FORENSIC TOXICOLOGY LABORATORY
OFFICE OF CHIEF MEDICAL EXAMINER
CITY OF NEW YORK

SCREENING FOR DRUGS OF ABUSE IN URINE, WHOLE BLOOD, SERUM AND VITREOUS HUMOR by ENZYME-LINKED IMMUNOSORBENT ASSAY on the DYNEX DSX MICROTITER PLATE ANALYZER

PRINCIPLE

Enzyme-linked immunosorbent assay (ELISA) is a microtiter plate based technology used to detect drugs of abuse It is a competitive, solid-phase, heterogeneous, enzyme immunoassay using antibodies immobilized on the surface of a microtiter plate. Free drug and drugs conjugated to an enzyme compete for binding to the antibody. After a short incubation, the plate is washed to remove all unbound enzyme conjugate and sample debris. A substrate solution is added which produces a colored product in the presence of enzyme. A stopping reagent is added to end the reaction. The intensity of the color produced is measured as OD at 450 nm via a spectrophotometer. Measurements taken are inversely proportional to the amount of free drug present in the original sample.

SPECIMENS

Whole blood

- **ALL** blood based specimens **MUST** be diluted 1:5 with OraSure Forensic Specimen Diluent
- Dilutions are stable for **ONE WEEK** from date of preparation; dilutions **MUST** be discarded after one week.

> ***Note**: Calibrators and controls for the cannabinoids assay must be diluted on the day of the assay.*

Urine

Urine specimens must be diluted before being aliquoted into the microtiter plate. Dilutions of either 1:5 or 1:60 (depending on the kit/assay type) are made with OraSure Forensic Diluent . This can be accomplished in one of two ways: automatically on the instrument; off-line before being placed on the instrument.

- If dilutions are made off line, they are stable for **ONE WEEK** from date of preparation; dilutions **MUST** be discarded after one week.

Serum

- **ALL** serum specimens **MUST** be diluted 1:5 with OraSure Forensic Specimen Diluent
- Dilutions are stable for **ONE WEEK** from date of preparation; dilutions **MUST** be discarded after one week.

> ***Note**: It is **extremely** important to eliminate bubbles; the presence of a bubble could produce a false negative result.*

Vitreous Humor

- Vitreous humor samples are run undiluted.

REAGENTS AND MATERIALS

Micro-Plate Assays

1. OraSure Technologies, Inc. Micro-Plate ELISA drugs of abuse test kits for Opiates, Cocaine Metabolite (BE), Barbiturates, Amphetamine Specific, Methamphetamine Specific, Benzodiazepines. Cannabinoids, Oxycodone and Methadone.
2. Antibody immobilized on polystyrene wells (distinct for each assay).

   Drug class specific enzyme conjugate labeled with horseradish peroxidase

   Substrate reagent which contains 3,3',5,5'- tetramethyl-benzidine

   Stopping reagent which contains 2 N sulfuric acid. (Sold separately)

3. Store kits at 2-8^{o}C until expiration date indicated on the kit label.

Plate Validation Calibrators

1. OraSure Negative Calibrator (Serum or Oral Fluid)
2. OraSure Cutoff Calibrator (Serum or Oral Fluid)

> *Note: No OraSure negative and cutoff calibrators are provided with the Oxycodone kit.*

Calibrators

Whole Blood

1. Negative calibrator (NCAL): UTAK Blank Blood
2. Cutoff Calibrator (CUTOFF): Prepared internally in UTAK Blank Blood
3. Negative control (NCTRL): Prepared internally in UTAK Blank Blood (1/2 cutoff)
4. Positive control (PCTRL): Prepared internally in UTAK Blank Blood (2 times cutoff)

Urine

1. Negative calibrator (NCAL): Certified Negative Urine
2. Cutoff Calibrator (CUTOFF): Prepared internally in Certified Negative Urine
3. Negative control (NCTRL): Prepared internally in Certified Negative Urine (1/2 cutoff)
4. Positive control (PCTRL): Prepared internally in Certified Negative Urine (2 times cutoff)

Serum

1. Negative calibrator (NCAL): UTAK Serum
2. Cutoff Calibrator (CUTOFF): Prepared internally in UTAK Serum
3. Negative control (NCTRL): Prepared internally in UTAK Serum (1/2 cutoff)
4. Positive control (PCTRL): Prepared internally in UTAK Serum (2 times cutoff)

Vitreous Humor

1. Negative calibrator (NCAL): Certified Negative Vitreous Humor
2. Cutoff Calibrator (CUTOFF): Prepared internally in Certified Negative Vitreous Humor
3. Negative control (NCTRL): Prepared internally in Certified Negative Vitreous Humor (1/2 cutoff)
4. Positive control (PCTRL): Prepared internally in Certified Negative Vitreous Humor (2 times cutoff)

Quality Control

In addition to the reagents above which are routinely run at the beginning of the assay, positive and negative internal controls are run after every 22 unknown samples and at the end of the assay.

REAGENT PREPARATION

Cocaine Metabolite

1. Using a volumetric pipette, add 2 mL of Conjugate Diluent to the vial of lyophilized Stock Conjugate.
2. Replace the stopper and gently mix the contents of the vial by inversion for 10 minutes. Alternatively, after addition of diluent, the conjugate vial may be left refrigerated overnight to reconstitute. Mix gently before use.
3. Using a calibrated pipette, add the volume of reconstituted Stock Conjugate specified on the Conjugate Dilution Instructions for this kit lot to the Conjugate Diluent bottle.

NOTE: *The Orasure Conjugate Dilution Instructions for OTI Cocaine Micro-Plate EIA for the current lot will be kept in a sheet protector at the end of this SOP. The calculations used to determine the amount of re-constituted conjugate to put back into the diluent will be shown on this sheet.*

4. Replace the lid on the bottle and gently mix the contents by inversion for 1 minute. Allow the reagent to equilibrate for 30 minutes at room temperature or overnight at 2-8^0 C.
5. Label the kit indicating that conjugate was prepared, when and by whom.

Note: *This conjugate dilution is stable for **3 months** when stored at 2-8^0 C and may be used in the OraSure Cocaine Metabolite Micro-Plate assay as needed.*

Opiates, Barbiturates, Benzodiazepines, Amphetamine Specific, Methamphetamine Specific, Cannabinoids, Oxycodone and Methadone

All reagents are ready to use.

GENERAL INSTRUCTIONS FOR ALL KITS

1. Allow all reagents and samples to come to **room temperature** (20-27^0 C) before use.
2. Ensure that kits are not outdated and that kit lot numbers are properly entered in the Access program on the Database computer.
3. Do **NOT** mix reagents or plates from different kits or manufacturers.
4. Do **NOT** use reagents past the expiration date.
5. Do **NOT** freeze reagents.
6. Proper handling of all reagents is strongly advised. It is suggested that disposable materials be used to avoid contamination of Substrate Reagent. Discard Substrate Reagent if **obvious blue color** develops.
7. Do **NOT** mouth pipette reagents. Handle all specimens and reagents as if potentially infectious.
8. Do **NOT** add sodium azide to samples as a preservative!
9. Keep all containers closed when not in use to avoid contamination.
10. It is suggested that all OraSure (STC) reagents be kept out of direct sunlight whenever possible.
11. The Stopping Reagent is corrosive; handle with care.

INSTRUMENTATION AND EQUIPMENT

1. Test tubes: 12 x 75 mm disposable glass.
2. Plastic containers for reagents and calibrators/controls.
3. Two mL volumetric pipet for reconstitution of BE conjugate.
4. Calibrated adjustable pipette (20-200 microliters) with disposable tips for specimen dilutions and addition of Cocaine Stock Conjugate to Conjugate Diluent bottle.
5. Calibrated repipettor or equivalent to dispense appropriate amounts of Forensic Diluent.
6. Reagent and specimen sample tips manufactured specifically for the DYNEX DSX.
7. Deep well plates for urine dilutions on instrument.

8. DYNEX DSX Automatic Sample Processor and Microplate Analyzer

The DYNEX Analyzer is an automated laboratory microplate assay-processing unit working in conjunction with specifically designed Microsoft WindowsXP software. The open architecture is programmed to perform multiple analyses of most ELISA microplate assays. The following ELISA microplate drugs of abuse assay protocols have been programmed for automated processing in specific profiling formats: Amphetamine Specific, Methamphetamine Specific, Barbiturates, Cannabinoids (THC), Benzodiazepines, Cocaine Metabolite, Oxycodone, Morphine/Opiates and Methadone.

***Note**: See manual section with hard copy of automated assay protocol steps and the profiles programmed for automated testing of multiple protocols per assay specific validated applications. See also the OraSure (STC) Application Technical Bulletin, which describes the assay specific application per the desired assay specific cutoff.*

DYNEX DSX (DSX) OPERATION

1. Turn on DSX instrument.
2. Turn on Computer

***Note**: If steps 1 and 2 are not done in order there an error message screen will appear (DSX Communication Module Error). Click on the Recovery Options button and select "Try to self test communications module again" (#2).*

3. Press and hold "Control" and "Alt" and hit "Delete". This will bring up the network login screen.
4. Use "OCMETOX" for the login name followed by the appropriate password.
5. Double click on the Revelation DSX icon on the desktop.
6. A screen appears with "Connect to DSX using Port 1" selected. Select "Do It". If this is not actively done within 30 seconds, it will continue automatically.
7. On the Revelation DSX Logon screen, enter User Name and Password and click OK The self-test on all modules will start. The results appear on screen. Scroll down to see if all tests have passed. Print the self test using the Printer icon on the toolbar. Follow directions for a double sided copy.

ORDER WORKLIST PANEL USING BUTTONS (PANEL OF DRUGS)

1. Click the button with the worklist name desired (Urine or Blood). The buttons are at the top of the screen under the first row of tool bars
2. The Sample Batch Selection screen appears with "Add assays using a new batch of samples" selected. Accept by clicking OK.

***Note:** The choice in the "Sample Caddy Definition in use" field is "Example rack". Do **NOT** change this choice.*

3. The Edit Worklist-Sample Batch 1 screen shows the assays programmed for a urine (or blood) worklist and their plate assignments. (For single analyte assays, see the end of this SOP)

***Note**: the buttons were not named well when programming. To see the test name hold the cursor over the button.*

4. Place the cursor in the field to the right of Sample ID field A1. Hold down the left mouse button and drag the cursor from the upper left corner of the grid to the lower right corner so that the boxes under all the tests are highlighted. Release the mouse button. The previously highlighted boxes will now contain a checkmark indicating that a test has been assigned. Scroll to the next page and repeat until there are checkmarks in all positions for the number of samples being analyzed (from A1 to C12 for a 48 well run)
5. On the same screen, in the "First Autosample ID" box enter the number "1". Click on Auto Assign Sample IDs. The number in the window should change to 41 for a full run of samples. The Sample ID fields are now numbered 1 through X (where X is the last sample to be entered, 40 for a run encompassing A1 to C12)
6. Scroll up to Sample ID number 1. Enter the unknown sample number. **Press TAB** to move to the next field. When all entries are completed click OK.

***Note: DO NOT TOUCH THE ENTER KEY** during the specimen data entry process. Touching ENTER will disrupt the programming requiring a completely new start-up.*

7. Click green PLAY button (green triangle). Wait while the timeline is building.
8. The Down Arrow at the upper left with turn blue when the time line is finished.
9. View timeline. Click Fast Forward (double green triangle) to start loading consumables.
10. A screen named "Lot Specific Data and Runtime Variable Entry" will appear. The first four fields under the heading Plate say Test 1, Test 2, Test 3, Test 4. These need to be edited to match the information listed in the Assay Kits field in the middle of the screen. The assays are listed in the in the correct assay order.
11. Edit the Test 1 field to match the first assay listed using the following format:

    Date - Matching assay type (add "Blood" for whole blood assays)

    Example: The first test listed in the box at the bottom is amphetamines

    Edit "Test 1" to read: A(B)040909-amphet

***Note**: A or B at the beginning of the format identifies the instrument used.*

12. Enter the lot number of the selected kit in the Number field and enter the expiration date in the Expires field.

***Note**: To move to next field use **TAB** or click cursor in the field.*

Continue until all four assay fields, lot numbers and expiration dates have been entered. Click the green checkmark to accept.

13. The Load Sample Rack screen appears. It shows all the specimens scheduled. Load the samples in the sample caddies starting at the back left moving left to right in each rack. The last sample will be in the front right position for that set of samples. Click the green checkmark to accept.

***Note**: Check specimens and reagents for bubbles when loading the instrument. It is **extremely** important to eliminate bubbles; the presence of a bubble could produce a false negative result.*

14. The Load Fluid Screen appears. This screen has the following headers.

    Fluid – name of reagent to add

    Bottle – the type of consumable used to hold the fluid

    Volume – the volume of reagent to be pipetted

    Dead Volume – the volume of reagent not available for sampling

    Bottle Volume – the total of pipetted volume and dead volume. MINIMUM amount of reagent needed for proper pipetting.

    ***Note**: Ensure that there is always more than enough fluid present for each need.*

    Loaded – shows yes or no depending on status

15. Follow prompts to load consumables (a duplicate of the instrument is shown on the screen with arrows pointing to the proper placement of the consumable described). When prompted to load plates, name the files as above [date – assay (Blood)]. Be SURE the plate identifier is unique.
16. To load an assay plate, remove the plate holder from the plate carrier. Insert the assay plate with position A1 at the front left corner. Angle the plates into the holders starting from the front to the back

    ***Note**: For a full panel of 32 calibrators, controls and unknown samples, there should be four well strips in a plate starting from the left side*

17. Replace the loaded plate holder on the carrier; repeat for each assay.

***Note**: the plate holders are removed to insert plates so that the positioning of the plate carrier on the instrument is not disrupted by pressing plates into it.*

18. Make sure the tip waste container and washer waste bottle are empty.
19. If green, the Fast Forward button can be pressed to start the run. If not selected, the run will start after an elapsed time.
20. When the first set of four plates is completed a status line on the screen flashes “3 plate(s) are ready to start form Sample Batch 1. Click Play button to start now.”
21. Take off the used plates and set aside.
22. Remove reagents specific to completed assays.
23. Change the STC controls from serum to oral fluid.

24. Refill the reagents common to all assays (forensic diluent, substrate, stop reagent).
25. Ensure there are sufficient sample and reagent tips when prompted. Click OK.
26. Empty the tip waste container. (This can also be done at the end of a session.)
27. Click on Forward (green triangle) and wait for timeline to build.
28. Click on Fast Forward (double green triangle)
29. Fill in the Lot Specific and Runtime Variable Entry screen as before. Click green checkmark when done.
30. Load appropriate plates and reagents as before following the prompts for proper placement.
31. Start run as before.
32. The complete run is finished when the timeline says “System Paused” and the Stop button is red (i.e. not gray). To end the session, click on Stop button. The instrument will then “home” itself.
33. Click on File in the top tool bar. Choose “Recent Plates”. The **four** most recent assays will be displayed. Select assay to print.
34. Click on the blue arrow to toggle to the screen displaying the data for the assay chosen. Click on print icon and OK to order a hardcopy of the data. Follow the directions for double sided printing.
35. Repeat from step 33 until the four most recent assays are printed.
36. To print the remaining data files, click on “File” and then “Open”.
37. At the bottom of the screen which appears, change the “Files of Type” field to Plate Files [*dat]. Scroll through the plates until the file to be printed is found.
38. Double click the file name and the data appears on the screen
39. Click on the Printer icon to print as before.
40. Click and the file appears on the screen

SINGLE ANALYTE ASSAY PROCEDURE

Cannabinoids in urine or whole blood and opiates in whole blood can, as necessary, be run as stand alone assays. To perform these assays, the following SOP changes or additional steps are outlined below.

OPIATES IN WHOLE BLOOD OR CANNABINOIDS IN WHOLE BLOOD OR URINE

1. At SOP step 3, click on the Blood panel button. The unneeded assays must be deleted.
2. Position the cursor over a button in the panel. If the assay displayed is NOT the assay of choice (opiates or cannabinoids), left click and chose “delete assay”. The button will be removed. Continue until only the assay of choice button is displayed.

3. Highlight and click on the appropriate number of test boxes so that a check mark appears.
4. Continue as in Step 5 above.

RESULTS ANALYSIS AND INTERPRETATION – FIRST LEVEL REVIEW

1. The hard copy report first identifies the specific session test protocol(s) that was performed, the session name, the profile name, the micro-plate carriage location, date, and name of user that performed this session.
2. Displacements are calculated by the instrument programming follows:

> ***Note:*** *Displacements or other assay related failures are printed on the assay data printouts.*

a. $\frac{\text{STC Negative Calibrator OD - STC Cutoff Calibrator OD}}{\text{STC Negative Calibrator OD}}$

b. $\frac{\text{Mean Whole Blood NCAL OD - Mean Whole Blood CUTOFF OD}}{\text{Mean Whole Blood NCAL OD}}$

3. Record displacements as a percent in the QC log book along with the acceptable ranges for both (found on the kit specific package inserts). If displacements do not meet criteria, consult a supervisor.
4. The next portion of the hard copy report information relates to the programmed validation criteria for the specific assay and whole blood calibrators.

The instrument automatically validates each assay and interprets the sample results as:

Positive Results: Any sample with an OD less than or equal to the mean of the whole blood cutoff calibrators is considered a positive.

Negative Results: Any sample with an OD greater than the mean of the whole blood cutoff calibrators is considered a negative.

5. Following the calibrator validation, the report lists calibrator and micro-plate positions, ODs, and the result interpretation call as positive or negative.
6. The report continues with micro-plate positions of the samples, the sample ID number, and the result interpretation call as positive or negative.

ACCEPTANCE CRITERIA

1. All positive quality controls must be positive and all negative quality controls must be negative. Consult a supervisor if there are discrepant results.
2. If the OD of any matrix positive control is not less than the OD of the matrix cutoff calibrator, the run will automatically be rejected. A new matrix cutoff calibrator and matirx positive and negative controls will be used and the run repeated.

3. If the OD of the matrix cutoff calibrator is not less than the OD of the matrix negative control, the run will automatically be rejected. A new matrix cutoff calibrator and matrix positive and negative controls will be used and the run repeated.

SECOND LEVEL REVIEW

1. The section supervisor will ensure that result analysis and interpretation performed by the first level reviewer is correct. He/she will sign off on the data review. Copies of all results will be made.
2. Results are reported to the case file following reporting criteria of the SOP.

THIRD LEVEL REVIEW (FINAL REVIEW)

The third and final level review can only be performed by the Laboratory Manager. He/she will review the data for the entire case according to all established criteria. They will ensure that screening, confirmatory and quantitative analysis on the case have been completed and reported accurately. As needed, they will also schedule additional analysis and contact the Medical Examiner on the case to discuss any findings and / or review case history.

REPORTING CRITERIA

CUTOFFS: The administrative cutoff concentration for each ELISA drug screen is the analyte concentration documented in the ELISA Quality Control Standard Operating Procedure.

1. Test results with an OD equal to or less than that of the mean cutoff calibrator OD are positive and are reported as detected for the specified drug.

Schedule of appropriate confirmations or substantiations.

1. For all tests except cannabinoids, schedule confirmation testing in the appropriate specimens.
2. For positive **urine** cannabinoids, schedule a cannbinoid test in blood. If the subsequent blood result is negative, repeat both the urine and blood tests.
3. For positive **blood** cannabinoids, schedule a repeat analysis.

Positive Quality Control Failure:

1. Negative result for positive control: OD near cutoff (-10%):

   Positive result for unknown specimens - report as detected (these tests are subject to confirmation).

Negative result for unknown specimen: OD between negative control OD (50% of cutoff) and cutoff calibrator OD – **REPEAT**.

Negative result for unknown specimen: OD greater than negative control OD (50% of cutoff) - report as not detected.

***Note**: for assays which routinely have few positive results (e.g. barbiturates) the entire assay may be repeated in the interest of efficiency*

2. Negative result for positive control: OD greater than negative control (50% of cutoff):

   Positive result for unknown specimens - report as detected (these tests are subject to confirmation).

   Repeat all unknown specimens with a negative result.

Negative Quality Control Failure

1. Repeat ALL unknown specimens.
2. The reporting criminalist may, at their discretion, refer a sample for testing by a different analytical methodology if the OD of the sample is negative but within 10% of the cutoff. The result should be reported as negative with a notation that the result is close to the cutoff.

Displacement Deviations

1. if the kit displacement is below the accepted range (35% to 75%) consult a supervisor.
2. If the blood displacement is below the accepted range (35% to 75%) consult a supervisor

Note: High displacements are acceptable except if OD readings are atypical.

3. If the % c.v. for the cutoff calibrator is greater than 10% consult a supervisor. The supervisor may choose one of the duplicates as the cutoff but only if the chosen cutoff OD creates more positive determinations which will then be subject to confirmation testing.

INTERFERENCES

See assay specific package inserts and supplementary OraSure (STC) Technical Bulletins for specificity and cross-reactivity data for each assay.

MISCELLANEOUS

1. Consult with section supervisor on troubleshooting, special protocols, non-routine preventive maintenance procedures, and other inquiries.

2. Additional assistance may be necessary from OraSure Technologies (OTI).
3. Inform the section supervisor when contacting the manufacturers for assistance.
4. Protocol for assay setup may be located in the package inserts in conjunction with the Applications Technical Bulletin.

DAILY MAINTENANCE

1. Verify that the self-test passes.
2. Empty and clean the tip waste container
3. Empty and clean (as necessary) the liquid waste container.

WEEKLY MAINTENANCE

1. Empty the wash buffer containers and clean them with several rinses of deionized water.
2. Remove and clean the waste tip chute. If desired, disinfect with 70% alcohol.

MONTHLY MAINTENANCE

1. Clean wash head. Refer to DSX Operators Manual, page 87.

***Note**: The wash head is also cleaned as necessary when indicated by assay results.*

SIX-MONTH MAINTENANCE

***Note**: This should be part of preventive maintenance from the current service contract vendor. Assure that this has been checked and/or done during service and preventive maintenance calls.*

1. Replace the dispense tubing
2. Replace the aspiration tubing.

REFERENCES:

OraSure (OTI) Enzyme Immunoassay Package Inserts

DYNEX DSX system manual

REVISION HISTORY

Ver.11.01.2013	Revision history implemented
Ver.04.21.2015	Additions to acceptance criteria
Ver.09.15.2015	Inclusion of First, Second and Third Level review