
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/Immunoanalysis Freedom EVO ELISA Analyzer
- Mechanical pipettes
- 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
- Immunoanalysis TMB Substrate Reagent
- Immunoanalysis TMBZ Substrate Reagent
- Immunoanalysis Stop Reagent
- Immunoanalysis ELISA Buffer

4.5 Primary Reference Materials

- Benzoylcegonine, 1 mg/mL
- Carisoprodol, 1 mg/mL
- (-)-delta-9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC-COOH), 5 µg/mL
- (±)-Methadone, 1 mg/mL
- d-Methamphetamine, 1mg/mL
- Morphine, 1 mg/mL
- Nordiazepam, 1 mg/mL
- Phenobarbital, 1 mg/mL
- *cis*-Tramadol HCl, 1 mg/mL

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- Zolpidem, 1 mg/mL

4.6 Critical Reagents

- Externally supplied blood containing 2000 ng/mL phenobarbital, 100 ng/mL nordiazepam, 100 ng/mL morphine, 100 ng/mL benzoylecgonine, 100 ng/mL methadone, 50 ng/mL THC-COOH, 100 ng/mL tramadol HCl, 200 ng/mL methamphetamine, 8 µg/mL carisoprodol, and 100 ng/mL Zolpidem
- Negative blood
- Immunoassay Blood Calibration Reference Materials
- Immunoassay Urine Calibration Reference Materials
- Immunoassay Blood Verification Reference Materials
- Immunoassay Urine Verification Reference Materials
- Immunalysis 96 well coated microplates for barbiturates, benzodiazepines, carisoprodol, and cocaine metabolite, metabolites of delta-9-THC, methamphetamine/MDMA, methadone, opiates, tramadol, and zolpidem.
- Immunalysis Enzyme Conjugate that is matched to the above microplates.

4.7 Prepared Reagents - reagents may be prepared in any amount provided that the component ratios are kept constant.

4.7.1 Immunoassay Blood Calibration Solution

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

- 0.5 mL of 1 mg/mL Phenobarbital
- 0.025 mL of 1 mg/mL Nordiazepam
- 0.025 mL of 1 mg/mL Morphine
- 0.025 mL of 1 mg/mL Benzoylecgonine
- 12.5 µL of 1 mg/mL (±)-Methadone
- 2.5 mL of 5 µg/mL (-)-THC-COOH
- 2.0 mL of 1 mg/mL Carisoprodol
- 0.050 mL of 1 mg/mL d-Methamphetamine
- 0.025 mL of 1 mg/mL Tramadol HCl
- 0.025 mL of 1 mg/mL Zolpidem

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 calibration (see 5.4).

4.7.2 Immunoassay Urine Calibration Solution

4.7.2.1 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 µL of 1 mg/mL Methadone
- 75 µL of 1 mg/mL Benzoylecgonine
- 50 µL of 1 mg/mL Nordiazepam

4.7.2.2 Dilute the flask 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.

4.7.3 Immunoassay Urine Verification Solution

4.7.3.1 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 µL of 1 mg/mL Methadone
- 75 µL of 1 mg/mL Benzoylecgonine
- 50 µL of 1 mg/mL Nordiazepam

4.7.3.2 Dilute the flask 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.

4.7.4 Immunoassay Blood Verification Solution

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

- 0.5 mL of 1 mg/mL Phenobarbital
- 0.025 mL of 1 mg/mL Nordiazepam
- 0.025 mL of 1 mg/mL Morphine
- 0.025 mL of 1 mg/mL Benzoylecgonine

- 0.025 mL of 1 mg/mL ($\pm$)-Methadone
- 2.5 mL of 5 μ g/mL (-)-THC-COOH
- 2.0 mL of 1 mg/mL Carisoprodol
- 0.050 mL of 1 mg/mL d-Methamphetamine
- 0.025 mL of 1 mg/mL Tramadol HCl
- 0.025 mL of 1 mg/mL Zolpidem

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 calibration (see 5.4).

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.

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- 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 (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**).

5.2.4.2 The shutdown shall be noted in the maintenance log.

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 **Calibrations**

5.4.1 Perform calibration with every sample plate that is analyzed.

5.4.2 **Standards and Controls**

5.4.2.1 **Blood/Urine Calibration Standard**

5.4.2.1.1 At the time of calibration, add 0.100 mL of the appropriate Immunoassay Calibration Solution to a test tube.

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

5.4.2.1.2.1 The concentration of the blood calibration standard is 1000 ng/mL phenobarbital, 50 ng/mL nordiazepam, 50 ng/mL morphine, 50 ng/mL benzoylecgonine, 25 ng/mL methadone, 25 ng/mL THC-COOH, 50 ng/mL tramadol HCl, 100 ng/mL methamphetamine, 4 µg/mL carisoprodol, and 50 ng/mL zolpidem.

5.4.2.1.2.2 The concentration of the urine calibration standard is 300 ng/mL phenobarbital, 100 ng/mL nordiazepam, 300 ng/mL morphine, 150 ng/mL benzoylecgonine, 150 ng/mL methadone, and 300 ng/mL of methamphetamine.

5.4.2.1.3 Cap and vortex the test tube.

5.4.2.1.4 Prepare as directed in **5.6**. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

5.4.2.2 Low Positive Blood Calibration Standard

- 5.4.2.2.1 This standard is only used for the evaluation of the benzodiazepine and opiate assays.
- 5.4.2.2.2 At the time of calibration, add 0.050 mL of the Immunoassay Calibration Solution to a test tube.
- 5.4.2.2.3 Add 4.95 mL of negative blood to the test tube.
- 5.4.2.2.4 The concentration of the calibration standard is 500 ng/mL phenobarbital, 25 ng/mL nordiazepam, 25 ng/mL morphine, 25 ng/mL benzoylecgonine, 12.5 ng/mL methadone, 12.5 ng/mL THC-COOH, 25 ng/mL tramadol HCl, 50 ng/mL methamphetamine, 2 µg/mL carisoprodol, and 25 ng/mL zolpidem.
- 5.4.2.2.5 Cap and vortex the test tube.
- 5.4.2.2.6 Prepare as directed in 5.6. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

5.4.2.3 Negative Calibration Standard

- 5.4.2.3.1 Prepare a negative standard as described in 5.6 using the appropriate negative matrix.

5.4.2.4 Urine Verification Standard

- 5.4.2.4.1 At the time of calibration add 0.200 mL of the urine Immunoassay Verification Solution to a test tube.
- 5.4.2.4.2 Add 4.8 mL of negative urine to the test tube.
 - 5.4.2.4.2.1 The concentration of the 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 methamphetamine.
- 5.4.2.4.3 Cap and vortex the test tube.
- 5.4.2.4.4 Prepare as directed in 5.6. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

- 5.4.2.5 **Blood Verification Standard** - purchased or prepared to produce a blood solution containing 2000 ng/mL phenobarbital, 100 ng/mL nordiazepam, 100 ng/mL morphine, 100 ng/mL benzoylecgonine, 100 ng/mL methadone, 50 ng/mL THC-COOH, 100 ng/mL tramadol HCl, 200 ng/mL, methamphetamine, 8 µg/mL carisoprodol, and 100 ng/mL zolpidem.

5.4.2.5.1 Prepared

- 5.4.2.5.1.1** At the time of calibration add 0.200 mL of the blood Immunoassay Verification Solution to a test tube.
- 5.4.2.5.1.2** Add 4.8 mL of negative blood to the test tube
- 5.4.2.5.1.3** Cap and vortex the test tube.
- 5.4.2.5.1.4** Prepare as directed in **5.6**. Dispose of any unused portion according to the State Crime Laboratory Safety Manual.

5.4.2.5.2 Purchased

- 5.4.2.5.2.1** Use an Immunoassay Blood Verification Standard Critical Reagent certified to meet the requirements in **5.4.2.5**.
- 5.4.2.5.2.2** Prepare as directed in **5.6**.

5.4.3 Kit Verification

- 5.4.3.1** Prior to use with casework, each new lot of microplates shall be evaluated by the ELISA Key Operator or designee to establish acceptability (by evaluating the absorbencies for the positive blood calibration standard, low positive calibration standard, negative blood calibration standard, and the blood verification standard).

- 5.4.3.2** The samples listed in **5.4.3.1** shall be prepared and analyzed in triplicate in accordance with **5.6** and the results shall be analyzed statistically through the use of a Microsoft Excel spreadsheet. This spreadsheet shall determine the mean, standard deviation, a 99.73 % confidence interval and the % Coefficient of Variation (CV). The acceptable range shall be determined by the use of a student t-table to determine a 99.73 % confidence interval about the mean.

- 5.4.3.2.1** The formula for the determination of the confidence interval is:

$$= \pm (k_{99.73\%} * \text{std. dev.}) / \sqrt{n}$$

Where:

$k_{99.73\%} = 5.507$ for degree of freedom (d_f) = 5

n = number of absorbencies = 6

- 5.4.3.3** The assays shall meet the following acceptance criteria prior to approval for use with casework.

- 5.4.3.3.1** No results shall lie outside of the acceptable range calculated using the formula in **5.4.3.2.1**.

- 5.4.3.3.2** The % CV shall be less than 20 %.

5.4.3.3.3 All verification standard absorbencies must be lower than the average of the positive calibrator.

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

5.4.4 Calibration Verification

5.4.4.1 The average absorbance of the verification standard duplicates must be evaluated as positive for each assay to be reported.

5.4.5 The calibration data shall include the lot numbers and expiration dates of each microplate, enzyme conjugate, TMB/TMBZ substrate, stop reagent and ELISA buffer, the lot number of Immunoassay Calibration Solution, the lot number of Immunoassay Verification Solution, the lot number of Negative blood/urine, the Calibration, Verification Standard and individual absorbencies, average absorbencies.

5.4.6 The calibration data shall be reviewed by another qualified forensic scientist and, if acceptable, approved in the Toxicology Unit section object repository in FA with a file name beginning with "ElisaQC" (capitalization optional) and the date in yyymmdd format with no space between them. A suffix will be added to the file name to distinguish multiple runs performed on the same day.

5.5 Instrument Setup

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

5.5.2 Check printer and add paper if needed.

5.5.3 Prime the system executing the "system fluid prime" script in the Evoware software.

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

5.5.5 Check and fill appropriate conjugate vials. Ensure that the enzyme conjugate lot matches the microplate lot being used.

5.5.6 Create a plate layout in NaviTrak and position the microplates to correspond.

5.6 Application of Procedure on Evidence

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

5.6.2 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).

5.6.3 Cap and vortex for approximately 10 seconds. Ensure that there is no foam on the surface of the liquid.

5.6.4 Arrange sample tubes into appropriate sample racks. For each plate, each sample, matrix calibration standard, low calibration standard (for blood immunoassay), matrix negative standard, and matrix verification standard must be prepared in duplicate and run concurrently.

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

5.6.6 Calculations

5.6.6.1 An inverse relationship exists between absorbance and concentration.

5.6.6.2 The absorbance value of the duplicates shall be averaged for evaluation.

5.6.7 For each assay, evaluate the average absorbance value of the duplicates.

5.6.7.1 For average absorbance values that are at or below the average absorbance of the calibration standard, the result is positive.

5.6.7.2 In accordance with the limitations outlined in [Technical Procedure for Toxicology Analysis](#) (6.2), the benzodiazepines and opiates blood assays results shall be evaluated by the following criteria:

5.6.7.2.1 For average absorbance values that are above the average absorbance of the calibration standard and at or less than the average absorbance of the low blood positive calibration standard value, the result is elevated.

5.6.7.2.2 For average absorbance values that are greater than the low blood positive calibration standard value, the result is negative.

5.6.7.3 For blood assays not listed in **5.6.7.2** and all urine assays the results shall be evaluated by the following criteria:

5.6.7.3.1 For average absorbance values that are greater than the positive calibration standard value, the result is negative.

5.6.7.4 Record the calibration data, the individual absorbance values and the average absorbance of each case sample in the case record.

5.6.7.5 Record instrument use in the instrument log.

5.6.8 Reporting

5.6.8.1 Refer to the [Technical Procedure for Toxicology Analysis](#).

5.7 Uncertainty of Measurement – N/A

6.0 Limitations

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- 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

Immunalysis Tecan Freedom EVO 75 Workstation Operating Procedures

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

<http://www.immunalysis.com/images/stories/labs/cross%20reactivities2.pdf>

Levine, Barry, ed. *Principles of Forensic Toxicology*. 2nd edition. AACCC 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