

Laboratory Procedure Manual

<i>Analyte:</i>	HIV-1/HIV-2 Antibody HIV-1 p24 Antigen Enzyme Immunoassay
<i>Matrix:</i>	Serum/Plasma
<i>Method:</i>	Bio-Rad Genetic Systems HIV Combo Ag/Ab EIA
<i>As performed by:</i>	<i>HIV Laboratory Branch Division of HIV/AIDS Prevention National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention</i>
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<i>First Published:</i>	<i>October 2007</i>
<i>Revised:</i>	<i>October 2014</i>

Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each protocol before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

File Name	Variable Name	SAS Label
HIV_I	LBXHIVC	HIV-1, 2 Combo Test

Genetic Systems Combo Ag/Ab EIA

1. Summary of Test Principle and Clinical Relevance- EIA

The **Genetic Systems Combo Ag/Ab EIA** marketed by BioRad is an enzyme immunoassay that simultaneously determines the presence of the human immunodeficiency virus (HIV) p24 antigen and antibodies to HIV Type 1 (HIV-1 groups M and O) and HIV Type 2 (HIV-2) in human serum or plasma. It is indicated as a screening test for serum or plasma specimens and as an aid in the diagnosis of infection with HIV-1 and/or HIV-2.

Summary and Explanation of the Test

Acquired immunodeficiency syndrome (AIDS) is caused by viruses transmitted by sexual contact, exposure to blood (including sharing contaminated needles and syringes) or certain blood products, or transmitted from an infected mother to her fetus or child during the perinatal period.¹ Additionally, transmission of these viruses can occur through tissue transplantation.² HIV-1 has been isolated from patients with AIDS and AIDS-related complex (ARC).³⁻⁵ HIV-1 was thought to be the sole causative agent of these syndromes until 1986, when a second type of HIV-2 was isolated and also reported to cause AIDS.⁶⁻⁷ Since the initial discovery, hundreds of cases of HIV-2 infection have been documented worldwide, including cases of AIDS related to HIV-2.⁸ In the United States, there have been more than 80 cases of infection with HIV-2 reported, including three potential blood donors.⁹⁻¹⁵

HIV-2 is similar to, but distinct from, HIV-1. Both viruses have similar morphology and lymphotropism¹⁶, and the modes of transmission appear to be identical.^{8,17} HIV-1 and HIV-2 genomes exhibit about 60% homology in conserved genes such as group-specific antigen (*gag*) and polymerase (*pol*), and 39-45% homology in the envelope (*env*) genes.¹⁸ Serologic studies have also shown that the core proteins (*gag*) of HIV-1 and HIV-2 display frequent cross-reactivity whereas the envelope proteins are more type-specific.¹⁹

Within the two major HIV types, there is significant variation, as well. By analyzing sequences of representative strains, HIV-1 has been divided into four groups: group M (for major), including at least 9 subtypes, 3 sub-subtypes of A, and 2 sub-subtypes of F (A1, A2, A3, B, C, D, F1, F2, G, H, J, and K); group O (for outlier); group N (for non-M, non-O), and group P.²⁰⁻²⁴ Similarly, HIV-2 strains have been classified into at least five subtypes (A through E).²⁵ Some HIV-1 variants share ≤50% homology in their *env* genes with those *env* sequences of more common prototype strains.

Despite some degree of immunological cross-reactivity between types and subtypes of HIV, reliable detection of the more divergent strains may only be achieved by incorporating specific protein sequences into the assay design. In one study, detection of HIV-2-positive samples by licensed HIV-1 antibody kits ranged from 60% to 91%, depending on the test used.²⁶ Detection of HIV-1 Group O samples by HIV-1 and HIV-1/HIV-2 assays varied from 0% to 100% in studies with U.S.-licensed and European test kits.^{27,28} The GS HIV Combo Ag/Ab EIA incorporates highly conserved recombinant proteins and synthetic peptide sequences representing HIV-1 (groups M and O) and HIV-2,²⁹⁻³⁵ as well as monoclonal antibodies specific for HIV-1 p24 antigen. The assay was developed to improve sensitivity and specificity of detection of HIV-1 p24 antigen and antibodies to HIV-1 and/or HIV-2 as an aid in the diagnosis of HIV infection. HIV antigens and antibodies appear and are detectable at different stages of seroconversion and of the infection.^{36,37}

The GS HIV Combo Ag/Ab EIA allows for the simultaneous detection of HIV p24 antigen and anti-HIV-1 (M and O groups) and anti-HIV-2 antibodies. This test has been developed to significantly reduce the serological window for detection of HIV.

Reactive specimens may contain HIV-1 p24 antigen or antibodies to either HIV-1 or HIV-2. Therefore, additional, more specific or supplemental tests for HIV-1 and HIV-2 such as NAT, Western blot, or immunofluorescence, must be performed to verify the presence of HIV-1 p24 antigen or antibodies to HIV-1 or HIV-2. Recommendations for appropriate use of such additional tests may be issued periodically by the United States Public Health Service

Biological Principles of the Procedure

The GS HIV Combo Ag/Ab EIA is an enzyme immunoassay based on the principle of the sandwich technique for the qualitative detection of HIV-1 p24 antigen and detection of envelope antibodies associated with HIV-1 and/or HIV-2 virus in human serum or plasma. The solid phase is coated with:

- Monoclonal antibodies against HIV-1 p24 antigen
- HIV antigens: HIV-1 gp160 recombinant protein, a synthetic peptide mimicking a totally artificial (i.e. encoded by no existing virus) HIV-1 group O-specific epitope, and a peptide mimicking the immunodominant epitope of the HIV-2 envelope protein.

The conjugates are based upon the use of:

- Biotinylated polyclonal antibodies to HIV p24 Ag (Conjugate 1)
- Peroxidase-conjugated streptavidin and peroxidase-conjugated HIV-1 antigens gp41 and gp36 peptides mimicking the immunodominant epitopes of the HIV-1 and HIV-2 envelope glycoproteins, and the same synthetic peptide mimicking a totally artificial HIV-1 group O specific epitope used for the solid phase) (Conjugate 2)

During the assay procedure, Conjugate 1 (biotinylated polyclonal antibody to HIV p24 Ag) is added to the microplate wells, followed by the addition of samples to be assayed, as well as controls and a calibrator. If present, HIV p24 antigen binds to the monoclonal antibody on the solid phase and also binds to the Conjugate 1. HIV-1 and/or HIV-2 antibodies, if present, bind to the antigens immobilized on the solid phase. The addition of Conjugate 1 and sample is validated through a color change from yellow-green to blue. After incubation, excess sample is removed by a wash step. Next, Conjugate 2 is added. Peroxidase-labeled streptavidin reacts with biotinylated Ab-Ag-Ab complexes; peroxidase-labeled HIV-1 and HIV-2 antigens bind to the IgG, IgM or IgA antibodies captured on the solid phase. After incubation, unbound Conjugate 2 is removed by washing. Working TMB Solution is added to the plate and allowed to incubate. A blue or blue-green color develops in proportion to the amount of HIV antibody and/or antigen present in the sample. Color development is stopped by the addition of acid, which changes the blue-green color to yellow. The optical absorbances of specimens, controls, and the calibrator are determined spectrophotometrically at a wavelength of 450 nm with a 615 to 630 nm reference.

2. Safety Precautions

1. Do not use any kit components beyond their stated expiration dates.
2. The reagents that may be used with different lots of the GS HIV Combo Ag/Ab EIA kit are the Chromogen (R9), Substrate Buffer (R8), Wash Solution Concentrate (R2), and Stopping Solution (R10). Do not mix any other reagents from different lots. Any lot number of the following reagents may be used with this assay provided they have the correct catalog number and are not used beyond their labeled expiration dates:
 - Chromogen (R9) - Catalog # 26182
 - Substrate Buffer (R8) - Catalog # 26181
 - Wash Solution Concentrate (R2) - Catalog # 25261
 - Stopping Solution (R10) - Catalog # 25260
3. The tabs at the end of the microwell strips are labeled with product code "JJ". Do not use strips that have other product codes with this kit.
4. Exercise care when opening vials and removing aliquots to avoid microbial contamination of the reagents.
5. Do not allow the microplate to dry between the end of a washing step and addition of reagents.
6. Do not pre-dilute samples in Conjugate 1 (R6) before adding to the microwell plate.
7. The waiting time between dispensing Conjugate 1 and adding the samples may not exceed 30minutes.
8. Use a clean, disposable container for the Working Conjugate 2 Solution. Exposure of this Conjugate to sodium azide will result in its inactivation.
9. Avoid exposing Chromogen or the Working TMB Solution to strong light during storage or incubation. Do not allow the chromogen solutions to come into contact with an oxidizing agent.
10. The enzyme reaction is very sensitive to metal ions. Do not allow any metal element to come into contact with the various conjugate or substrate solutions.
11. Do not carry out the test in the presence of reactive vapors (acid, alkaline, aldehyde vapors) or dust that could alter the enzyme activity of the conjugates.
12. Use clean, polypropylene containers (do not use polystyrene containers) to prepare and store the Working TMB Solution. If glassware must be used, pre-rinse thoroughly with 1N sulfuric or hydrochloric acid followed by at least three washes of deionized water. Be sure that no acid residue remains on the glassware.
13. Bring all reagents to room temperature before use.
14. Clinical samples may contain very high levels of HIV antibody. Therefore, care must be exercised when dispensing samples to avoid cross contamination through aerosols or carryover. For manual pipetting of controls and specimens, use an individual pipette tip for each sample and do not allow other parts of the pipetting device to touch the rim or interior of the specimen container. Consider using new stoppers/caps to seal specimen tubes after use, to avoid errors or contamination of the work area while recapping tubes. Handle the Negative Control and Positive Controls in the same manner as patient specimens.
15. If a specimen or reagent is inadvertently not added to a well, the assay results will read negative. Reagents of this kit have been color-coded to enable confirmation of the addition of specimens/controls and Working Conjugate Solution.
16. Inadequate adherence to package insert instructions may result in erroneous results.
17. Use only adequately calibrated equipment with this assay.
18. Use of dedicated equipment is recommended if equipment performance validations have not precluded the possibility of cross-contamination.
19. The GS HIV Combo Ag/Ab EIA performance is highly dependent upon incubation times and temperatures and effective washing. Temperatures outside of the validated ranges may result in

invalid assays. Incubation temperatures should be carefully monitored using calibrated thermometers, or equivalent.

Seven WARNINGS FOR USERS

For In Vitro Diagnostic Use

Warning: FDA has approved this test for use with serum and plasma specimens only. Use of this test kit with specimens other than those specifically approved for use with this test kit may result in inaccurate test results.

This test is not intended for use in children younger than 2 years of age.

1. Wear protective clothing, including lab coat, eye/face protection and disposable gloves (synthetic, non-latex gloves are recommended) while handling kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
2. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
3. Do not pipette by mouth.
4. This test kit should be handled only by qualified personnel trained in laboratory procedures and who are familiar with their potential hazards. Wear appropriate protective clothing, gloves and eye/face protection and handle appropriately with the requisite Good Laboratory Practices. The following is a list of potential chemical hazards contained in some reagents (refer to product description chart in the Reagents section):
 - a. ProClin 300 (0.1% or 0.5%) and ProClin 950 (0.16%) are biocidal preservatives that are irritating to eyes and skin, may be detrimental if enough is ingested, and may cause sensitization by skin contact; prolonged or repeated exposure may cause allergic reaction in certain sensitive individuals.
 - b. 0.005% Gentamicin Sulfate, a biocidal preservative, which is a known reproductive toxin, photosensitizer and sensitizer; prolonged or repeated exposure may cause allergic reaction in certain sensitive individuals.
 - c. The 1 N Sulfuric Acid (H₂SO₄) Stopping Solution is irritating to skin and severely irritating or corrosive to eyes, depending on the amount and length of exposure; greater exposures can cause eye damage, including permanent impairment of vision. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Keep away from strong bases, reducing agents and metals; do not pour water into this component. Waste from this material is considered hazardous acidic waste; however, if permitted by local, regional, and national regulations, it might be neutralized to pH 6-8 for non-hazardous disposal if operators are trained and equipped to do so.
5. The GS HIV Combo Ag/Ab EIA contains human blood components. No known test method can offer complete assurance that infectious agents are absent. Therefore, all human blood derivatives, reagents and human specimens should be handled as if capable of transmitting infectious disease, following recommended Universal Precautions for bloodborne pathogens as defined by OSHA40, the guidelines from the current CDC/NIH Biosafety in Microbiological and Biomedical Laboratories⁴¹ and/or local, regional and national regulations.^{42,43} The following human blood derivatives are found in this kit:

- a. Human source material used in the preparation of the Negative Control (C0) and Cutoff Calibrator (C4), and as a diluent for the HIV-1 Ab Positive Control (C1) and HIV-2/O Ab Positive Control (C2), has been tested and found nonreactive for Hepatitis B surface antigen (HBsAg), and antibodies to Hepatitis C virus (HCV Ab) and human immunodeficiency virus (HIV-1 and HIV-2).
 - b. Human source material, containing HIV-1 and HIV-2 human antibody used in the preparation of the HIV Ab Positive Controls (C1 and C2) has been heat-treated. It has been tested and found nonreactive for Hepatitis B surface antigen (HBsAg) and antibodies to Hepatitis C virus (HCV Ab).
 - c. The HIV-1 viral lysate used in the HIV Ag Positive Control (C3) has been inactivated using a chaotropic agent and heat.
6. Biological spills: Human source material spills should be treated as potentially infectious. Spills not containing acid should be immediately decontaminated, including the spill area, materials and any contaminated surfaces or equipment, with an appropriate chemical disinfectant that is effective for the potential biohazards relative to the samples involved (commonly a 1:10 dilution of household bleach, 70-80% Ethanol or Isopropanol, an iodophor [such as 0.5% Wescodyne™ Plus, EPA Registration #4959-16-52], or a phenolic, etc.), and wiped dry.⁴⁴⁻⁴⁷ Spills containing acid should be appropriately absorbed (wiped up) or neutralized, the area flushed with water and wiped dry; materials used to absorb the spill may require biohazardous waste disposal. Then the area should be decontaminated with one of the chemical disinfectants.

NOTE: DO NOT PLACE SOLUTIONS CONTAINING BLEACH INTO THE AUTOCLAVE.

7. Dispose of all specimens and material used to perform the test as though they contain an infectious agent. Laboratory, chemical or biohazardous wastes must be handled and discarded in accordance with all local, regional and national regulations.

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 to Westat's ISIS computer system weekly and transferred from there to NCHS.

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

Serum or plasma specimens may be used in the test. The following tube types and anticoagulants, including those in both glass and plastic tubes, may be used: serum tubes, serum separator tubes (SSTs) with and without activator, potassium EDTA, sodium citrate, sodium and lithium heparin, and plasma separator tubes (PSTs). Samples that are collected into anticoagulant tubes should be filled as labeling indicates to avoid improper dilution. Specimens with observable particulate matter should be clarified by centrifugation prior to testing. Suspended fibrin particles or aggregates may yield falsely positive results. Do not heat-inactivate the samples. Extensive hemolysis may affect test performance. The potential interfering substances at the levels tested below did not produce a change in clinical

interpretation. Studies with these substances included HIV negative samples and those spiked with both low and high levels of HIV-1 antigen, HIV-1 antibody, and HIV-2 antibody. In these studies the samples were spiked with the substances at two levels, including the upper levels listed here that were tested in 7 samples each.

Hemolyzed: 500 mg/dL of hemoglobin
Lipemic: 1000 mg/dL of triglycerides
Icteric: 20 mg/dL of bilirubin
Proteinemic: 12 g/dL of protein

Samples may be stored for no longer than 2 days at room temperature or 7 days at 2-8°C, including the time that samples are in transit. Minimize room temperature storage of samples to the shortest time possible in order to preserve maximum p24 antigen reactivity. For long-term storage, the specimens should be removed from the clot, red blood cells, or separator gel and should be frozen at -20°C or colder. Samples should not be used if they have incurred more than 4 freeze/thaw cycles. Mix samples thoroughly after thawing

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

Not applicable for this procedure

6. **Equipment and Instrumentation, Materials, Reagent Preparation, Calibrators (Standards), and Controls**

A. **Working Conjugate 2 Solution (R7a + R7b)**

Note: The lyophilized Conjugate 2 (R7a) may be supplied as a small vial (18 mL) or large vial (40 mL). Check the vial label to determine the size before proceeding.

Bring Conjugate Diluent (R7b) to room temperature. Gently tap the vial of the lyophilized Conjugate 2 (R7a) to remove any substance from the rubber cap. Carefully remove the cap and prepare as follows:

for the **small** Lyophilized Conjugate vial (R7a), add **18 mL** of Conjugate Diluent (R7b)

OR

for the **large** Lyophilized Conjugate vial (R7a), add **40 mL** of Conjugate Diluent (R7b).

Replace the cap and let stand for 10 minutes, while gently shaking and inverting from time to time to help the dissolution. Working Conjugate 2 Solution is stable for 15 hours at room temperature, and for 4 weeks if stored at 2-8°C. Studies have demonstrated no adverse effects from cycling the Working Conjugate Solution between 2-8°C and room temperature (18-30°C) multiple times (e.g., 5 cycles of 3 hours at room temperature followed by 2-8°C). Working Conjugate 2 Solution that is stored frozen (-20°C or colder) may be used until the expiration date of the kit. It may be frozen and thawed 10 times. Note the Concentrate lot number and date and time of preparation of the Working Conjugate Solution. Prepare a user log to record the time and temperature of Working Conjugate Solution storage, to ensure that the recommended limits are not exceeded. Always mix working solution by inverting just prior to use.

One vial of prepared Working Conjugate 2 Solution provides enough reagents for testing of approximately 1 plate (18 mL vial) or 2½ plates (40 mL vial). For testing of additional microwell strips, prepare additional Working Conjugate 2 Solution. Ensure that the volume of prepared reagent will be adequate for the entire run.

B. Working TMB Solution (R8 + R9)

Bring Chromogen and Substrate Buffer to room temperature. Invert the Chromogen and Substrate Buffer to mix before using. Prepare a 1:11 dilution for each strip to be tested by mixing 100 µL of Chromogen to 1 mL of Substrate Buffer in a clean, **polypropylene** container (**do not use a polystyrene container**). Note Chromogen lot number, date and time of preparation, and date and time of expiration (8 hours from preparation) on container. Mix Working Solution gently when combined and again just prior to use. Working TMB Solution should be kept in the dark at room temperature and used within 8 hours.

Chromogen should be colorless to slightly yellow. Any other color indicates that the reagent is contaminated and should not be used. The Working TMB Solution should also be colorless. A distinct blue color indicates that the reagent is contaminated and should be discarded. Prepare fresh Working TMB Solution in a clean container as needed.

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 run. Extra Chromogen is provided. Use the following table as a guide:

Preparation of Working TMB Solution by Number of Strips Used

Number of Strips To be used	1	2	3	4	5	6	7	8	9	10	11	12*	24**
Amount of Chromogen (µL)	100	200	300	400	500	600	700	800	900	1000	1100	1200	2400
Amount of Substrate Buffer (mL)	1	2	3	4	5	6	7	8	9	10	11	12	24

* 1 Complete Plate ** 2 Complete Plates

7. Calibration and Calibration Verification Procedures

A. Validation of Results

Calculate the mean absorbance of the Cutoff Calibrator (CCx)

Determine the mean absorbance for the Cutoff Calibrator (CCx) by dividing the sum of the absorbance values by the number of acceptable Calibrators. The individual absorbance values of the Cutoff Calibrator must be greater than 0.000 AU and less than 0.170 AU. One Cutoff Calibrator absorbance value may be discarded if it is outside this range. The CCx̄ may be calculated from the two remaining absorbance values. The mean of the absorbance of the Cutoff Calibrators (C4) should be less than 0.150.

Cutoff Calibrator

<u>Sample Number</u>	<u>Absorbance</u>
1	0.075
2	0.083
3	<u>0.081</u>
Total Absorbance =	0.239
Total Absorbance/ 3 calibrators	= 0.239/3
	= 0.080 (CCx)

Calculate the cutoff value

Determine the cutoff value by adding the CCx to 0.200 as shown in the example below:

$$CCx^- = 0.080$$

$$\text{Cutoff Value} = 0.080 + 0.200 = 0.280$$

B. Assay Validation

A run is valid if the following criteria are met:

- The absorbance of Negative Control (C0) must be greater than 0.000 and less than the assay cutoff (OD $0.000 < C0 < \text{cutoff}$).
- The absorbance of HIV-1 Ab Positive Control (C1) must be greater than 0.700 AU (OD $C1 > 0.700$).
- The absorbance of HIV-2/O Ab Positive Control (C2) must be greater than 0.700 AU (OD $C2 > 0.700$).
- The absorbance of HIV-1 Ag Positive Control (C3) must be greater than 0.700 AU (OD $C3 > 0.700$).
- The absorbance of each Cutoff Calibrator (C4) should be greater than 0.000 and less than 0.170 AU (OD $0.000 < C4 < 0.170$). One Cutoff Calibrator value may be discarded. If two or more Cutoff Calibrator values are out of limit, the assay must be repeated.
- The mean of the absorbance of the Cutoff Calibrators (C4) should be less than 0.150.

If any of these criteria have not been met, the assay is invalid and must be repeated.

Materials Required But Not Provided

1. Precision pipettes that deliver 20 to 200 μL , 1 mL, 10 mL, 25 mL and 50 mL, as needed (accurate within $\pm 10\%$), and corresponding pipette tips; multichannel pipettors capable of delivering 25 μL , 75 μL , 80 μL and 100 μL are optional.
2. Appropriately sized graduated cylinders (25 mL, 100 mL, 1000 mL capacity).
3. Dry-heat incubator capable of maintaining $37 \pm 2^\circ\text{C}$.

4. Microwell plate or strip washer qualified for use with this assay. The washer must be capable of dispensing 400 μ L per well, cycling 5 times, and soaking for 30-60 seconds between each wash.
5. Microwell strip reader qualified for use with this assay. The spectrophotometer should have the following specifications at wavelengths 450 nm and 615 to 630 nm:

- Bandwidth: 10 nm HBW (Half Band Width) or equivalent
- Absorbance Range: 0 to 2 AU (Absorbance Units)
- Repeatability: \pm (0.5% + 0.005) AU
- Linearity or Accuracy: 1% from 0 to 2.0 AU

The instrument should contain a reference filter for reading at 615 to 630 nm.

6. The GS HIV Combo Ag/Ab EIA is approved for use with the Bio-Rad EVOLIS™ Automated Microplate System.
7. Household bleach (5% to 8% sodium hypochlorite) which may be diluted to a minimum concentration of 10% bleach (or 0.5% sodium hypochlorite). Alternative disinfectants include 70-80% ethanol or 0.5% Wescodyne™ Plus.
8. Paper towels or absorbent pads for blotting.
9. Labeled null strips for testing partial plates.
10. Clean polypropylene container for preparation of Working TMB Solution (**do not use polystyrene**).
11. Deionized or distilled water. Clinical laboratory reagent water is acceptable.⁴⁸
12. Gloves.
13. Laboratory timer.
14. EIA reagent reservoirs (optional).

8. Procedure Operating Instructions; Calculations; Interpretation of Results

Preliminary statements

1. The expected run time for this procedure is approximately 2.5 - 3 hours from initiation of the first incubation step. Each run of this assay must proceed to completion without interruption after it has been started. The allowable time from start of sample pipetting to start of incubation is \leq 1 hour.
2. One Negative Control, one HIV-1 Ab Positive Control, one HIV-2/O Ab Positive Control, one HIV Ag Positive Control, and three wells of Cutoff Calibrator must be run on each plate. The cutoff for patient samples is determined by the calibrators on each individual plate, and assay validity is determined by the controls on each plate.
3. Do not splash controls, specimens, or reagents between microwells of the plate.
4. Cover plates for each incubation step using plate sealers provided or other appropriate means to minimize evaporation.
5. Avoid exposure of the plates to light during the final incubation step (following the addition of Working TMB Solution).

Adhere to the recommended time constraints for the use of the Working TMB Solution (8hours), Working Conjugate 2 Solution (15 hours at room temperature, 4 weeks at 2-8°C, or until the expiration of the kit at -20°C or colder), and Working Wash Solution (4 weeks).

6. Avoid the formation of air bubbles in each microwell.
7. Avoid bumping plates containing liquid reagents (especially Working Conjugate 2 Solution) to prevent adherence of liquid to the plate sealer and/or top edges of the microwells.
8. Adequate washing of the microwells with a validated microplate washer is essential to eliminate non-specific binding.

Dry residue from the plate blocking process may be visible in the microwells. This material will not affect assay results. Before reading the plates, carefully wipe the bottom of the plates to remove any material that remains on the outside of the wells, and ensure that all strips have been pressed firmly into place.

Reagent	Preparation	Storage	Lot specific
Working Conjugate 2 Solution (R7a + R7b)	Small volume (18 mL) Add 18 mL of Conjugate Diluent to the small lyophilized conjugate vial Large volume (40 mL) Add 40 mL of Conjugate Diluent to the large lyophilized conjugate vial	15 hours at room temperature. 4 weeks if stored at 2-8°C Kit expiration date when stored at -20°C or colder. Up to 10 freeze/thaw cycles permitted	Yes
Working TMB Solution (R8 + R9)	1:11 dilution (100 µL of Chromogen into 1 mL of Substrate Buffer for each strip to be tested)	Room temperature (18-30°C) for up to 8 hours. Store in the dark	No
Working Wash Solution (R2)	1:30 dilution (one part Wash Solution Concentrate [30X] to 29 parts of water)	Room temperature for up to 4 weeks (not to exceed the expiration date of the 30X Wash Solution Concentrate)	No
Stopping Solution (R10)	Use as supplied	2-30°C for shelf life of reagent	No

EIA Procedure

1. Perform equipment maintenance and calibration, where necessary, as required by the manufacturer.
2. Bring all of the reagents to room temperature before beginning the assay procedure.
3. Prepare Working Conjugate 2 Solution, Working TMB Solution, and Working Wash Solution if not previously prepared. Mix gently by inversion. Mix again just before use.
4. Remove strips not needed for the assay and replace them with labeled null strips, if necessary.
5. Microwell strips not needed for the assay may be returned to the plate pouch and sealed, and then used at a later time. Strips from different plates can only be mixed to assemble full or partial plates if they are from the same plate lot, and have come from plates that have

previously been tested with kit controls and yielded valid runs. When assembling a plate that contains strips from a newly opened, previously untested plate, one of these strips should be placed at the beginning of the plate and tested with the kit controls.

6. If sample identity is not maintained by an automatic procedure, identify the individual wells for each specimen or control on a data sheet.
7. Add 25 μL of Conjugate 1 (R6) to each well.
8. Within 30 minutes, add 75 μL of the controls, calibrator, and the specimens to the appropriate wells. Do not pre-dilute samples in Conjugate 1 (R6) before adding to the microwell plate. One Negative Control, one HIV-1 Ab Positive Control, one HIV-2/O Ab Positive Control, one HIV-1 Ag Positive Control, and three wells of Cutoff Calibrator must be assayed on each plate or partial plate of specimens.

NOTE: After addition of samples, controls and calibrator, wells containing Conjugate 1 turn from yellow-green to blue. It is possible to verify the presence of sample in the wells by spectrophotometric reading at 620 nm. (Refer to section 10: Spectrophotometric Verification of Sample and Reagent Pipetting) Cover the microwell plate with a plate sealer or use other means to minimize evaporation.

9. Incubate the plate for 60 ± 5 minutes at $37 \pm 2^\circ\text{C}$. At the end of the incubation period, carefully remove the plate cover, if used, and aspirate the fluid from each well into a biohazard container. Wash the microwell plate or strip a minimum of five times with the Wash Solution (at least 400 μL /well/wash). Soak each well for 30 to 60 seconds between each wash cycle. Aspirate the Wash Solution after each wash. After the last wash, if excess liquid remains, blot the inverted plate on clean, absorbent paper towels.

NOTE: Grasp the plate holder firmly at the center of the long sides before inverting to blot.

10. Add 100 μL of Working Conjugate 2 Solution (R7a + R7b) into all wells. The Conjugate must be mixed well before use.

NOTE: The Working Conjugate 2 Solution is colored red.

It is possible to verify the presence of Conjugate 2 in the wells by spectrophotometric reading at 450/620 nm. Refer to section 10: Spectrophotometric Verification of Sample and Reagent Pipetting. Cover the microwell plate with a plate sealer or use other means to minimize evaporation.

11. Incubate the plate for 30 ± 5 minutes at room temperature (18-30°C).

At the end of the incubation period, carefully remove the plate cover, if used, and aspirate the fluid from each well into a biohazard container. Wash the microwell plate or strip a minimum of five times with the Wash Solution (at least 400 μL /well/wash). Soak each well for 30 to 60 seconds between each wash cycle. Aspirate the Wash Solution after each wash. After the last wash, if excess liquid remains, blot the inverted plate on clean, absorbent paper towels.

NOTE: Grasp the plate holder firmly at the center of the long sides before inverting to blot.

12. Add 80 μL of the Working TMB Solution to each well containing a specimen, control, or calibrator. Incubate plates in the dark for 30 ± 5 minutes at room temperature (18-30°C). Use of a plate sealer or cover is optional.

13. Carefully remove the plate cover, if used, and add 100 μ L of Stopping Solution to each well to terminate the reaction. Use the same sequence and rate of distribution as for the Working TMB Solution addition. Tap the plate gently, or use other means to assure complete mixing. Complete mixing is required for acceptable results.
14. Read absorbance within 30 minutes after adding the Stopping Solution, using the 450 nm filter with 615 nm to 630 nm as the reference.

9. Procedure Operating Instructions; Calculations; Interpretation of Results

The presence or absence of detectable HIV-1 antigen or antibodies to HIV-1 and/or HIV-2 is determined by comparing the absorbance measured for each sample to the calculated cutoff value.

Samples with absorbance values that are <0.000 must be repeated. Those with values greater than the upper linearity limits of the reader should be reported as reactive.

Specimens with absorbance values less than the cutoff value are considered nonreactive by the GS HIV Combo Ag/Ab EIA and may be considered negative for HIV-1 (M and O Groups) and HIV-2 antibodies and HIV-1 antigen.

Samples with absorbance values equal to or greater than the cutoff value are considered reactive by the GS HIV Combo Ag/Ab EIA. **Initially reactive specimens must be retested in duplicate to validate the initial test results.** If, after repeat testing, the absorbance values of both duplicate specimens are less than the cutoff value, the original specimen may be considered non-repeatedly reactive and negative for HIV-1 (Groups M and O) and HIV-2 antibodies, as well as HIV p24 antigen. If, after repeat testing, the absorbance value of either of the duplicates is greater than or equal to the cutoff value, the specimen must be considered repeatedly reactive.

Repeatedly reactive specimens must be investigated by additional, more specific, or supplemental tests. Refer to CDC guidelines for the current recommended HIV testing algorithm.

Limitations of the Procedure

1. The GS HIV Combo Ag/Ab EIA Procedure and the Interpretation of Results must be followed closely when testing for the presence of HIV-1 antigen or antibodies to HIV-1 and/or HIV-2 in plasma or serum specimens. The user of this 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 and temperature of the incubation steps. Testing of other body specimens, pooled blood or processed plasma, and products made from such pools is not recommended.
2. The GS HIV Combo Ag/Ab EIA detects circulating antibodies to HIV-1 (Groups M and O) and HIV-2, and it also detects HIV-1 antigen. Thus, it is useful 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 or HIV-2.⁴⁹ **Repeatedly reactive specimens must be investigated by additional, more specific, or supplemental tests.** Recommendations for appropriate use of

such additional tests may be issued periodically by the United States Public Health Service. For individuals who are confirmed positive for HIV antigen or antibodies, appropriate counseling and medical evaluation should be offered. Both confirmation of the test result on a freshly drawn sample and counseling should be considered an important part of testing for HIV antigen and antibody to HIV-1 and HIV-2.

3. AIDS and AIDS-related conditions are clinical syndromes and their diagnosis can only be established clinically. Testing alone cannot be used to diagnose AIDS, even if the recommended investigation of repeatedly reactive specimens suggests a high probability that HIV antigen or antibody to HIV-1 or HIV-2 is present.
4. 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 and/or HIV-2.
5. Negative results can occur if the quantity of marker present in the sample is too low for the detection limits of the assay, or if the marker which is detected is not present during the stage of disease in which a sample is collected.
6. 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.
7. The risk of an asymptomatic person with a repeatedly reactive test result developing AIDS or an AIDS-related condition is not known, as the course of HIV infection may vary among individual patients and may be altered by antiretroviral therapy.^{50,51} However, in a prospective study, AIDS developed in 51% of homosexual men after 10 years of infection.⁵²
8. Data obtained from testing persons both at increased and at low risk for HIV-1 and/or HIV-2 infection suggest that repeatedly reactive specimens with high reactivity on the GS HIV Combo Ag/Ab EIA may be more likely to demonstrate the presence of antibodies to HIV-1 (Groups M and O) and/or HIV-2 by additional, more specific, or supplemental testing.⁵³ Borderline reactivity is more frequently nonspecific, especially in samples obtained from persons at low risk for infection with HIV-1 or HIV-2; however, the presence of antibodies to HIV-1 and/or HIV-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 (e.g. 3 to 6 months).⁵⁴
9. The performance of this assay has not been established for individuals younger than 2 years of age. It is generally recognized that detection of HIV antibody in infants born to seropositive mothers is not adequate to diagnose HIV infection in the infant, since maternal IgG frequently persists for as long as 18 months after birth. Nearly all infants born to HIV-infected mothers passively acquire maternal antibody. Supplemental assays designed specifically for neonatal specimens may be helpful in resolving such cases, including HIV nucleic acid tests or viral culture.⁵⁵

10. An absorbance value of less than 0.000 AU may indicate a procedural or instrument error that should be evaluated. That result is invalid and that specimen must be re-run.
11. Factors that can affect the validity of results include failure to add the specimen or reagents to the well, inadequate washing of microplate wells, failure to follow stated incubation times and temperatures, addition of wrong reagents to wells, the presence of metals, or splashing of bleach into wells, improper washing of microwell plates, cross-contamination of nonreactive specimens with HIV antigen or antibody from a high-titered specimen, contamination of the Chromogen or Working TMB Solution by oxidizing agents (sodium hypochlorite, hydrogen peroxide, etc.), and contamination of the Stopping Solution.
12. A person who has antibodies to HIV is presumed to be infected with the virus, except that a person who has participated in an HIV vaccine study may develop antibodies to the vaccine and may or may not be infected with HIV. Clinical correlation is indicated with appropriate counseling, medical evaluation, and possibly additional testing to decide whether a diagnosis of HIV infection is accurate.
13. Heat-inactivated samples may affect the quality of the results, and should not be used.

10. Reference Ranges (Normal Values)

A normal sample from an uninfected individual is negative for HIV antibodies.

11. Critical Call Results (Panic Values)

Not applicable to this assay method.

12. Specimen Storage and Handling during Testing

Specimens are stored at $\leq -20^{\circ}\text{C}$ until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at $\leq -20^{\circ}\text{C}$.

13. Alternate Methods for Performing Test or Storing Specimens If Test System Fails

If the analytical system fails, it is preferable to store specimens at $\leq -20^{\circ}\text{C}$ until the system is returned to functionality.

14. Test Result Reporting System; Protocol for Reporting Critical Calls (If Applicable)

Not applicable to this assay method.

15. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Standard record keeping involves using the computerized database and the hard copy results themselves to track specimens. Records are maintained indefinitely. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

For the NHANES study, residual serum is retained at $\leq -70^{\circ}\text{C}$ for 1 year and then returned to the NCHS serum bank.

16. Summary Statistics and QC Graphs

Qualitative assays are assays with a positive, negative or borderline/indeterminate result. The absorbance values for each kit control are graphed using Levey-Jennings charts to check for any possible kit degradation during storage and to monitor changes in new kit lots.

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