Laboratory Procedure Manual

Analyte: **HIV Antibody / HIV Western Blot Confirmatory Test**

Matrix: **Serum**

Method: **Bio-Rad Laboratories HIV-1/HIV-2 Peptide EIA and Calypte HIV-1 Western Blot Kit**

as performed by: *HIV Immunology and Diagnostics Branch*
Division of AIDS, STD and TB Laboratory Research
National Center for Infectious Diseases

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Public Release Data Set Information

This document details the Lab Protocol for NHANES 1999–2000 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label (and SI units)
lab03	LBDHI	HIV antibody test result

HIV-1/HIV-2

1. Summary of Test Principle and Clinical Relevance- EIA

The BioRad HIV-1/HIV-2 Peptide EIA is manufactured using synthetheic peptides derived from highly conserved, immunodominant regions of the enc (envelop) and pol (polymerase) gene products for both HIV-1and HIV-2.

During the assay, specimens are evaluated for the presence of HIV-1 and HiV-2 antibodies by interaction with the absorbed peptides in the wells. Specimens are diluted in specimen diluent and added to each well, and the plates are incubated and washed. If antibodies to either HIV-1 or HIV-2 are present, they bind to the adsorbed antigen and are not removed by washing. The working conjugate solution, peroxidase-labeled goat anti-human immunoglobulin is then added to the wells and binds with the antibody-antigen complex, if present. Unbound conjugate is removed by a wash step. Working chromogen solution is then added to the plate and allowed to incubate. A blue or blue-green color develops in proportion to the amount of antibody that has been bound to the antigen-coated plate. The enzyme reaction is stopped by the addition of acid, which results in a color change to yellow. The optical absorbance of controls and specimens is determined with a spectrophotometer with wavelength set at 450nm.

All repeatedly positive specimens are confirmed by Western blot (method at the end of this section)

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. 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 to 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 useed in the current test run should be sealed in the foil bag with desciccant and stored at 2-8°C.
i. Remove reagents from the refrigerator storage approximately 60 minutes 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. For the manual pipetting of controls and specimens, use individual pipette tips to eliminate carryover of samples.
k. Handle negative and positive controls in the same manner as patient specimens.
l. If a specimen is inadvertently not added to well, the assay result will read nonreactive.
m. Inadequate adherence to package insert instructions may result in erroneous or invalid results.
n. The BioRad Systems HIV- I /HIV-2 Peptide EIA performance is highly dependent upon incubation times and temperatures. Temperatures outside of the validated ranges may result in invalid assays. Incubation temperatures should be carefully monitored using calibrated thermometers, or equivalent.
o. Use only adequately calibrated equipment with this assay.
p. Use of dedicated equipment is recommended if equipment performance validations have not precluded the possibility of cross-contamination.

WARNINGS FOR USERS

WARNING for *in vitro* diagnostic users: FDA has licensed this test for use with serum and plasma specimens only. Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.

a. The HIV-1 and HrV-2 positive controls are heat-treated to inactivate viruses. However, handle all the reagents as though capable of transmitting infection. All tests should be conducted using the precautions recommended for bloodborne pathogens, as defined by OSHA regulations.

b. Do not pipette by mouth.

c. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.

d. Wear protective clothing and disposable gloves while handling, the kit reagents. Wash hands thoroughly after performing the test.

e. Handle Chromogen Reagent with care since DMSO is readily absorbed through the skin.

f. The Stopping Reagent is an acid. Wipe up spills immediately and flush the area with water. If the Stopping Reagent contacts the skin or eyes, flush with copious amounts of water and seek medical attention.

g. BIOLOGICAL SPILLS: Spills not containing acid should wiped thoroughly with an effective disinfectant. Disinfectants that can be used include (but are not limited to) a solution of 10% bleach (O.5% solution of sodium hypochlorite),70% ethanol, or 0.5% Wescodyne. Spills containing acid should be wiped dry. The area of the spill should be wiped with one of chemical disinfectants. Materials used to wipe up spills should be disposed of as biohazardous waste. Note: DO NOT PLACE SOLUTIONS CONTAINING BEACH IN THE AUTOCLAVE.

h. Dispose of all specimens and materials used to perform the test as though they contain an infectious agent. Disposal should comply with all applicable waste disposal requirements.

i. Sodium azide is included as a preservative in the positive and negative controls. Sodium azide has been reported to form lead or copper azide in laboratory plumbing. These azides are explosive. To prevent azide build-up, flush plumbing, with a large volume of water if solutions containing azide are disposed of in the sink after biological inactivation.

3. **Computerization; Data System Management**

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. Serum or plasma may be used in the test.
b. The following anticoagulants have all been evaluated and found to be acceptable: EDTA, heparin, sodium citrate, CPD, CPDA 1, and ACD. Specimens which are collected into anticoagulant tubes should completely fill the tube as label indicates to avoid improper dilution. Specimens with observable particulate matter should be cleared by centrifugation prior to testing.
c. No clinically significant effect on assay results has been detected with increased levels of protein,

lipids, bilirubin, hemolysis, or microbiological contaminants, or after heat inactivation of patient samples.

d. Specimens may be stored at 2–8°C for days. For long, term storage, the specimens should be frozen (at 20°C or colder). Samples should not be used if they have incurred more than 5 freeze-thaw cycles. Mix samples thoroughly after thawing.

e. If specimens are to be shipped, they should be packed in compliance with Federal Regulations covering the transportation of etiologic agents. Studies have demonstrated that specimens may be shipped refrigerated (2–-8°C) or at ambient temperature for up to days. For shipments that are in transit for more than 7days, specimens should be kept frozen (20°C or lower).

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) Working Conjugate Solution

Bring conjugate diluent to room temperature. Invert diluent and conjugate concentrate to mix before using. Prepare a 1:101 dilution for each strip to be tested by adding 10 µl of conjugate concentrate to I ml of conjugate diluent in clean container. Note: Concentrate lot number, date and time of preparation and time of expiration of the working conjugate solution. Mix working solution prior to use. Working solution is stable for 8 hours at room temperature.

Return conjugate-concentrate to the refrigerator immediately after use. To avoid contamination of Conjugate, wear clean gloves and do not touch tips of pipettes. Store working, conjugate solution at room temperature until use. Avoid prolonged exposure to light. Do not add all the concentrate to diluent. Prepare only the amount of reagent to be used within 8 hours**,** ensuring that the volume of diluted reagent will be adequate for the entire plate(s).

(2) Working Chromagen Solution

Bring chromogen reagent and chromogen diluent to room temperature. Invert the chromogen reagent and chromogen diluent to mix before using, Prepare a 1:101 dilution for each strip to be tested by adding l0 ml of chromogen reagent to i ml of chromogen diluent in a clean polypropylene container. (DO NOT USE A POLYSTYRENE CONTAINER). Note chromogen reagent lot number, date and time of preparation and time of expiration of the working chromogen solution. Mix working solution gently when combined. Working, chromogen solution should be kept in the dark at room temperature prior to use. If solution remains crystalline after warming, do not use. Chromogen reagent should be colorless to very pale yellow. Any other color indicates that the reagent is contaminated and should not be used. The working chromogen solution should be colorless. A distinct blue color indicates that the reagent is contaminated. Discard the working chromogen solution and prepare fresh reagent in a clean container. Prepare only the amount of the reagent to be used within 8 hours, ensuring that the volume of diluted reagent will be adequate for the entire plate(s). Extra chromogen reagent is provided.

(3) Working Wash Solution

Prepare working wash solution as needed by adding one part wash solution concentrate (30×) to 29 parts of water (e.g. 120 ml of wash solution to 3480 ml of water). Use deionized or distilled water. The working wash solution can be stored at room temperature for four weeks. Note lot number, date prepared and expiration date. Discard if no foaming is evident in the working- wash solution. Prepare a sufficient quantity of working wash solution to complete a fell plate run.

(4) Preparation of Quality Control Materials

Determine the mean absorbance for the negative and positive controls by dividing the sum of their absorbance values by the number of acceptable controls.

Mean Negative Control absorbance value (NCx)

The individual negative control absorbance values must be greater than equal to 0.020 AU and less than or equal to 0. 140 AU. One negative control absorbance value may be discarded if it is outside this range. The NCx may be calculated from the two remaining values.

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

HIV-1 Negative Control (HIV-1 NCx)

Sample Number	Absorbance
1	0.095
2	0.110
3	0.102
	0.307

Total absorbance = 0.307 = 0.102 (HIV-1 NCx)
3 3

Mean HIV-1 Positive Control absorbance value (HIV-1 PCx)
Determine the mean of the HIV-1 positive control as shown in the example below.

HIV-1 Positive Control (HIV-1 PCx)

Sample Number	Absorbance
1	1.435
2	1.501
	2.936

Total absorbance = 2.936 = 1.468 (HIV-1PCx)
2 2

The HIV-1 PCx must be greater than or equal to 0.900 AU and each positive control absorbance value must be within the reproducibility range of 0.65 to 1.35 times the PCx. No positive control absorbance values may be discarded. Both the HIV-1 positive control absorbance values are within the reproducibility range of 0.65 to 1.35 times the PCx , as shown by the following calculation:

0.65 x {HIV-1 PCx} = 0.65 x 1.468 = 0.954
1.35 x {HIV-1 PCx} = 1.35 x 1.468 = 1.092

Therefore the acceptable range is 0.954–1.982.

Mean HIV-2 Positive Control absorbance value (HIV-2 PCx)
Determine the mean of the HIV-2 positive control as shown in the example below.

HIV-2 Positive Control (HIV-2 PCx)

Sample Number	Absorbance
1	1.070
2	1.123
	2.201

Total absorbance = 2.201 = 1.101 (HIV-2 PCx)
2 2

The HIV- 2 PCX must be greater than or equal to 0.700 AU, and each positive control absorbance value must be within the reproducibility range of 0.65–I.35 times the PCX. No positive control absorbance value above are within the reproducibly range of 0.65 to 1.35 times the PCX. No positive control absorbance values may be discarded.

Both of the HIV-2 positive control absorbance values above are within the reproducibility range of 0.65 to 1.35 times the PCX as shown by the following calculation:

0.65 x (HFV-2 PCX) = 0.65 x 1.I01 = 0716
1.35 x (HIV-2 PCX) = 1.35 x 1.101 = 1.486

Therefore, the acceptable range is 0.716–1.486

Cut-off value:
Determine the cutoff by adding 0.240 to NCx, as shown in the example below:

NCx = 0.102
Cuttoff Value = 0.102 + 0.240 = 0.342

Validity Criteria
A run is valid if the following criteria are met:

- The absorbance value of each negative control is greater than or equal to 0.020 AU and less than or equal to 0.140 AU. One negative control value may be discarded, and the mean of negative controls (NCx) may be calculated from the two remaining values. If two or more negative controls are out of limit, the plate is invalid and must be repeated.

- The mean absorbance of the HIV-1 positive control is equal to or greater than 0.900 AU, and the individual absorbance values are within the reproducibility range of 0.65–1.35 times the HIV-2 positive control mean. No HIV-2 positive control values may be discarded. If the HIV-2 positive control mean is less than 0.700 AU, the plate is invalid and must be repeated.

d. Other Materials

Materials required but not provided:

(1) Precision pipettes to deliver 0–20 µl, 20–200 µl, 1 ml, 5 ml and 10 ml (accurate within +10%) or automated pipettor-dilutor; appropriately sized graduated cylinders.

(2) Pipette tips Dry heat incubator capable of maintaining 37 + 1°C.

(3) Dry heat incubator capable of maintaining 37 + 1°C

(4) Calibrated thermometer

(5) Biorad systems microwell plate or strip washer or an equivalent. The washer must be capable of dispensing at least 350 µl per well and cycling 5 times.

(6) Biorad systems microwell plate or strip reader or an equivalent. The spectrophotometer should have the following specifications at wavelength 450 nm:

Bandwidth: 10 nm HBW (half band width) or equivalent

Absorbance range: 0–2.0 AU (absorbance units)

Repeatability: + (0.5% sodium hypochlorite)

Linearity or accuracy: 1% from 0 to 2.0 AU

The instrument should contain a reference filter for reading at 615–630 nm. An instrument without a reference filter can be used: however areas in the bottom of the wells that are opaque, scratched or irregular may cause absorbance readings that are falsely elevated.

(7) Household bleach (5% to 8% sodium hypochlorite). Alternative disinfectants include: 70% ethanol or 0.5% Wescodyne (West Chemical Products).

(8) Paper towels or absorbent pads for blotting.

(9) Null strips, for testing partial plates.

(10) Clean polypropylene container for preparation of working chromogen solution. (Do not use polystyrene) Clean container for preparation of working conjugate solution.

(11) Deionized or distilled water. Clinical laboratory reagent water is acceptable. Store the water in nonmetallic containers.

(12) Gloves

(13) Laboratory timer

(14) EIA regent reservoirs (optional)

e. Instrumentation

(1) See number 6 above.

7. Calibration and Calibration Verification Procedures

1. Perform equipment maintenance and calibration, where necessary, as required by the manufacture.

2. Bring all of the reagents except the HIV-1/HIV-2 peptide EIA conjugate concentrate to room temperature before beginning the assay procedure.

3. Prepare working wash solution and working chromogen solution. See reagent preparation section. Mix gently prior to use.

4. Remove any strips from the microwell plate(s) not needed for the assay run and replace with null strips, if necessary.

5. If sample identity is not maintained by an automatic procedure, label or identify the individual wells for each specimen or control on a data sheet.

6. Dilute specimens 1:10 in the specimen diluent (for example, dilute 15 µl of specimen in 135 µl of specimen diluent). When pipetting manually use a separate disposable pipette tip for each specimen. Two separate dilutions of both HIV-1 and HIV-2 positive controls and three separate dilutions of negative control should be assayed with each plate or partial plate of specimens. Mix each diluted specimen and control thoroughly. Mix to avoid foaming of the diluent. All microwell plates containing controls and specimens must be subjected to the same process and incubation times.

7. Add 100 µl of the diluted serum or plasma or control to the appropriate well or if doing in well dilutions, combine 10 µl of specimen or control with 900 µl of specimen diluent.

8. Cover the micro-well plate with a plate sealer or use other means to minimize evaporation and incubate the plate for 30–33 minutes at 37 + 1°C.

9. At the end of the incubation period, carefully remove the plate cover and aspirate the fluid in each well into a biohazard container. Wash the microwell plate or strip a minimum of five times with the wash solution (at least 350 µl/well/wash). Aspirate the wash solution after each wash. After the wash, aspirate the liquid completely or blot the inverted plate on clean absorbent paper towels, if necessary. Note: grasp the plate holder firmly at the center of the long sides before inverting to blot.

10. Add 100 µl of working conjugate solution to each well.

11. Cover the microwell plate with a fresh plate sealer or use other means to minimize evaporation and incubate the plate for 30–33 minutes at 37 + 1°C.

12. At the end of the incubation period, carefully remove the plate cover and a aspirate the fluid in each well into a biohazard container. Wash the microwell plate or strip a minimum of five times with the wash solution (at least 350 µl/well/wash). Aspirate the wash solution after each wash. After the last

wash, aspirate the liquid completely or blot the inverted plate on clean, absorbent paper towels. Note: grasp the plate holder firmly at the center of the long sides before inverting the blot.

13. Add 100 µl of the working chromogen solution per well. Cover the microwell plate with fresh plate sealer or use other means to minimize evaporation. Incubate plates in the dark for 30–33 minutes at room temperature (15–30°C).

14. Carefully remove the plate cover and add 100 µl of stopping reagent to each well to terminate the reaction. Tap the plate gently, or use other means to assure complete mixing.

15. Read absorbance within 30 min after reading the stopping reagent, using 450 nm filter with 615- to 630-nm filter as the reference (blank on air). Ensure that all strips are firmly into place before reading.

Decontamination
Dispose of all specimens and materials used to perform the test as though they contain an infectious agent.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

The presence or absorbance of antibodies to HIV-1 and or HiV-2 is determined by relating the absorbance value of the specimens to the cutoff value. The cutoff value is determined by adding 0.240 to the mean absorbance value of the negative controls.

1. Specimens with absorbance values less than the cutoff value are considered nonreactive by the Biorad systems HIV-1/HIV-2 Peptide EIA and may be considered negative for antibody to HIV-1 and HIV-2. Further testing is not required.

2. An absorbance value of less than 0.000 AU may indicate a procedural or instrument error which should be evaluated. That result is invalid and that specimen must be re-run.

3. Specimens with absorbance values equal to or greater than the cutoff value are considered initially reactive to the Biorad Systems HIV-1/HIV-2 peptide EIA should be retested in duplicate before interpretation. When tube dilutions are used to mix the specimen with specimen diluent, prepare a new dilution of the specimen for retesting. If after repeat testing, the absorbance of either or both duplicate specimens with values greater that the upper linearity limits of the reader should be reported as negative.

4. Initially reactive specimens that do not react in either of the duplicate repeat tests are considered negative for antibodies to HIV-1 and HIV-2.

5. If the specimen is repeatedly reactive, the probability that antibodies to HIV-1 and or / HIV-2 are present is high, especially for specimens obtained from subjects at increased risk for HIV-1/HIV-2 infection or for specimens with very high absorbance values. In most settings, it is appropriate to investigate repeatedly reactive specimens by additional more specific or supplemental tests, such as Western blot or immunofluorescence. Specimens that are repeatedly reactive by the Biorad Systems HIV-1/HIV-2 Peptide EIA and are found to be positive for antibodies to HIV-1 by additional, more specific or supplemental testing but negative or indeterminate for antibodies to HIV-2 are considered positive for antibodies to HIV-1.

6. Specimens that are repeatedly reactive by the Biorad Systems HIV-1/HIV-2 Peptide EIA and are found to be positive for antibodies to HIV-2 by additional, more specific or supplemental testing but negative or indeterminate for antibodies to HIV-1 are considered positive for antibodies to HIV-2

7. Specimens that are repeatedly reactive by the Biorad Systems HIV-1/HIV-2 Peptide EIA and are found to be positive for antibodies to HIV 1 and HIV-2 by additional, more specific or supplemental testing may contain antibodies that cross-react with both virus types, or may be indicative of a dual infection with both HIV-1 and HIV-2.

8. The interpretation of results of specimens found to be repeatedly reactive by Biorad systems HIV-1/HIV-2 Peptide EIA and negative or indeterminate on additional, more specific testing for antibodies to both HIV-1 and HIV-2 is unclear. Clarification may sometimes be obtained by testing another specimen taken three to six months later.

9. Limitations of the Procedure

1. The Biorad HIV-1 and HIV-2 Peptide EIA procedure and the interpretation of Results must be followed closely when testing for the presence of antibodies to HIV-1 and/or HIV-2 in plasma or serum. The user of the kit is advised to read the package insert carefully prior to conducting the test. In particular, the test procedure must be carefully followed for sample and reagent pipetting, plate washing, and time temperature of the incubation steps. Data regarding the interpretation were derived from testing serum or plasma samples. Insufficient data are available to interpret test performed on other body specimens, pooled blood or processed plasma, and products made from such pools: testing of these specimens is not recommended.

2. The Biorad Systems HIV-1/Hiv-2 Peptide EIA detects circulating antibodies to HIV-1 and HIV-2 and thus is useful in screening blood and plasma donated for transfusion and further manufacture, in evaluating patients with signs or symptoms of AIDS, and in establishing prior infection with HIV-1 or HIV-2. Clinical studies continue to clarify and refine the interpretation and medical significance of the presence of antibodies to HIV-1/2. Repeatedly reactive specimens must be investigated by additional tests.

3. A negative test result at any point in the investigation of individual subjects does not preclude the possibility of exposure to or infection with HIV-1/2.

4. False negative results can occur if the quantity of the marker present in the sample is too low for the detection limits of the assay, or if the maker which is detected is not present during the stage of disease in which a sample is collected.

5. Failure to add specimen or reagent as instructed in the procedure could result in a falsely negative test. Repeat testing should be considered where there is clinical suspicion of infection or procedural error.

6. Data obtained from testing persons both at increased and at low risk for HIV-1/2 infection suggest that repeatedly reactive specimens with high reactivity on the Biorad Systems HIV-1/2 Peptide EIA may be more likely to demonstrate the presence of antibodies to HIV-1/2 by additional, more specific or supplemental testing. Borderline reactivity is more frequently nonspecific, especially in samples obtained from persons at low risk for infection with HIV-2 or HIV-2, however the presence of antibodies to HIV-1/2 in some of these specimens can be demonstrated by additional, more specific or supplemental testing, or by testing a subsequent sample drawn at a later date (eg. 3 or 6 months).

7. An absorbance value of less than 0.000 AU may indicate a procedural or instrument error which should be evaluated. The result is invalid and the specimen must be e-run

8. Factors that can affect the validity of results include failure to add the specimen to the well, inadequate washing of microplate wells, the presence of metals, or the splashing of bleach into wells.

9. Non-repeatedly reactive specimens can be caused by: Improper washing of microplate wells, during the initial test; cross-contamination of non-reactive specimens with HIV antibody from a high-titered specimen; contamination of the chromogen reagent solution by oxidizing agents (sodium hypochlorite, hydrogen peroxide, etc.) ; contamination of the stopping reagent.

10. Summary Statistics and QC graphs

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

References

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2. Delmonico FL, Snydman DR. Organ donor screening fro infectious diseases. Transplantation. 1998:65 (5):603-610.

3. Schumaacher RT, Garret PE, Tegtmeier GE, Thomad D. Comparative detection of anti-HIV in early HIV seroconversion. J Clin Immunoassay. 1988:11;130-134.

4. Gnann JW, McCormick, Mitchell S, Nelson J, Oldstone MBA: Synthetic peptide immunoassay distinguishes HIV type 1 and HIV type 2 infections. Science. 1987:237;1346-1349.

HIV-1 Western Blot

1. Summary of Test Principle and Clinical Relevance- Western Blot

The enzyme-linked immunosorbent blot technique (Western Blot) has been used to select antibodies to HIV-1 recognized as the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS). The combination of electrophoretic separation of complex mixtures of antigens with the highly sensitive immunoblotting technique has been useful in characterizing the antigenic profile of HIV-1 and describing the immune response to this virus in exposed or infected persons. Separate kits are used for urine or serum and plasma but the principles apply to both kits.

The Calypte HIV-1 Western Blot Kit, is manufactured by Calypte Corporation from HIV-1 propagated in an H9/HTLV-IIIb T-Lymphocyte cell line. The partially purified virus is inactivated by treatment with psoralen and ultraviolet light, and detergent disruption. Specific HIV-1 proteins are fractionated according to molecular weight by electrophoresis on a polyacrylamide slab gel in the presence of sodium dodecysulfate (SDS). The separated HIV-1 proteins are electrotransferred from gel to nitrocellulose membrane which is then washed, blocked (to minimize nonspecific immunoglobulin binding), and packaged. Individual nitrocellulose strips are incubated with serum or plasma specimens, or controls. During incubation, if HIV-2 antibodies are present in the specimen, they will bind to the viral antigens bound to the nitrocellulose strips. The strips are washed again to remove unbound material. Visualization of the human immunoglobulin specifically bound to HIV-1 proteins is accomplished in situ using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with horseradish peroxidase (HRP), and the HRP substrate 4-chloro-1-naphthol. If antibodies to any of the major HIV-1 antigens are present in the specimen in sufficient concentration, bands corresponding to the position of one or more of the following HIV-1 proteins (p) or glycoprotiens (gp) will be seen in the nitrocellulose strip: p17, p24, p31, gp41, p51, p55, p66, gp120, gp160 (number refers to apparent molecular weight in kilodaltons).

2. Precautions

a. Handle assay specimens, strips and reactive and non-reactive controls as if capable of transmitting an infectious agent. Inactivated HIV-1 antigen has been electrophoresed and transferred onto nitrocellulose. Weakly and strongly reactive controls have been inactivated by heat treatment. In addition, plasma used to produce the controls was shown to be non-reactive for hepatitis B surface antigen. However, no known test method can offer assurance that products derived from human blood will not transmit infectious agents. Therefore, thses components must be handled as if they are capable of transmitting infectious agents.
b. Do not pipette by mouth.
c. Wear disposable gloves throughout the test procedure. Dispose of gloves as biohazard waste. Thoroughly wash hands after handling tests.
d. Wipe spills promptly with a 1% sodium hypochlorite solution (1:5 dilution of liquid household bleach) Contaminated materials should be disposed of as biohazard waste.
e. Dispose of all specimens and materials in the Calypte HIV-1 Western Blot Kit procedure as biologic waste. The recommended of disposal is autoclaving for a minimum of 1 hour at 121 degrees C. Disposable materials may be incinerated. Mix liquid wastes with an equal volume of 5% sodium hypochlorite solution allowing at least 60 minutes for disinfection.
f. Do not permit substrate, especially 4-chloro-1-naphthol to contact the skin. If contact occurs, flush with water.
g. The controls contain sodium azide as a preservative. If these materials, either concentrated or diluted, are to be disposed of through a sink or other common plumbing systems, flush with generous amounts of water to prevent accumulation of potentially explosive compounds.
h. Avoid use of metal instruments in contact with substrate B and working substrate solution since metals can cause reduction in H2O2.

WARNING: FDA has licensed this test for use with serum and plasma specimens only (separate kit for urine). Use of this licensed test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.

a. Do not interchange reagents between kit lots.

b. Do not use kit beyond its expiration date. The date is printed on kit boxes

c. Avoid contamination of reagents, when opening and withdrawing aliquiots from the primary vials. Keep all reagents refrigerated (2-8C) when not in use

d. Do not interchange vial or bottle caps or stoppers; this will lead to cross contamination of reagents. Designate specific reservoirs for specific reagents.

e. Grossly contaminated specimens or strips may result in the development of dark spots on the strip which could not be interpreted. Careful attention must be given to the storage of specimens and kits to prevent this problem.

f. Shield working substrate solution from sunlight during preparation and use within 30 minutes of mixing.

g. Use reagent grade water (deionized water which is free of bacteria) to dilute reagents in order to avoid substances which may interfere with the assay.

h. Do not remove nitrocellulose strips from the storage tube until immediately before use. To prevent moisture from condensing inside the strip tube, open only after the strips have reached room temperature (approximately 30 minutes). Close the tube immediately after removing strips for use.

i. Allow all kit reagents and materials to reach room temperature before use (approximately 30 minutes).

j. Use only the controls supplied in the kit.

k. Do not cut strips. Narrower strips can lead to misinterpretation because strips may flip-over in the incubation tray, or artifacts in the reaction zones may be mistaken for possible bands or may prevent recognition of positive bands**.**

l. Measure all reagents. Use extreme care and calibrated pipettors with good quality tips when preparing working conjugate solutions**.**

3. **Computerization; Data System Management**

HIV western blot 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. The Calypte HIV-1 Western Blot kit may be used with human serum or plasma (separate kit for urine). Reliability of test results with grossly lipemic, hemolyzed or cloudy specimens is not known.

b. Specimens may be stored at 2–8°C for up to two weeks. For longer intervals, the specimens should be frozen (at –18°C or colder).

c. Avoid multiple freeze/thaw cycles. Mix samples thoroughly after thawing.

d. If specimens are to be shipped, they should be packed in compliance with Federal Regulations covering

the transportation of etiologic agents.

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. Reagents

(1) Nitrocellulose strips
Each nitrocellulose strip contain separated, bound antigenic proteins from partially purified inactivated HIV-1, insufficient quantity to detect human antibodies. Bovine protein is present as a blocking agent. Strips are consecutively numbered (1 through 27).

(2) Non-Reactive Control
Normal serum non-reactive for HIV-1 antibodies and hepatitis B surface antigen. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

(3) Strongly Reactive Control
Inactivated human serum containing a high titer of antibodies to HIV-1 antigens. Non-reactive for HBsAg. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

(4) Weakly Reactive Control
Inactivated human serum containing a low titer of antibodies to HIV-1 antigens. Non-reactive for HBsAg. Contains 0.1% sodium azide and 0.005% thimerosal as preservatives.

(5) Wash Buffer
Supplied as a 20X concentrate. When diluted contains 0.02M tris, 0.1 M NaCl, 0.3% Tween 20 , and 0.005% thimerosal as preservative at pH 7.4.

(6) Blotting Buffer
Supplied as a 10X concentrate. When diluted contains 0.02 M tris, 0.1 M NaCl, heat inactivated normal goat serum , and 0.01% thimerosal as preservative at ph 7.4.

(7) Conjugate 1
Biotinulated Goat anti-human IgG (heavy and light chain) antibodies. Contains 0.002% thimerosal as a preservative.

(8) Conjugate 2
Avidin conjugated horseradish peroxidase. Contains 0.01% thimerosal as a preservative.

(9) Substrate A
7.8 mM solution of 40 chloro-I-naphthol in an alcohol solution.

(10) Substrate B
Aqueous hydrogen peroxide solution (0.02%) in citrate buffer.

(11) Blotting power
Nonfat dry milk

Note: Allow reagents to reach room temperature before use (approximately 30 minutes).

b. Reagent Preparation

1. Diluted Wash Buffer
    a. Dilute 1 volume of wash buffer (20X) with 19 volumes reagent grade water. Mix well.
    b. Dilute wash buffer may be stored at room temperature for 3 months.
2. Working Blotting Buffer
    a. Working blotting buffer should be prepared fresh prior to use.
    b. Dilute 1 volume blotting buffer (10x) with 9 volumes of reagent grade water. Mix well
    c. Use 1.0 g of Blotting power per 20 ml of the diluted blotting buffer prepared in step 2B above. Mix thoroughly to dissolve the power. If the entire kit is to be used within five days, add 9.0 g to 180 ml of diluted Blotting buffer. Store at 2-8 degrees C.
3. Working conjugate 1 solution
    a. Refer to the supplemental instruction sheet for the dilution appropriate for the conjugate lot supplied with the kit.
    b. Working conjugate 1 solution should be prepared fresh prior to use.
4. Working conjugate 2 solution
    a. Refer to the supplemental instructions sheet for the dilution appropriate for the conjugate lot supplied with the kit.
    b. Working conjugate 1 solution should be prepared fresh prior to use.
5. Working substrate solution
    a. Working substrate solution should be prepared fresh prior to use.
    b. Prepare working substrate solution by mixing equal volumes of substrate A and substrate B. Mix well.

Reagents required (in nLs) for Various Number of strips.

	1	2	6	9	15	20	27
Diluted Wash Buffer	20.0	60.0	120.0	180.0	300.0	400.0	540.0
Blotting power	6.0g	0.9 g	1.8g	2.7g	4.5g	6.0g	8.1g
Working Blotting buffer	6.0	18.0	36.0	54.0	90.0	120.0	162.0
Working conjugate1**	2.0	6.0	12.0	18.0	30.0	40.0	54.0
Working conjugate 2**	2.0	6.0	12.0	18.0	30.0	40.0	54.0
Substrate A	1.0	3.0	6.0	9.0	15.0	20.0	27.0
Substrate B	1.0	3.0	6.0	9.0	15.0	20.0	27.0

- Minimum volumes. Prepare a slight excess of each solution to compensate for loss during pipetting.
- ** See supplemental Instructions sheet for dilution calculation

Storage Instructions

1. Store Cambridge Biotech HIV – 1 Western Blot Kits and /or individual reagent at 2-8 C.
2. Unused Nitrocellulose strips should be kept dry and in the dark, in their storage tube at 2-8 degrees C

Indications of Instability or deterioration of reagents

Changes in the physical appearance of the reagents supplied may indicate instability of deterioration of these materials. Substrate A should be colorless. If substrate A shows a color it has become oxidized and should not be used.

Materials provided

Each Calypte HIV-1 Western Blot Kit contains:

Nitrocellulose Strips	27 strips
Non-Reactive Control	1 vial (green) 160uL/vial
Weakly Reactive Control	1 vial (lavender 160uL/vial

Strongly Reactive Control	1 vial (red) 160uL/vial
Wash Buffer (20x)	1 Bottle (60mL/bottle)
Blotting Buffer (10x)	1 Bottle (18 mL/bottle
Conjugate 1	1 vial (Blue) 160uL/vial
Conjugate 2	1 vial (Black) 160uL/vial
Substrate A	1 Bottle (30 mL/bottle)
Substrate B	1 Bottle (30 mL/bottle)
Blotting Power	1 Package (9.0+ g, minimum)
Incubation Trays	3 Trays (9 well trays)

Materials Required – Not provided

Rocker or rotary platform
Pipettors and tips
Tweezers and forceps
20 to 30 well incubation tray (in lieu of the small trays provided)

7. Quality Control

The non-reactive and weakly reactive controls must be included with each run, regardless of the number of specimens tested or nitrocellulose strips used. The strongly reactive control is used to establish criteria for reactivity of bands and is to be included with the first run of specimens for each kit. The strongly reactive control need not be included in subsequent runs unless the strip is misplaces or faded

In order for the results obtained from any run of specimens be considered to be valid, the following criteria must be met:

1. Non-reactive control: No bands should be visible on the nitrocellulose strip used to test the non-reactive control.

2. Strongly reactive control: All relevant molecular weight bands must be visible on the nitrocellulose strip used to test the strongly reactive control. These bands are p17, p24, p31, gp41, p51, and gp160. A gp 20 band may also be seen but is not a requirement for acceptable performance.

3. Weakly reactive control: The nitrocellulose strip used to test the weakly reactive control provides a measure of the sensitivity of the Cambridge Biotech HIV-1 Western Blot Kit and must exhibit bands at p24 and gp160. Additional weak bands may appear but are not required to demonstrate acceptable performance.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

Caution: When handling the incubation tray supplied with the kits, take care not to splash or mix specimens. Remove the lid carefully to prevent moisture which may condense on the lid from falling into the tray. Do not handle samples or sample loaded pipette tips over uncovered incubation trays. Splashing or aerosols may lead to cross-contamination of sample wells.

1. Bring all reagents to room temperature prior to use (approximately 30 minutes).

2. Add 2.0 ml of diluted wash buffer to each well to be used.

3. Using forceps, carefully remove a nitrocellulose strip from the vial and place numbered side up into a well containing diluted wash buffer.

4. Place the tray on a rocker or rotary platform for 5 to 10 minutes at room temperature, then remove the buffer by aspiration.

5. Add 2.0 ml of working blotting buffer to each well.

6. Add 20 uL of each undiluted specimen or control to a well containing its assigned strip in working blotting buffer. Caution: use a different pipette tip for each sample.
7. Cover the tray and incubate on the rocker or rotary platform overnight (14-20 hours) at room temperature (20-28 degrees C).

8. Carefully uncover the tray to avoid splashing or mixing specimens. Remove condensation or droplets on the incubation tray lid by rinsing with diluted wash buffer or wiping with absorbent towels.

9. Aspirate the mixture from the wells into a trap containing disinfectant. Rinse aspirator tip with diluted wash buffer or deionized water between samples to avoid cross contamination.

10. To each strip, add 2.0 ml of diluted wash buffer and rock by hand several times. Remove buffer by aspiration.

11. Add 2.0 mL of diluted wash buffer to each strip for a minimum of 5 minutes. Aspirate the wash buffer between washes. Repeat a second time. Perform all wash steps at room temperature on a rocking rotary platform.

12. Add 2.0 mL of working conjugate 1 solution (prepared as directed in supplemental instructions) to each well. Incubate for 60 minutes at room temperature on the rocker or rotary platform.

13. Aspirate the conjugate from the wells. Wash each strip three times for 5 minutes as in Step 11.

14. Add 2.0 mL of working conjugate 2 solution prepared as directed in supplemental instructions) to each well. Incubate for 60 minutes at room temperature on the rocker or rotary platform.

15. Aspirate the conjugate from the wells. Wash each strip three times for 5 minutes as in Step 11.

16. Add 2.0 mL of the working substrate solution to each well and incubate at room temperature on the rocker or rotary platform for 10 to 15 minutes (or until weak positives exhibits p24 and gp 160 bands)

17. Aspirate the substrate and stop the reaction by rinsing the strips several times with distilled or deionized water.

Note: Some specimens may cause spots to form on the strip due to precipitation. A cotton swab dipped in reagent grade water can be used to carefully remove the spots and allow for better visualization of results.

Air dry the strips between absorbent paper towels and score as directed in the Interpretation of Results section. For best results and consistency, strips should be scored soon after drying. When mounting with tape, do not tape over developed bands. This will cause bands to fade.

18. If desired, the strips may be photographed using high resolution film. Developed strips will retain their color if stored in the dark. Exposure to light and air will eventually cause bands to fade.

9. Interpretation of Results

The presence or absence of antibodies to HIV-1 in specimens and the identity of any antibodies present are determined by comparison of each nitrocellulose strip to the strips used for the non-reactive and weakly reactive controls tested with that run, and the strip used for the strongly reactive control tested once with the kit.

The interpretation process requires three steps. First, each band which appears on the test strip must be identified based on the strongly reactive control strip. Second, each band is assigned a reactivity score based on its intensity. Third, the strip is interpreted based on the combination of band pattern and reactivity.

The major HIV-1 gene products that have been identified are as follows:

gp 160 - precursor of ENV glycoprotein
gp 120 -outer ENV glycoprotein
p66 - reverse transcriptase component of POL translate.
p55 – precursor of GAG proteins
gp 41 – transmembrane ENV glycoprotein
p31 – endonuclease component of POL translate
p24 – GAG protein
p17 – GAG protein

Note : The gp 160 band may, in many cases, represent a multimer of gp41. However, the presence of gp120 has been verified using specific mono and polyclonal antibodies. The primary response of most env reactive antibody to Western blot is to the transmembrane part whether it is a tetramer or derived from the precursor.

Intensity of bands present on strips used to test specific specimens may be scored as follows:

Intensity of band	**Reactivity Score**
Absent	–
Less than the intensity of p24 on the weakly Reactive control strip	+/–
At least as intense as p24 on the weakly reactive control strip but less intense than p24 on the strongly reactive control strip	+
Greater than or equal to the intensity of p24 on the strongly reactive control strip	++

Using the strongly reactive control as a reference for position and the p24 band on the weakly reactive control strip as a reference for intensity, each band on a strip should be assigned a reactivity score. When analyzing test specimens, it is helpful to place the control strips side by side with unknown strips to facilitate the assignment of molecular weights and intensities of each band. The results of blotting is then interpreted as negative, indeterminate or positive based on the pattern which is present, according to the following table:

Pattern	Interpretation
No bands present	Negative
Any bands present but pattern does not meet criteria for positive	Indeterminate
Any two or more of the following bands present: p24, gp41 and gp120.160. Each band had a Reactivity score of + or greater. Commonly, the bands at gp 41 or gp 160 is diffuse. Other viral bands may or may not be present	Positive

The positive criteria follow the recommendations of the Centers for Disease Control and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD). These publications along with others have suggested that the additional requirement for p31 reactivity is unnecessary.

Clinical studies with the Calypte HIV-1 Western Blot Kit have indicated that it is inappropriate to assign a positive interpretation to strips which display bands but lack any two of p24, gp41, gp120/160 with a reactivity score of + or greater for each band present. It is known that persons who have recently seroconverted may display incomplete patterns but will develop increased reactivity (both numbers and intensity of bands) when followed for a period of for up to six months. Most blots with positive results will have other virus-specific bands present including p17, p31, p66, gp120.

Conversely, persons at low risk for infection may have nonspecific reactions on the blot particularly in regions corresponding to p17, p24, p55 and p66, which will persist but which do not evolve into more extensive patterns over time. Although nonspecific reactivity may sometimes be attributed to autoantibodies, it is possible that in some cases the pattern may represent cross reaction with another human retrovirus. Persons with HIV-1 infection may also present incomplete patterns due to the natural history of AIDS or other immunodeficiency states. In particular, it has been noted that AIDS patients lose antibody reactions to p24 and p31, and in particular, infants may fail to seroconvert. In addition, infants may test positive for HIV-1 due to passive transfer of maternal antibodies which may persist for several months. Also, infected patients with malignancies and patients receiving immunosuppressive drugs may fail to develop a positive pattern.

Since reactivity of any degree with any of the virus-specific proteins (i.e. p24, p31, p66/51 or gp41/130/160) identified on the strip is presumptive evidence of antibodies to HIV-1, any such result (interpreted as Indeterminate) must be taken as suspicious and should trigger repeat testing and follow-up testing. Indeterminate assay results must not be considered positive or negative. The correct evaluation in such situations must be based on the subsequent blot testing and clinical evaluation. In such cases, indeterminate blots may offer useful information.

10. Summary Statistics and QC graphs

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.