immunoassay Urine Calibration Standard** is 300 ng/mL phenobarbital, 100 ng/mL oxazepam, 300 ng/mL morphine, 150 ng/mL benzoylecgonine, 150 ng/mL methadone, and 300 ng/mL of d-methamphetamine.

5.4.2.2.3 Cap and vortex the test tube.

5.4.2.2.4 **Prepare on day of use.**

5.4.2.2.5 Prepare as directed in **5.7**. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

5.4.2.3 Prepared Blood/Urine Verification Standards

5.4.2.3.1 Add 0.100 mL of the appropriate Immunoassay Verification Solution to a test tube.

5.4.2.3.2 Add 4.9 mL of the appropriate negative matrix to the test tube.

5.4.2.3.2.1 The final concentration of the **Immunoassay Blood Verification Standard I** is 600 ng/mL phenobarbital, 100 ng/mL nordiazepam, 100 ng/mL morphine, 100 ng/mL benzoylecgonine, 50 ng/mL methadone, and 40 ng/mL d-amphetamine.

5.4.2.3.2.2 The final concentration of the **Immunoassay Blood Verification Standard II** is 50 ng/mL THC-COOH, 100 ng/mL tramadol HCl, 40 ng/mL d-methamphetamine, 1000 ng/mL carisoprodol, 40 ng/mL zolpidem, and 50 ng/mL oxycodone.

5.4.2.3.2.3 The final concentration of the **Immunoassay Urine Verification Standard** is 600 ng/mL phenobarbital, 200 ng/mL nordiazepam, 600 ng/mL morphine, 300 ng/mL benzoylecgonine, 300 ng/mL methadone, and 600 ng/mL of d-methamphetamine.

5.4.2.3.3 Cap and vortex the test tube.

5.4.2.3.4 **Prepare on day of use.**

5.4.2.3.5 Prepare as directed in **5.7**. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

5.4.2.4 Purchased Blood Verification Standards

5.4.2.4.1 Use an Immunoassay Blood Verification Standard I Critical Reagent certified to meet the requirements in **5.4.2.3.2.1**.

5.4.2.4.2 Use an Immunoassay Blood Verification Standard II Critical Reagent certified to meet the requirements in **5.4.2.3.2.2**.

5.4.2.4.3 **Mix well before use.**

5.4.2.4.4 **Expiration: 25 days after thawed.**

5.4.2.4.5 Prepare as directed in **5.7**.

5.5 Critical Reagent QC check

5.5.1 Prior to use with casework, each new lot of microplates, conjugates, negative blood/urine, calibration solution, verification solution, and purchased verification standard shall be evaluated by the ELISA Key Operator or designee to establish acceptability.

5.5.2 For each new lot of microplate/conjugate: Three solutions of the blood calibration standards (**5.4.2.2**) and the blood verification standards (**5.4.2.3 or 5.4.2.4**) shall be prepared and analyzed along with a negative blood calibration standard in accordance with **5.7** and the results shall be analyzed statistically through the use of a Microsoft Excel spreadsheet. This spreadsheet shall determine the mean, standard deviation, and the % Coefficient of Variation (CV).

5.5.3 For each new lot of negative blood/urine, calibration solution, and/or verification solution: Three negative calibration standards (**5.4.2.1**), calibration standards (**5.4.2.2**) and/or verification standards (**5.4.2.3**) shall be prepared and analyzed as samples in accordance with **5.7** and the results shall be analyzed statistically through the use of a Microsoft Excel spreadsheet. This spreadsheet shall determine the mean, standard deviation, and the CV.

5.5.4 The verification shall meet the following acceptance criteria prior to approval for use with casework.

5.5.4.1 The % CV of the % b/b_0 shall be less than 20 %.

5.5.4.2 All verification standards % b/b_0 must be lower than the average of the positive calibrator.

5.5.5 An electronic copy of the spreadsheet shall be placed into the Resource Manager in Forensic Advantage under the appropriate kit or reagent lot. The Toxicology Technical Leader shall approve the verification and document approval in the Resource Manager.

5.6 Instrument Setup

5.6.1 Check system liquid container and fill with deionized water as needed.

5.6.2 Prime the system executing the “system fluid prime” script in the Evoware software.

5.6.3 Fill the appropriate reagent troughs with TMB substrate, TMBZ substrate and stop reagent. Ensure that no color develops.

5.6.4 Check and fill appropriate conjugate test tubes. Ensure that the enzyme conjugate lot matches the microplate lot being used.

5.6.5 Create a plate layout in NaviTrak and position the Micro-plates and the conjugate test tubes to correspond.

5.7 Application of Procedure on Samples

5.7.1 In duplicate, pipette 0.25 mL of each sample to be analyzed into a disposable glass test tube.

5.7.2 Using a pipette, add 2.5 mL of deionized water into each tube. (ELISA Buffer may be used in place of the deionized water provided that it is used for all samples and standards and is noted in the case record).

5.7.3 Vortex for approximately 10 seconds. Ensure that there is no foam on the surface of the liquid.

5.7.4 Arrange the duplicate standard and case sample tubes into the appropriate sample racks in the following order:

- Blood / Urine Negative Calibration Standard
- Blood / Urine Calibration Standard
- Blood / Urine Verification Standard
- Blood / Urine case samples

5.7.5 Follow steps outlined in "Immunalysis Standard Operating Procedure for EVO 75/2 with NaviTrak-OS™ V1.4."

5.7.6 Record instrument use in the instrument log.

5.8 Data and Verification Acceptance Criteria

5.8.1 An inverse relationship exists between absorbance and concentration.

5.8.2 For each assay, evaluate the % b/b_0 value.

5.8.2.1 For % b/b_0 values that are less than or equal to the b/b_0 of the calibration standard, the result is positive.

5.8.2.2 For % b/b_0 values that are greater than the b/b_0 of the calibration standard, the result is negative.

5.8.3 The verification standard % b/b_0 of an assay must be evaluated as positive for data from that assay to be used for reporting.

5.8.4 If a verification standard fails to meet the acceptance criteria in **5.8.3**, the assay must be repeated.

5.8.5 Create a workstation in Forensic Advantage (FA), containing the following:

- Lot numbers and expiration dates of the paired Micro-plate and enzyme conjugate.
- Lot number and expiration date of TMB/TMBZ substrate.
- Lot number and expiration date of stop reagent.
- Lot number and expiration date of ELISA buffer (if applicable).
- Lot number and expiration date of Immunoassay Calibration Solution.
- Lot number and expiration date of Immunoassay Verification Solution or externally supplied Blood Verification Standard.
- Lot number and expiration date of negative blood/urine.

5.8.6 Create a quality control data packet for the run, including the following quality control data:

- Summary page with FA workstation reference
- Completed ELISA worksheet
- NaviTrak sequence list
- NaviTrak plate configuration printout
- Assay print outs showing the results of each Calibration and Verification Standard

5.8.7 The data packet shall be reviewed by another authorized forensic scientist and approved in the Toxicology Unit section object repository in FA with a file name beginning with "Elisa" (capitalization optional) and the date in yyyymmdd format with no space between them. A suffix will be added to the file name to distinguish multiple runs performed on the same day.

5.8.7.1 Example: ELISA20140728

5.8.8 Reporting

5.8.8.1 The case record shall contain the following:

- Approved Quality Control data packet
- Individual case report showing each assays % b/b_0

5.8.8.2 Refer to the Technical Procedure for Toxicology Analysis.

5.9 Calculations

5.9.1 The % b/b_0 is determined by taking the average absorbance value of the sample duplicates and dividing by the average absorbance value of the negative calibration sample and multiplying by 100.

5.10 Uncertainty of Measurement – N/A

6.0 Limitations

6.1 This is a preliminary drug screen. Refer to the Immunalysis website (see references) for substances known to test positive and negative with this technique.

6.2 Cross reactivity and interference with the enzyme process may cause false positive and false negative results. Refer to *Principles of Forensic Toxicology*, 2nd edition, pages 119-139.

7.0 Safety

7.1 Refer to Laboratory Safety Manual.

7.2 Ensure that the instrument cover is in the down position when the instrument is in use.

8.0 References

Immunalysis Tecan Freedom EVO 75 Workstation Operating Manual

Standard Operating Procedure for EVO 75/2 with NaviTrak-OS™ V1.4, Immunalysis.

http://immunalysis.com/wp-content/uploads/2013/04/Troubleshooting_chart_2010.pdf

Levine, Barry, ed. *Principles of Forensic Toxicology.* 2nd edition. AACC Press, 2006, 119-139.

9.0 Records

- ELISA Calibration Data
- Case Record
- ELISA Instrument Log

10.0 Attachments – N/A

Revision History		
Effective Date	Version Number	Reason
09/17/2012	1	J-18 Conversion to ISO format
10/26/2012	2	Changed procedure title; 1.0 - added wording; 5.2.3.2 and sub items - removed maintenance duplicated in 5.5, cleaned up wording, moved maintenance to 5.5.3 to be part of system set up; 5.2.3.3 sub items - removed reference to acid/base wash, cleaned up wording, removed cleaning the toothed racks since done under 5.2.3.2; 5.2.3.4 and sub items - changed Monthly to Six Months, removed reference to filter slide maintenance, removed 6% from description of bleach solution; 5.4.6 - added language to distinguish multiple runs on the same day; grammar
02/15/2013	3	2.0 - Changed scope for procedure merge 5.6.2 - restructured sentence 4.5, 4.7.1.1, 4.7.2.1, 4.7.3.1, 4.7.4.1 - Changed (±)-methamphetamine to d-methamphetamine
05/10/2013	4	4.7.1.3, 4.7.1.3.1, 4.7.2.3, 4.7.2.3.1, 4.7.3.3, 4.7.3.3.1, 4.7.4.3, and 4.7.4.3.1 - simplified lot number format, change also reflected in example 5.2.3.3 and 5.4.2.5.2.1 - corrected reference
11/15/2013	5	Added issuing authority to header
08/29/2014	6	4.5, 4.6 – Added components, modified concentrations, consolidated 4.7 – Added components, added verification standard requirement, modified prep volume instructions and create two different calibration and verification stock solutions., modified QC Check requirement 5.2.4.2 - Removed 5.4 – Removed 5.4.2.2, Consolidated prep instructions for Calibration and Verification and Purchased Verification Standards, modified to reflect added components and modified concentrations 5.4.3 – Now 5.5, removed 5.4.3.2, 5.4.3.2.1, and 5.4.3.3.1. Modified 5.4.3.1 to remove low positive calibration standard and to change to evaluating by % b/b_0 Inserted 5.5.2 and 5.5.3 5.4.4, 5.4.4.1, 5.4.5, and 5.4.6 – Moved to new 5.8 5.6.4 – removed low calibration standard and restructured, now 5.7.4 5.6.7.5 moved now 5.7.6 5.6.6 – Moved now 5.9 5.9.1- added

		5.6.7, 5.6.7.1, 5.6.7.2.2- moved to new 5.8 5.6.7.2, 5.6.7.3, and 5.6.7.3.1- removed due to consolidations 5.6.7.2.1 – Removed elevated criteria 5.6.8 - moved now 5.8.8 5.6.7.4 - moved to 5.8.8.1, modified to reflect data packet. Added 5.7, 5.7.3 – removed "Cap and" 8.0- removed duplicate reference
03/20/2015	7	4.1- Added diluter system to equipment 4.5 – Added component oxazepam 4.6 – Distinguished the components between the 2 externally purchased verifiers 4.7.1.1, 4.7.5.1, 5.4.2.2.2.1, 5.4.2.2.2.3 – removed nordiazepam and inserted oxazepam 5.2.2 – clarified post-maintenance criteria to include only one assay in triplicated needed 5.4.2.2.4, 5.4.2.3.4 – Changed expiration to Prepare day of use. 5.4.2.4.3, 5.4.2.4.4 – inserted and corrected numbering in section 8.0 – removed cross-reactivity reference, added troubleshooting reference