Laboratory Procedure Manual

Analyte: **HIV Antibody**

Matrix: **Urine**

Method: **Calypte HIV-1 Peptide Enzyme Immunoassay (EIA)**

as performed by: *HIV Immunology and Diagnostics Branch*
Division of AIDS, STD and TB Laboratory Research
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Public Release Data Set Information

Positive results are the Western blot confirmed test results. All samples were screened by enzyme immunoassay (EIA); therefore, the urine EIA method is included in this file. The Western blot used for serum samples is the same method used to confirm the urine EIA-repeated positive results (see the HIV Serum EIA-Western Blot file).

1. Summary of Test Principle and Clinical Relevance

The Calypte HIV-1 Urine Enzyme Immunoassay (EIA) utilizes a recombinant envelope protein of HIV-1 to detect the presence of antibodies to HIV-1 in human urine. The recombinant gp160 envelope protein in absorbed onto wells of a micro well plate. Urine specimens or urine controls which may contain antibodies to HIV-1, along with sample buffer, are added to the wells and incubated. If antibodies to the HIV-1 envelope protein are present in the specimen, they will bind to the antigen coated on the well. The sample buffer significantly reduced the non-specific binding of antibodies and other proteins to the well. A wash step removes any unbound material. Then a conjugate consisting of alkaline phosphatase chemically bound to goat anti-human immunoglobulin is added to each well and allowed to incubate. The conjugate will bind to HIV-1 antibodies which are bound to the immobilized antigen. A wash step removes any unbound conjugate. The substrate for the enzyme *p*-nitrophenylphosphate (p-NPP), is added to all the wells and incubated. If antibodies to HIV-1 are present in the specimen, the enzyme will cause the color to change from colorless to yellow. The intensity of the color is proportional to the amount of the HIV-1 antibody present in the test specimen or control. The reaction is terminated by the addition of a stop solution containing ethylenediaminetetraacetic acid (EDTA). The absorbance values are determined spectrophotometrically with a plate reader at a peak wavelength of 405 nm.

Using the positive and negative controls included with the test kit, two positive control wells and three negative control wells are tested with each plate or partial plate of specimens. A specimen is determined to be either reactive or non-reactive by comparing its absorbance value to a cutoff value which is calculated by adding the mean absorbance value of the negative controls to a value of 0.180.

Samples that are initially reactive should be tested in duplicate using the originally collected specimen. If after repeat testing, one or both of the duplicate tests are reactive, the specimen is considered repeatedly reactive in the test. Before a determination of HIV-1 status can be made, subjects with repeatedly reactive results should be further evaluated using the additional more specific Cambridge Biotech HIV-1 Western blot kit (PN98076).

All repeatedly positive specimens are confirmed by western blot. See method in the HIV serum method description.

Summary and Explanation of the Test

Acquired Immunodeficiency Syndrome (AIDS) is the result of the progressive loss of immunocompetence following infection with Human Immunodeficiency Virus (HIV). Individuals exposed to HIV may experience an initial acute phase illness characterized by flu-like symptoms. The acute phase is followed by a putative latency period of varying length, culminating in the onset of the symptoms of opportunistic infections which characterize AIDS. Most individuals infected by HIV develop antibodies to the major structural proteins of HIV. Detection of these antibodies in blood has long been considered a prerequisite for the diagnosis of AIDS. Recent evidence also demonstrates the detection of antibodies to HIV in the urine of HIV infected individuals.

There are several safety advantages to using urine specimens compared with the use of blood. Infectious HIV is unlikely to be present in urine and urine can be collected by a non-invasive procedure. The use of urine eliminates the risk of accidental needle stick exposure to blood borne pathogens during the collection of specimens. An HIV-1 urine antibody test may facilitate surveillance for HIV infection. With the availability of an HIV-1 Western Blot (Cambridge Biotech HIV-1 Western Blot kit- PN-98076) approved for use with urine specimens, urine specimens that are repeatedly reactive in the Urine HIV-1 EIA may be further tested for HIV-1 antibodies.

2. Precautions

a. The positive and negative controls are heat treated to inactivate viruses. However, handle assay specimens and controls as if capable of transmitting infectious agents. Use of good laboratory practices and CDC-NIH guidelines as recommended.
b. All test operators should adhere to the Occupational Safety and Health Administration (OSHA) regulations (29 CFR 19.10).

c. Keep testing area separate from areas in which blood or blood products for transfusion are stored.
d. Do not use reagents beyond the expiration date printed on the reagent label.
e. With the exception of Substrate Tablets, Substrate Diluent, Wash Solution, and Stop Solution, do not interchange reagents from different lots or kits.
f. Do not interchange bottle kits.
g. Mix all liquid reagents by gently inverting 3–5 times, just prior to use.
h. Prior to performing the test, bring to room temperature only as many strips of microwells as needed to perform the test run. Any strip of microwell which are not to be used in the current test run should be sealed in the foil bag with desiccant and stored at 2–8°C.
i. Remove reagents from the refrigerator storage approximately 60 min before beginning assay. Bring kit reagents to room temperature (15–30°C) prior to use. Return all kit components to their recommended storage conditions immediately after use.
j. Avoid microbial contamination and cross contamination of reagents and specimens. Use separate pipettes and/or pipette tips, and reagents reservoirs for each component of the kit and for each specimen to be tested.
k. Open the foil wrapping containing the Substrate Tablets by tearing at the indentation while holding the tablet within the foil. Do not touch the Substrate tablet with fingers. Invert the foil wrapper and drop the tablet(s) into the container used for preparation of substrate solution. Substrate solution which has turned yellow in color before use must be discarded. Avoid contact of the Substrate tablets with skin and mucous membranes. If this reagent comes into with skin or mucous membranes, flush thoroughly with water.
l. Do not reuse plate sealers.
m. Proceed immediately to add the next reagent after each wash step. Do not allow the microwells to dry out.
n. Mix specimens thoroughly by inversion before adding them to test wells.
o. Do not smoke, drink or eat in areas where specimens or kit reagent are being handles.
p. Do not pipette by mouth.
q. Avoid splashing or creating aerosols.
r. Wipe spills promptly with a 0.5% sodium hypochlorite solution (1:10 dilution of liquid household bleach). Do not place solutions containing bleach in the autoclave.
s. Dispose of all specimens and materials used in the procedure according to local regulations.
t. Some of the reagents in this kit contain sodium azide as a preservative. Sodium azide has been reported to form lead or copper azide in plumbing. These azides are explosive. Flush drains thoroughly after disposing of solutions cosodium azide to prevent azide build-up. Check with local regulations for disposal restrictions.
u. Wear protective clothing and disposable gloves while handling the kit reagents. Wash hands toughly after performing test.

3. **Computerization; Data System Management**

a. HIV antibody results are manually entered into a Microsoft Excel result file spreadsheet. After a run is complete and any additional corrections by the analyst are made, the Excel result file is prepared. Data is transmitted electronically weekly to Westat's ISIS computer system, and transferred from there to NCHS.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

a. It is recommended that specimens be collected according to the NCCLS tentative guidelines.
b. If specimens are to be shipped, they should be packaged and labeled in compliance with applicable federal and international regulations covering the transport of clinical specimens. Do not allow urine specimens to freeze during shipping.
c. Specimens visibly contaminated with bacteria, blood or sediment may give inaccurate test results.
d. Store the urine refrigerated at 2–8°C. Do not freeze the urine.
e. Test urine specimens as soon after collection as possible. Only urine specimens without preservatives or specimens preserved with Stabilur (R.P. Cargille Laboratories, Inc. Cedar Grove IL) may be used in this assay. Unpreserved or Stabilur-preserved urine specimens may be stored

at room temperature (15–30°C) up to 55 days or for up to one year at 2–8°C.

f. Polyethylene or polypropylene containers are recommended for shipping or storing urine specimens

5. Procedures for Microscopic Examinations; Criteria for Rejection of Inadequately Prepared Slides

Not applicable for this procedure

6. Preparation of Reagents, Calibration (Standards), Controls, and All Other Materials; Equipment and Instrumentation

a. Reagent Preparation

(1) Microwell Plate

Remove coated microwell plate from its foil bag. Remove any unneeded strips from the plate frame, reseal them in the foil bag along with the desiccant, and return the foil bag to 2–8°C. If using a 96-well plate, dilute 50 µl of conjugate concentrate into 11 mL of conjugant diluent. For partial plates, use the following table. Mix the prepared conjugate solution thoroughly by gently inverting 3–5 times.

Preparation of Conjugate Solution

No of coated strips	1	2	3	4	5	6	7	8	9	10	11	12
Volume of Conjugate Concentrate (µL)	10	10	20	20	20	30	30	40	40	40	50	50
Volume of Conjugate Diluent (mL)	2.2	2.2	4.4	4.4	4.4	6.6	6.6	8.8	8.8	8.8	11	11

Conjugate Solution

Substrate solution mast be freshly prepared each time the assay is performed by dissolving substrate tablets in substrate diluent (see HIV-1 Urine EIA Procedure Step 14). To test a complete 96-well plate, dissolve three substrate tablets into 15 mL of substrate diluent. For partial plates, use the following table. Mix the prepared substrate solution thoroughly before use. Vortex if necessary. Undissolved material should be visible.

Preparation of Substrate Solution

No of coated strips	1	2	3	4	5	6	7	8	9	10	11	12
Number of Substrate Tablets	1	1	1	1	1	2	2	2	2	2	2	3
Volume of Substrate Diluent (mL)	5	5	5	5	5	10	10	10	10	10	10	15

Note: See warnings, interpretations of results, limitation of the procedure and performance characteristics sections for information on:

1. Reduced sensitivity and specificity of testing urine specimens compared with testing blood specimens. The Calypte HIV-1 Urine EIA gave a false negative rate of 1.3% for a combined populations of AIDS patients and other HIV-1 seropositive rate of 0.9% in low risk populations,

9.7% false-positive rate for patients at high risk for HIV-1 infection, 14% false-positive rate when testing subjects with unknown risk factors for HIV-1 and 18% false-positive rate patients with other medical conditions.

2. The requirement for laboratories using the test to provide ordering physicians with Subject Information Brochures and stickers.

3. The requirement for the test subject to sign or initial the "Subject Information Brochure" sticker and to affix the sticker to the urine collection container. Laboratory testing should be performed only specimens which have a signed or initialed sticker on the urine collection container.

4. Reporting test results to the ordering physician or someone under the supervision of the ordering physician.

5. Results from this test are not to be used for screening potential blood donors.

Warnings

For *In Vitro* diagnostic use

FDA has licensed this test for use with urine specimens only. Use of this licensed test kit with other types of specimens may result in inaccurate results.

1. Only urine specimens without preservatives or specimens preserved with Stabilur may be used in this assay.

2. Specimens preserved with Stabilur may have lowered absorbance values and reduced sensitivity.

3. HIV-1 antibody testing of urine specimens has reduced sensitivity and specificity compared with HIV-1 antibody of blood specimens (see Performance Characteristics section details.)

4. Due to the possibility of false-positive (i.e. EIA repeatedly reactive) HIV-1 test results, subjects with repeatedly reactive results should be further evaluated using only the additional, more specific Cambridge Biotech HIV-1 Western Blot, kit part number 98076. (Rockville, MD)

7. Precautions

1. The positive and negative controls are heat-treated to inactive viruses. However, handle assay specimens and controls as if capable of transmitting infectious agents. Use good laboratory working practices and CDC-NIH guidelines is recommended.

2. With the exception of substrate tablets, substrate diluent, wash solution and stop solution, do not interchange reagents from different lots of kits.

3. Do not interchange bottle caps.

4. Mix all liquid reagents by gently inverting 3–5 times, just prior to use.

5. Prior to performing the test, bring to room temperature only as many strips of microwells as are needed to perform the test run. Any strips of microwells which are not to be used in the current test run should be sealed in the foil bag with descant and stored at 2–8°C.

6. Remove reagents from refrigerator storage approximately 60 min before beginning assay. Bring kit reagents to room temperature (15–30°C) prior to use. Return all kit components to their recommended storage conditions immediately after use.

7. Proceed immediately to add the next reagent after each wash step. Do not allow the microwells to dry out.

8. Storage

The recommended storage condition for this kit and its components is 2–8°C. Allow approximately 60 min for kit reagents and components to reach room temperature (15–30°C) prior to beginning the assay. Return reagents to their labeled storage condition immediately after use.

9. HIV-1 Urine EIA Procedure

Caution: Proper reagent preparation is critical. Refer to Reagent Preparation section as appropriate throughout this procedure. Once the test has been started, each step must be performed without delay according to the package insert.

1. Allow approximately 60 min for the kit reagents to reach room temperature (15–30°C) prior to use. Return reagents to their labeled storage condition immediately after use.

2. Prepare the 96-well plate for use according to Reagent Preparation (Step 1).

3. Prepare 1× wash solution according to reagent preparations (Step 2).

4. Identify well positions for each specimen on a date sheet, plate map or by an automated bar-code identification system. Each plate, or partial plate of test specimens must include 2 positive control wells and 3 negative control wells.

5. Gently mix the bottle of sample buffer by inverting 3–5 times. Only remove as much sample buffer as required for immediate testing process. Add 25 µL of sample buffer to each coated microwell that will contain a specimen or control. Do not return unused portion to the bottle.

6. Add 200 µL of each specimen or control to the bottom of each microwell according to the positions identified in Step 4. All microwell containing controls and test specimens must be subjected to the same process and incubating conditions. Use a separate pipette tip for each specimen or each different control, to minimize the potential for cross-contamination.

7. Cover each plate securely with a plate sealer and ensure that the edges are completely sealed.

8. Incubate the plates at 37C ± 1°C for 60 min ± 5 min.

9. Prepare conjugate solution according to reagent preparation (step 3).

10. Washing Procedure:

    a. At the end of the incubation, carefully remove the plate sealer, avoiding splashing, and discard it in an appropriate waste receptacle.
    b. Completely aspirate the liquid from the microwells by using a plate washer or hand-held aspirator connected to a vacuum source.
    c. Fill the microwells with 1X wash solution (approximately 350 µL) and immediately aspirate.
    d. Repeat step 10 an additional 5 times for a total of 6 washes.
    e. Grasp the microwell plate firmly by the long sides in order to secure the strips firmly in the plate frame. Blot the plate by inverting the plate vigorously tapping it on a clean absorbent towel.

11. Dispense 100 µl of conjugate solution into each coated microwell which received a specimen or control.

12. Cover each plate securely with a plate sealer and ensure that the edges are completely sealed.

13. Incubate at 37 ± 1°C for 60 minutes ± 2 min.

14. Prepare substrate solution according to Reagent Preparation (Step 4) as soon as the conjugate incubation has started. Ensure that the tablets have completely dissolved before using the solution.

15. Perform a wash procedure as in Step 10.

16. Dispense 100 µL of substrate solution into each microwell which received a specimen or control. Do not use the substrate solution if it had developed a yellow color or if the tablets have not completely dissolved.

17. Cover each plate securely with a plate sealer and ensure that the edges are completely sealed.

18. Incubate at 37 ± 1°C for 30 min ± 2 min.

19. At the end of the incubation, carefully remove the plate sealer, avoiding splashing, and discard it in an appropriate waste receptacle.

20. Add 50 µL of stop solution to each microwell which received a specimen or control.

21. Blank the microwell reader on air and read the plate at 405 nm. If using a dual filter instrument, the recommended reference wavelength is 630 nm. Plates should be read within 30 min of adding stop solution.

22. Read and record the absorbance of all wells.

Calculation of Results:

The presence or absence of antibodies to HIV-1 is determined by comparing the absorbance value of the specimen to the cutoff value. The cutoff value is determined by adding 0.180 to the mean absorbance of the three negative controls.

Negative Control Values

1. The individual negative control absorbance values must less than or equal to 0.200. One negative control absorbance value may be discarded if it is outside this range. The mean may be calculated from the two remaining values. If two or more negative control values are greater than 0.200, the assay is invalid and must be repeated.

2. Determine the mean of the negative controls as shown in the example below.

Negative Control

Sample Number	Absorbance	Total absorbance	= 0.291	= 0.097
1	0.113	3	3	
2	0.091			
3	0.087			
	0.291			

Positive Control Values

Each Positive Control must have an absorbance value in the range of 1.200 to 2.700. No Positive Control value may be discarded. If any Positive Control value is less than 1.200 or greater than 2.700 the assay must be repeated.

Cutoff Value

The cutoff value is 0.180 plus the mean absorbance of the Negative Controls.
Example: 0.180 + 0.097= 0.277

Interpretation of Results

Note:
There is reduced sensitivity and specificity with testing urine specimens compared with testing blood specimens.

1. Specimens with absorbance values less than the cutoff value are considered noncreative by the criteria of the Calypte HIV-1 Urine EIA and may be considered negative for antibody to HIV-1 and HIV-2. Further testing is not required.

2. Specimens with absorbance values equal to or greater than the cutoff value are considered initially reactive by the criteria of Calypte HIV-1 Urine but should be retested in duplicate before interpretation.

3. Initially reactive specimens that do not react in either of the duplicate repeat tests are considered negative for antibodies to HIV-1.

5. If either duplicative retest is reactive, the specimen is considered repeatedly reactive

   Prior to interpretation, supplemental testing must be performed on repeatedly reactive specimens using only the Cambridge Biotech HIV-1 Western Blot Kit (PN 98076). Refer to the Cambridge Biotech HIV-1 Western Blot Kit package insert for the testing requirements for urine specimens.

Limitations of the Procedure

Note: This test is less sensitive and specific than the test using blood.

1. False negative results occur more frequently when testing urine specimens compared with testing blood specimens. See Performance Characteristics for details. False negative results (the subject is infected, but the urine specimen test is negative) may be the result of antibody levels in urine which are below the sensitivity (lower limit of detection) of this procedure. This may occur, for example, during the early phase of infection, or when there is a disease condition in which urine can not be concentrated by the kidney.

2. False-positive results (i.e. the subject is not infected but the urine test is repeatedly EIA positive) occur more frequently when testing urine specimens compared to blood specimens. See Performance Characteristics sections for details. Supplemental testing of repeatedly reactive urine specimens should be performed using only the Cambridge HIV-1 Western Blot Kit before the HIV-1 status of an individual can be determined.

3. The Calypte HIV-1 Urine EIA procedure and the interpretation of results must be followed closely when testing for the presence of antibodies to HIV-1 from individuals. Data regarding the interpretation were derived from testing individual's urine specimens. Insufficient data are available to interpret tests performed on other body specimens or pooled urine. Testing of these specimens is not recommended.

4. Studies to determine the performance characteristics of the Calypte HIV-1 Urine EIA in subjects younger than 18 years of age have not been performed.

References

1. Bauer G. Johnson J, Lewis G, Gottfried T et al. Antibodies to HIV in urine of children of HIV-1 infected women. Lancet. 1992:340;559.

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3. Cao Y, Hosein B, Borkowsky W, Mirabile M et. al. Antibodies to Human Immunodeficiency Virus Type 1 in the urine specimens of HIV-1 seropositive individuals. AIDS Res. Hum. Retro. 1989:5;311-319

4. Connell, JA, Parry JV, Mortimer PP et. al. Preliminary report: Accurate assays for anti-HIV in urine. Lancet. 1990:335;1366-1369.

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