

TENNESSEE BUREAU OF INVESTIGATION

Forensic Services Division

Toxicology Quality Assurance and Procedures Manual

8.3 Enzyme-Linked Immunosorbent Assay (ELISA) Procedure



8.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) PROCEDURE

8.3.1 Purpose

To routinely perform presumptive testing for opiates (including oxycodone/oxymorphone), buprenorphine, cannabinoids, cocaine/benzoylecgonine, barbiturates, benzodiazepines, methamphetamine, and fentanyl in submitted evidence by sample dilution followed by instrumental analysis with a Tecan EVO 75.

8.3.2 Specimen Requirements

Acceptable samples for this analysis include blood and urine. For additional samples, see Alternative Matrices (section 6.6).

8.3.3 Apparatus and Equipment

13 x 100 mm or 12 x 75 mm glass culture tubes
16 x 100 mm glass culture tubes (with screw caps and without)
Volumetric pipettes and disposable tips/Hamilton Dilutor
Assorted volumetric glassware
Sample mixer (vortex)
Tecan EVO 75 (including reagent troughs)

8.3.4 Reagents and Standards

Negative blood, urine or other matrix as needed
Drug Standards (indicated below)
DI Water (H₂O)
Methanol (CH₃OH)
100mM Phosphate-buffered saline (PBS) Buffer
Reagent Kits (coated microplates, TMB, Stop solution, conjugates)
1.0 N HCl (made in-house from concentrated HCl)
1.0 N NaOH (purchased concentration)

8.3.5 Standard and Reagent Preparation

The following are examples of how to prepare the standards and reagents used in this procedure.



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8.3.5.1 Prepared Standards

ELISA Stock Reference Standard Solutions

- **Morphine [1000 ng/mL]**
Pipette 50 μ L of morphine [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Buprenorphine [500 ng/mL]**
Pipette 25 μ L of buprenorphine [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **THC-COOH [1000 ng/mL]**
Pipette 50 μ L of THC-COOH [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Benzoylcegonine [1000 ng/mL]**
Pipette 50 μ L of benzoylcegonine [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Secobarbital [10,000 ng/mL]**
Pipette 500 μ L of secobarbital [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Oxazepam [1000 ng/mL]**
Pipette 50 μ L of oxazepam [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with the methanol or reagent alcohol.
- **Methamphetamine [1000 ng/mL]**
Pipette 50 μ L of methamphetamine [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Oxycodone [1000 ng/mL]**
Pipette 50 μ L of oxycodone [1 mg/mL] certified reference material into a 50 mL volumetric flask and dilute to volume with methanol or reagent alcohol.
- **Fentanyl [100 ng/mL]**
Pipette 10 μ L of fentanyl [1 mg/mL] certified reference material into a 100 mL volumetric flask and dilute to volume with the methanol or reagent alcohol.



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Note: The stock reference standard solutions expire one year from the date made or at the expiration date provided by the manufacturer, whichever occurs first. All Stock Reference Standard Solutions should be made on the same day from new reference materials.

ELISA Working Reference Control Standards

To make the Working Reference Control Standard for the 6 drug mixture listed below, add all of the following amounts of ELISA Stock Reference Standard Solutions to a 10 mL culture tube and dilute to a final volume of 5 mL (4,440 μ L) with negative blood/PBS buffer (1:1) or negative urine/PBS buffer (1:1).

DRUG	STD SOLUTION	AMOUNT USED	FINAL CONCENTRATION
Morphine	1000 ng/mL	50 μ L	10 ng/mL
Buprenorphine	500 ng/mL	10 μ L	1 ng/mL
THC-COOH	1000 ng/mL	50 μ L	10 ng/mL
Benzoylecgonine	1000 ng/mL	250 μ L	50 ng/mL
Secobarbital	10000 ng/mL	125 μ L	250 ng/mL
Oxazepam	1000 ng/mL	75 μ L	15 ng/mL

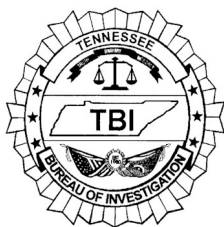
To make the Working Reference Control Standard for the 3 drug mixture listed below, add all of the following amounts of ELISA Stock Reference Standard Solutions to a 10 mL culture tube and dilute to a final volume of 5 mL (4,775 μ L) with negative blood/PBS buffer (1:1) or negative urine/PBS buffer (1:1).

DRUG	STD SOLUTION	AMOUNT USED	FINAL CONCENTRATION
Methamphetamine	1000 ng/mL	125 μ L	25 ng/mL
Oxycodone	1000 ng/mL	50 μ L	10 ng/mL
Fentanyl	100 ng/mL	50 μ L	1 ng/mL

Note: Based on validation studies, it is not necessary to make matrix matching controls.

A negative control shall be made at the same time from the negative blood or negative urine and PBS buffer lot number used to make the working reference control standards and shall be analyzed concurrently.

Note: The working reference control standard and the negative control expire two months from the date made (not to exceed the expiration date of the stock solution) or at the expiration date provided by the manufacturer, whichever occurs first.



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When the working reference control standard is nearing expiration, a new working reference control standard shall be made and ran concurrently (in duplicate) with the working reference control standard currently in use. The difference data mean for each assay for the new working reference control standard should be within +/- 20% of the difference data mean for the corresponding assay in the working reference control standard currently in use. The difference data mean values (for both new and old controls) shall be documented in the appropriate Instrument Record Book every time new controls are made.

If the new working reference control standard is slightly outside of the +/- 20% acceptance criteria of the control currently in use, then the scientist/technician shall make a low positive control (50% below the concentration of the control standard concentration) and a high positive control (100% above the concentration of the control standard concentration). If the ranges of the low positive control, positive control and high positive control (difference data mean +/- 2 standard deviations for each) do not overlap, then the new working reference control standard will be considered fit for use. See ELISA Validation notebook for examples.

8.3.5.2 Prepared Reagents

Negative Blood/PBS Buffer 1:1

Add 2.5 mL of negative blood to 2.5 mL of PBS buffer in a culture tube, cover, and mix.

Negative Urine/PBS Buffer 1:1

Add 2.5 mL of negative urine to 2.5 mL of PBS buffer in a culture tube, cover, and mix.

1.0 N HCl

Add 82 mL concentrated hydrochloric acid to H₂O and dilute to 1000 mL.

8.3.6 Procedure

1. Allow all working reference control standards, case samples, and reagents to equilibrate to room temperature before beginning procedure.
2. Label, check and load/unload all samples in accordance with the "Sample Pipetting Check List" (see Appendix section).
3. Vortex and pipette 100 µL of positive control, negative control, or corresponding case sample into the appropriately labeled 13 X 100 or 12 x 75 mm glass culture tube.
4. Add 900 µL of PBS Buffer and vortex for approximately 10 seconds.
5. Place the labeled 13 X 100 or 12 x 75 mm culture tubes into the appropriate positions of the Tecan EVO 75 sample racks and slide the sample racks into the appropriate slots on the instrument.

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6. Place the reagents/conjugates and 96 well microplates in the appropriate locations on the instrument.
7. Analyze the samples by Tecan EVO 75 ELISA instrumentation.

8.3.7 Quality Control

8.3.7.1 The positive control will be analyzed in duplicate at the beginning of the run (positions A1 and B1 on the well plate). The negative control will be analyzed in duplicate immediately following the positive control (positions C1 and D1). The difference data mean of the positive control must be less than the difference data mean for the negative control in order for results to be reported (PC<NC). See 6.15 for additional criteria.

8.3.7.2 The difference data variation coefficient for both the positive and negative controls shall not exceed 20%.

8.3.8 Interpretation of Results

8.3.8.1 Results shall be listed on the ELISA Drug Screening Report, generated by the instrument, as either “positive” or “negative” indicating the presumptive presence or absence of a compound within one or more drug classes.

8.3.8.2 Results that are presumptively positive shall be confirmed with testing appropriate for that drug class, as available within the TBI Toxicology Unit.

8.3.8.3 The ELISA provides semi-quantitative presumptive results based upon the cut-off concentration of each assay’s target compound. Different drugs within each drug class may be more or less reactive to the assay than the target compound. Therefore, consideration shall be given to cases where the reported difference data may not be positive but are less than the difference data mean for the known negative controls that appear on the ELISA Drug Screening Report (specific for each assay). Cases showing lower responses may require further confirmatory testing (i.e. decreased absorption rate but result reads “negative” for benzodiazepines). Cases showing a negative response do not preclude the presence of drugs for which the assay has low sensitivity. Each trained examiner shall use discretion when evaluating these circumstances.

8.3.9 Reporting

Results that are negative should be reported on the Official Toxicology Report (e.g., “Barbiturates presumptively not detected”). See 8.3.8.3 for exception.

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8.3.10 References

B.J.Perrigio and B. P. Joynt, Use of ELISA for the Detection of Common Drugs of Abuse in Forensic Whole Blood Samples. *Can. Soc. Forens. Sci. J.*, 28(4): 261-269, 1995.

Immunoanalysis, "Forensic Analysis: Drugs in Blood Specimens, Validation of Enzyme Linked Immunosorbent Assays (ELISA)" February 2014.

O'Conner, Torrance, and McKeown, ELISA Detection of Phenazepam, Etizolam, Pyrazolam, Flubromazepam, Diclazepam and Delorazepam in Blood Using Immunoanalysis Benzodiazepine Kit. *Journal of Analytical Toxicology*, 40:159-161, 2016.

Scientific Working Group for Forensic Toxicology Standard Practices for Method Validation in Forensic Toxicology (May 20, 2013).