Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens

Single Well Avidity Enzyme Immunoassay for Detection of Recent HIV-1 Infection Using Dried Blood Spot (DBS) Specimens Only

Cat. No. 1003

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Includes Two Packs:
3048 - Refrigerator Pack (Store at 2 to 8°C)
3039 - Freezer Pack (Store at -25 to -10°C)

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NAME AND INTENDED USE
The Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (“Sedia® HIV-1 LAg-Avidity EIA for DBS”) (Cat. No. 1003) is an in vitro quantitative limiting antigen (LAg) avidity enzyme immunoassay for distinguishing recent HIV-1 infections from those which are long-term using dried blood spot (DBS) specimens collected from HIV-1 infected persons. Persons with recently acquired HIV-1 infections typically have lower avidity HIV IgG than those with long-term infections. The test measures HIV-1 antibody avidity in blood samples [1, 40]. This assay kit is not for use with liquid serum, plasma or blood or with dried serum or dried plasma spots. Users who wish to test liquid serum or plasma specimens must use the Sedia® HIV-1 LAg-Avidity EIA (Cat. No. 1002). The Sedia® HIV-1 LAg-Avidity EIA for DBS is solely intended for research use only such as estimating HIV-1 incidence in a population, monitoring and evaluating intervention programs, and recognizing those high-incidence populations so that prevention research, vaccine trials, and resources are most appropriately utilized. This product is not intended for use in diagnostic procedures or for determining clinical outcome or treatment.

BACKGROUND OF HIV-1 INCIDENCE TESTING
The public health community needs to know where and among whom HIV is spreading and where intervention is most effective at reducing the spread of the HIV epidemic. Accurate assays for the detection of recent infections of HIV-1 have been sought as they may be used as a means to estimate HIV-1 incidence. Such information can therefore be a useful tool in surveillance, program planning, effectiveness of intervention programs and planning for vaccine or other prevention trials. A variety of laboratory-based assays have been evaluated as an alternative to longitudinal cohort studies to determine HIV-1 incidence. These earliest methods included desensitized or “less sensitive” commercial HIV immunoassays [2-9] where the lower titers of anti-HIV antibodies typical of recent infections were used as a basis for identifying those individuals likely to be recently infected. However, since desensitized commercial HIV immunoassays were developed by modifying commercial assays that employ HIV-1 subtype B antigen(s), the accuracy of the tests were sometimes found to be less accurate in populations containing primarily non-subtype B infections (8,9). To overcome this subtype bias liability and address the extreme sample dilution and assay variability problems also observed with desensitized assays, scientists at the U.S. Centers for Disease Control and Prevention (US CDC) developed the BED capture-EIA (BED CEIA), which employed a synthetic antigen containing sequences from multiple subtypes and a simple capture format to allow the measurement of the proportion of HIV-1 antibodies which increase over time after seroconversion [10]. The BED CEIA (currently available as the Sedia® HIV-1 BED Incidence EIA, Cat. No. 1000) has been used in several studies [11-16]. However, high false recency rates giving overestimations of HIV-1 incidence have been reported at varying levels with the BED CEIA depending on population [17-20] and consequently post-test adjustments have been proposed to improve the accuracy of those incidence estimates [21, 22].
Several studies to estimate HIV incidence rates have been conducted using laboratory assays based on antibody maturation as measured by antibody avidity [23-31]. These estimates have demonstrated low false recency rates in those studies conducted in U.S. populations [30, 31]. Some studies have suggested that further reduction of false recency rates may be possible by using an algorithm incorporating both BED CEIA and avidity assays [31, 32]. However, little is known about the performance of these avidity assays across multiple clades, and avidity assays based on commercial assays or based on a single antigenic subtype may demonstrate the same subtype bias described above for desensitized assays. As a result, US CDC developed avidity assays incorporating a new recombinant protein (“rIDR-M”) containing the major variants of gp41 immunodominant regions among the HIV-1 group M viruses including a one-well avidity assay using limiting amounts of antigen [1]. Testing performed on a number of well characterized samples by the US CDC indicate that subtype bias is minimized by the use of the multi-subtype antigen. Furthermore, results of the HIV-1 LAg-Avidity EIA in Africa have demonstrated a false recency rate of less than 1%, suggesting improved accuracy over previous technologies [41]. The technology for these assays was licensed by the US CDC to Sedia Biosciences and the one-well assay was commercialized by Sedia with modifications to enhance stability and reproducibility without affecting fundamental assay characteristics or accuracy. The licensed product is the Sedia® HIV-1 LAg-Avidity EIA available for liquid serum and plasma specimens (Catalog No. 1002) or for dried blood spot specimens (Cat. No. 1003).

SUMMARY AND EXPLANATION OF THE TEST
The Sedia® HIV-1 LAg-Avidity EIA for DBS measures HIV-1 antibody avidity and determines the recent/long term HIV-1 status by referencing the EIA numerical result against that of an internal calibrator specimen. The principle of the test is based on the observation that in response to exposure to the HIV-1 virus, the immune system produces low avidity HIV-1 antibodies early in the infection, and as time progresses, the immune system matures and produces high avidity HIV-1 antibodies. The amount of high avidity HIV-1 antibody present in the blood can therefore be used as an indication that the infection is a long-term one, instead of a recent one [1, 24-27, 40]. As the Sedia® HIV-1 LAg-Avidity EIA for DBS is based on the functional avidity or binding strength of the antibodies, the assay is likely to be less affected by disease state than other types of assays that have been previously used [33, 34]. The effect of antiretroviral therapy (ART) on assay performance has not been evaluated. Therefore, individuals on ART should be excluded from testing with this assay. The Calibrator and controls are selected to have an avidity such that specimens with normalized OD values (ODn) below 1.5 are classified as “recent” using the Sedia® HIV-1 LAg-Avidity EIA for DBS and have a mean duration of recent infection of 130 days (95% CI 118-142) [35].

The Sedia® HIV-1 LAg-Avidity EIA for DBS contains the same plasma controls and Calibrator contained in the Sedia® HIV-1 LAg-Avidity EIA but in the form of dried blood spots on filter paper cards. The Sedia® HIV-1 LAg-Avidity EIA for DBS also contains
a unique Sample Buffer-DBS which must be used for elution of both dried blood spot specimens and the Calibrator and controls spots as well as dilution of the conjugate.

PRINCIPLES OF THE PROCEDURE

1. The Sedia® HIV-1 LAg-Avidity EIA for DBS is a single well limiting antigen IgG capture enzyme immunoassay. During a sample incubation of 60 minutes at 37°C, both low and high avidity HIV-1 specific IgG is captured by a multi-subtype recombinant HIV-1 antigen (rIDR-M) coated in limiting concentration in the microplate wells.

2. Dissociation Buffer is added and incubated for 15 minutes at 37°C to preferentially remove low avidity IgG from the antigen-coated plate.

3. Goat anti-human IgG-HRP conjugate is incubated for 30 minutes at 37°C and binds to remaining IgG bound to microplate.

4. TMB substrate is incubated for 15 minutes at 25°C and color is generated with intensity proportional to the amount of HRP.

5. The optical density (OD) of each well is measured. The OD value is divided by the OD value of an internal kit calibrator to generate the normalized OD or “ODn”. The value of the ODn dictates whether a result needs to be confirmed and/or if the HIV infection is recent or long term.
FEATURES OF THE SEDIA® HIV-1 LAG-AVIDITY EIA FOR DBS

- New technology developed at the U.S. Centers for Disease Control and Prevention (CDC) and commercialized by Sedia Biosciences.
- The assay is based on the proven principles of limiting antigen avidity rather than extreme dilution or IgG ratios.
- Simple “single-well” method instead of the more complex 2-well methods used in other avidity assays results in higher reproducibility and increased number of specimens tested per plate.
- Custom multi-clade “rIDR-M” antigen provides for equivalent detection across all subtypes.
- Elution of the dried blood specimens requires an overnight incubation (i.e. minimum of 12 hours, but as long as 24 hours) at 2-8°C followed by warming at 37°C for one hour.
- Simple and fast test procedure (approximately 2 hours after elution steps) in a microtiter format allows for high throughput and excellent reproducibility.
- The assay uses microtiter plates with removable 1x8 strips and individual wells, allowing the use of exactly the number of wells needed, thus eliminating waste.
- The assay is for use with dried blood spot specimens. If liquid serum or plasma specimens are to be tested, the Sedia® HIV-1 LAg-Avidity EIA (Cat. No. 1002) should be used instead.
- Plasma used in the manufacture of the controls and Calibrator has been heat inactivated.
- The assay is manufactured to the exact specifications of the United States CDC by scientists with significant experience in HIV-1 incidence test development and manufacture.
- Each lot of the Sedia® HIV-1 LAg-Avidity EIA for DBS has gone thorough a stringent QC check both by Sedia and the CDC before release to customers.

GENERAL KIT INFORMATION

The Sedia® HIV-1 LAg-Avidity EIA for DBS is comprised of two component boxes of matching lot numbers that have separate temperature requirements (frozen and refrigerated). The kit contains two 96-well plates with twelve (12) 1 x 8 removable strips and all necessary reagents to run the assay. Each 1 x 8 strip may be further broken down into individual wells which may be inserted back into the plate frame so that only the exact number of wells required need be used. It is recommended that empty spaces in the plate frame be filled with used wells or strips if using dispensing and/or washing equipment that cannot be programmed on an individual well basis.

Each test plate requires 11 wells be allocated for the controls and Calibrator thus allowing for the test of up to 85 specimens in the initial screening mode. Specimens generating an ODn value below a threshold value of 2.0 must be confirmed by triplicate testing in a subsequent test run following the same procedure. Twenty eight (28) specimens can be tested on each plate in the confirmatory testing mode.
MATERIALS PROVIDED AND THEIR STORAGE

REFRIGERATOR PACK (#3048): Store contents at 2-8°C.

1. **HIV-1 rIDR-M Coated Microwell Plates** (Labeled “For Dried Blood Spot Specimens Only”) - Two (2) 96-well plates. Plates are provided in resealable foil pouches with desiccant.

2. **10X Wash Buffer Concentrate** - Two (2) bottles (100 mL each). Contains phosphate buffered saline, detergent and preservative. Note: The 10X Wash Buffer Concentrate found in this kit is identical to that found in the Sedia® HIV-1 LAg-Avidity EIA (Cat. No. 1002) and the Sedia® BED HIV-1 Incidence EIA (Cat. No. 1000), and may be used interchangeably regardless of lot number if two or more of these assays are being run by the lab.

3. **Sample Diluent-DBS** - One (1) bottle (175 mL). Contains phosphate buffered saline, detergent, blocking agents and preservative. For use only with dried blood spot testing.

4. **Dissociation Buffer** - One (1) bottle (55 mL). Contains dissociation agent in acidic buffer.

5. **TMB Substrate** - One (1) bottle (27 mL). Contains 3,3’,5,5’ tetramethylbenzidine (TMB) in acidic buffer.

6. **Stop Solution** - One (1) bottle (27 mL). Contains dilute acid solution.

7. **Plate Sealers** - One (1) pack of 25.

8. **Package Insert** - One (1).

FREEZER PACK (#3039): Store contents at -25 to -10°C.

Each Freezer Pack contains a resealable foil pouch containing DBS Control Cards (items 7 through 10) along with a humidity indicating desiccant packet and humidity indicator card. Conjugate is packaged separately in a vial under the foil pouch.

7. **Negative Control (NC, blue label)** - One (1) filter paper card containing 5 dried blood spots non-reactive for HBsAg and antibodies to HCV and HIV. Contains preservative.

8. **Calibrator (CAL, green label)** - One (1) filter paper card containing 5 dried blood spots reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.

9. **Low Positive Control (LPC, yellow label)** - One (1) filter paper card containing 5 dried blood spots reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
High Positive Control (HPC, red label) - One (1) filter paper card containing 5 dried blood spots reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.

Goat Anti-Human IgG-HRP Conjugate (purple cap and label) - One (1) vial (50 µl). Goat anti-human IgG conjugated to horseradish peroxidase (HRP). Contains glycerol, preservative and is concentrated to 1001X the Conjugate Working Solution.

WARNINGS AND PRECAUTIONS

1. The Sedia® HIV-1 LAg-Avidity EIA for DBS is intended for research use only, specifically for the determination of recency of HIV infection in populations. It is not intended for use in diagnostic procedures or for determining clinical outcome or treatment.

2. Use good laboratory working practices and universal precautions when handling the DBS Control Cards, specimens and materials that have been in contact with specimens. All blood cells and plasmas used in the DBS Control Cards are non-reactive for HBsAg and HCV antibody. Blood cells and negative plasma are non-reactive for HIV antibody. All plasmas have been inactivated. Although the DBS Control Cards are presumed to be non-infectious, they should still be treated as if potentially infectious as a precaution. Dispose of all exhausted DBS Control Cards and eluted controls and test specimens as biohazardous waste when finished.

3. It is critical that all aspects of the procedure be strictly adhered to, particularly timing and temperatures. Reagents should not be used after their expiration date.

4. The Sedia® HIV-1 LAg-Avidity EIA for DBS is comprised of two packs, a Freezer Pack (#3039) and a Refrigerator Pack (#3048) with matching lot numbers. It is critical that only packs with matching lot numbers are used together during the test operation. The 10X Wash Buffer Concentrate is lot number independent and may be used with other kit lots, including the Sedia® HIV-1 LAg-Avidity EIA (for liquid specimens)(Cat. No. 1002), and with the Sedia® BED HIV-1 Incidence EIA (Cat. No. 1000) if two or more of these assays are being run at the same time.

5. Promptly disinfect any spills using a 0.5% sodium hypochlorite solution (1:10 dilution of household liquid bleach) or equivalent. Thoroughly clean work surfaces both before and after the procedure.

6. Use only the Sample Diluent-DBS provided in this kit for elution of DBS Controls, Calibrator and specimens and dilution of the Conjugate. Use only the HIV-1 rIDR-M Coated Microwell Plates provided in this kit for testing the eluates of the DBS Controls and specimens. The Sample Diluent and Microplates found in the Sedia® HIV-1 LAg-Avidity EIA (for liquid specimens)(Cat. No. 1002) are not compatible with this assay.
7. Use plate washers, readers and incubators that have undergone proper installation, operational and performance qualification and are properly maintained to minimize assay variability. Because the Sedia® HIV-1 LAg-Avidity EIA for DBS semi-quantitatively measures avidity, the assay is more susceptible to significant variations in plate washing and incubator temperature zones. If equipment is not properly qualified, intra-plate variability may be increased. It is important to read results on a plate reader at 450 nm against a reference filter at 620-650 nm to obtain accurate OD and ODn values within acceptable ranges.

IMPORTANT PROCEDURAL NOTES

1. Do not use kit or kit components beyond the expiration dates specified on the product and component labeling. Storage of kit materials at temperatures except as specified may result in diminished assay performance and may give inaccurate results.

2. Remove the Refrigerator Pack (#3048), the kit controls and Calibrator in the Freezer Pack (#3029) from cold storage 60 minutes before the test is to be run. Allow all reagents to reach room temperature (15-30°C) before use. The Goat Anti-Human IgG-HRP conjugate concentrate should remain in the freezer until immediately before use. Place the TMB Substrate bottle in a 25°C (±2°C) incubator until use.

3. For accurate results, dried whole blood spot test specimens must be collected only on filter cards GE/Whatman #903 Protein Saver Cards (www.whatman.com). Dried serum or dried plasma spot specimens cannot be tested using this kit.

4. If using less than 96 wells, use only the required number of strips and/or wells and return unused strips/wells to the foil bag with desiccant, seal, and store at 2-8°C.

5. Use only calibrated pipettes and microliter syringes. Always use separate pipette tips, tubes and reagent reservoirs for each specimen or kit component. Do not interchange bottle or vial caps.

6. Keep the DBS Control Cards sealed in the original foil pouch with desiccant until use. Before opening the foil pouch containing the DBS Control Cards, allow the pouch to come to room temperature to avoid condensation on the cards or on the inside of the pouch. DBS Contol Cards must be kept dry at all times until added to Sample Diluent-DBS during the elution step. **Immediately after use**, reseal the foil pouch containing any partially used or unused cards along with desiccant and humidity card and return to storage at -25 to -10°C. The DBS Control Cards may only be used if all spots on the humidity indicator card included in the foil pouch are blue and the indicator particles in the desiccant pack are blue (both indicating the absence of moisture).

7. When performing replicate testing of DBS Control Cards and dried blood spot specimens, elute an individual disk (6 mm or 0.25 in. punch) for each replicate using the Sample Diluent-DBS provided in the kit. Be sure to use the same paper punch for all controls, Calibrator and specimens used for each plate, as commercial paper punches may vary in diameter and yield disks of significantly different surface area.
8. Add Sample Diluent-DBS to all disks in a rack of titer tubes at the same time, not as each disk is placed in its titertube. Elute all DBS Control Cards and dried test specimen disks at the same time and location, using the elution method described in the Assay Procedure below.

9. Carefully inspect each titertube after adding Sample Diluent-DBS to tubes containing dried blood spot disks to confirm that each disk is completely submerged in buffer. **It is critical that each disk be completely submerged.**

10. Carefully note and follow the 12-24 hour DBS elution time followed by the 1 hour warming incubation specified in the assay procedure.

11. Disk eluates should be used immediately after elution. Confirmatory or repeat testing should be done on freshly eluted disks.

12. Accurate preparation of the Conjugate Working Solution (as described in the Assay Procedure) in particular is **critical** as small inaccuracies may significantly impact absolute OD values. Use a positive displacement pipette or microliter syringe to accurately measure out the Goat Anti-Human IgG-HRP conjugate concentrate.

13. Mix all reagents immediately before use. Refrigerator Pack (#3048) reagents can be mixed by gentle inversion 3-5 times. Freezer Pack (#3039) reagents should be briefly vortexed. Wash Buffer Concentrate typically requires both mixing and warming in a water bath to dissolve salt crystals. The Goat Anti-Human IgG-HRP Conjugate concentrate may need to be flicked or spun down after vortexing as it often congregates in the cap after vortexing.

14. Preparation of dilutions of specimens, the kit controls and Calibrator requires thorough mixing and accurate measurement. Carefully mix the Sample Diluent-DBS with the sample in the titertube by drawing the mixture up and down in the multichannel pipette at least 4 times. Pipette slowly to avoid bubbles. Change pipette tips for each specimen. Filtered tips are recommended for specimen handling to minimize cross contamination. Unfiltered tips may be used for all subsequent steps.

15. The kit and its components should be returned to their recommended storage conditions after use.

16. When punching either control or specimen cards, punch a blank part of the card in between punches to cleanse the hole punch.

**MATERIALS REQUIRED BUT NOT PROVIDED**

- Polypropylene tubes with cap, 12-15 mL.
• Dried whole blood specimens collected on GE/Whatman #903 ProteinSaver Cards (GE/Whatman catalog number 10531018 (EU) or 10534612 (US), www.gelifesciences.com, +1-800-526-3593 or see website for country specific contact numbers). Dried serum or dried plasma specimens are unsuitable for this assay.
• Graduated cylinders, beakers and flasks.
• Serological pipettes with pipette bulb (or equivalent).
• Hole punch capable of punching 6 mm (0.236 in.) or 0.25 inch (6.35 mm) disks from the DBS Control Cards and dried blood spot specimen cards. Common commercially available paper punches are not controlled collection devices and may vary in diameter and are commonly sold as “6 mm”, “0.25 inch” or “0.25 in./6 mm” punches. To minimize variation due to disk punching, use the same punch for collecting both specimen punches and for Calibrator and control punches.
• Positive displacement pipette or microliter syringe capable of delivering 5-20 µL (e.g. Hamilton Co., +1-775-858-3000, www.hamiltoncompany.com, 25 µL syringe catalog no. 7643-01 with needle catalog no. 7770-02; Gilson Co., +1-608-836-1551, www.pipetman.com, 25 µL positive displacement pipette catalog no. F148502; or equivalent) to measure out Goat Anti-Human IgG-HRP conjugate concentrate.
• Vortex Mixer.
• Single-channel pipettes (2-100 µL) and multi-channel pipette (50-200 µL). Filtered tips are recommended for specimen handling.
• Reagent reservoir (e.g. VWR International, +1-800-932-5000, www.vwr.com, catalog number 53504-035 or equivalent).
• Incubators, one each at 37°C (±2°C) and 25°C (±2°C).
• Refrigerator or incubator at 2-8°C for dried blood spot elution.
• Microwell Plate Washer, either 96-well or strip.
• Spectrophotometer (Plate Reader) capable of reading 96-well plates at 450 nm with a reference filter at 620-650 nm.
• Timer.
• Deionized water.
• Household bleach (5-8% hypochlorite).
• Personal protection equipment (PPE) (disposable gloves, safety glasses, etc.).
• Biohazardous waste container.
• Optional: A pair of forceps or tweezers for handling filter card punches.

SPECIMEN COLLECTION AND PREPARATION

1. The Sedia® HIV-1 LAg Avidity EIA for DBS is intended for use with dried blood spot specimens only. If liquid serum or plasma specimens are to be tested, the Sedia® HIV-1 LAg-Avidity EIA (Cat. No. 1002) should be used instead. The Sedia® HIV-1 LAg Avidity EIA for DBS kit cannot be used to test dried serum or plasma spot specimens.

2. Dried blood spot specimens should be collected, handled, and stored according to NCCLS guidelines (43). DBS specimens should be kept in a dry environment or with desiccant at all times.
3. Specimens eluted with Sample Diluent-DBS should be tested within 24 hours of dilution. If retesting is required, fresh dilutions should be prepared prior to testing.

4. Multiple freeze-thaw cycles should be avoided for DBS Control Cards and DBS specimens.

5. Test performance with DBS specimens derived from grossly hemolyzed, lipemic or cloudy specimens has not been determined. DBS specimens should be prepared from freshly collected blood.

6. Dried blood spot specimens should be shipped in accordance with all local requirements and regulations for the transport of etiological agents.

**REAGENT PREPARATION**

1. Remove the Refrigerator Pack (#3048) and the kit controls and Calibrator in the Freezer Pack (#3039) from cold storage 60 minutes before the test is to be run. Allow all kit components to reach room temperature (15-30°C) before use except for the Goat Anti-Human IgG-HRP Conjugate and TMB Substrate as per note 2 below. Particular care should be taken to ensure that the pouched HIV-1 rIdR-M Coated Microwell Plates are brought to room temperature before being opened to avoid formation of condensate on the plates. Condensation on the unused portions of the plates may compromise the plates for future use. Subsequent use after re-storage of such strips or plates may result in erroneous results.

2. The Goat Anti-Human IgG-HRP conjugate concentrate should remain in the freezer until immediately before use. Place the TMB Substrate bottle in a 25°C (±2° C) incubator until use.

3. Accurate preparation of the Conjugate Working Solution is **critical** as small inaccuracies may significantly impact absolute OD values. Use a positive displacement pipette or microliter syringe to accurately measure out the Goat Anti-Human IgG-HRP conjugate concentrate.

4. Prepare 1X Wash Buffer as follows:
   a) Mix 100 mL of 10X Wash Buffer Concentrate and 900 mL of deionized water.
   b) Mix well using a magnetic stir bar and stir plate for at least 10 minutes.

**Notes:** *The 10X Wash Buffer Concentrate may require brief heating in a water bath to dissolve remaining salt crystals.*

*10X Wash Buffer Concentrate from Sedia® BED HIV-1 Incidence EIA (Cat. No. 1000) and from Sedia® HIV-1 LAg-Avidity EIA (Cat. No. 1002) may also be used in this assay. See page 6 and 7 above for details.*

*Store the 1X Wash Buffer at 2-30°C for a maximum of 1 month and then discard.*

5. Return kit components to recommended storage conditions after use.
ASSAY PROCEDURE

1.0 Creation of Plate Diagram or Map

The configuration of the HIV-1 rIDR-M coated Microwell Plate (“Avidity Plate”) shown below is recommended for the initial testing. Note that the Negative Control is tested in duplicate, the two other controls and Calibrator are tested in triplicate, and (during the initial testing) all specimens are tested singly. For confirmatory testing, the controls are tested in the same manner but each specimen is tested in triplicate (see plate map on page 22). If only partial plates are required, unused strips and/or wells can be removed and replaced with blanks (previously used strips or wells) if desired.

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**Note:** The recommended plate configuration is compatible with the Excel spreadsheet available for downloading at [http://www.sediabio.com/products/lag-avidity-eia](http://www.sediabio.com/products/lag-avidity-eia).

**Note:** Confirmatory testing requires specimens tested in triplicate. See Section 5.0, page 21.
2.0 Test Procedure

2.1 Set up an appropriately labeled blank set of titertubes in an 8 x 12 rack as an assay “pre-plate” for the elution of controls, the Calibrator, and specimens.

2.2 Remove the Freezer Pack (#3039) with the foil pouch containing the four (4) DBS Control Cards (Negative Control, Calibrator, Low Positive Control, and High Positive Control) from cold storage and allow to come to room temperature before opening the foil pouch. **Do not open DBS Controls Cards pouch unless at room temperature to avoid condensation on the cards and inside pouch.** Open the pouch and verify that all the spots on the included humidity indicator card are still blue or crystals in the desiccant pack are still blue, both confirming that the integrity of the cards has not been compromised due to moisture. If any spots on the humidity indicator card are pink or the desiccant indicator crystals are no longer blue, do not use the DBS Control Cards in that package.

2.3 Use a 6 mm or 0.25 inch hole punch to punch out all DBS Controls Card and specimen disks. Use the same hole punch for all disks prepared on a given assay plate. For each control/Calibrator card, punch out and place disks in titertubes as described in Steps 2.4-2.7 below. Each spot should yield 3-4 fully saturated punches if the user carefully positions the punch within each spot. To minimize cross-contamination, clean the paper punch between each control/specimen by punching clean paper 2-3 times before going to the next control/specimen. To further minimize cross-contamination, punch out disks directly to the titertubes. Minimize exposure of each DBS Control Card to ambient humidity. When finished with each DBS Control Card, immediately return all cards with useable spots from that same pack to the original foil pouch, ensure desiccant and humidity indicator card are enclosed, and seal the zip-lock on the pouch securely.

2.4 Punch out two disks from one of the the DBS Negative Control spots on a blue-labeled Negative Control DBS Control Card and place each disk into separate titertubes consistent with the plate map generated in Step 1.0. Punches can be punched directly into the titertube or transferred with tweezers, as shown. If tweezers are used, wipe tweezers with a clean tissue in between punches. Punches may need to be bent or crimped to allow them to fall to the bottom of the titer tube. Return the card to its pouch immediately after use and reseal with desiccant and humidity indicator.
2.5 Punch out three discs from one of the DBS Calibrator spots on a green-labeled Calibrator DBS Control Card and place each disk into separate titertubes consistent with the plate map generated in Step 1.0. Return the card to its pouch immediately after use and reseal with desiccant and humidity indicator.

2.6 Punch out three disks from one of the DBS Low Positive Control spots on a yellow-labeled DBS Low Positive Control Card and place each disk into separate titertubes consistent with the plate map generated in Step 1.0. Return the card to its pouch immediately after use and reseal with desiccant and humidity indicator.

2.7 Punch out three discs from one of the DBS High Positive Control spots on a red-labeled DBS High Positive Control Card and place each disk into separate titertubes consistent with the plate map generated in Step 1.0. Return the card to its pouch immediately after use and reseal with desiccant and humidity indicator.
2.8 Punch a single spot out for each dried specimen and add to a separate titertube consistent with the plate map generated in Step 1.0. Specimen cards should also be handled so as to minimize their exposure to moisture.

2.9 Once all disks from all specimens and controls have been placed into the titertubes, use a multichannel pipette to add 500 µL of Sample Diluent-DBS to each titertube that contains a DBS control, Calibrator, or specimen disk. Carefully evaluate each titer tube individually to confirm each disk is completely submerged in Sample Diluent-DBS. Use a clean pipette tip for each disk to push the disks so that they are completely submerged in Sample Diluent-DBS. Several disks can be pushed down at once using a multichannel pipette as long as the tips are changed before moving to the next row and each tube is individually inspected.

2.10 Cover the ELISA titertube rack. Incubate overnight (12-24 hours) at 2-8°C to allow for elution.

2.11 The next day, warm the eluted specimens for 1 hour (60±5 minutes) at 37°C (±2°C) before adding the specimens to the plate. During this time, bring the kit components to appropriate temperature as described in Reagent Preparation Step 1 on page 11.
2.12 A few minutes before the elution step (Step 2.11, pg. 15) is completed, use a multichannel pipette to add 50 µL of Sample Diluent - DBS to each well of an empty LAg-Avidity test plate.

2.13 Carefully mix the controls and specimens in the titertubes by gently withdrawing and expelling the fluid in the tips of a multichannel pipette 4-6 times. Avoid bubbles by proceeding slowly. Change pipette tips for each sample.

2.14 After mixing, use a multichannel pipette to transfer 100 µL of each specimen or control from the titertubes to the Avidity Plate. Be sure to use a new pipet tip for each specimen or control. If the frame for the Avidity Plate contains positions that do not contain controls or specimens, it is recommended to fill those positions with used "blank" wells or strips.

2.15 Apply a Plate Sealer to the Avidity Plate and incubate for 1 hour at 37°C (±2°C).

2.16 After the 1 hour sample incubation, wash the Avidity Plate 4 times (rotating the plate after the first 2 washes) with 1X Wash Buffer (see Reagent Preparation, page 11) using a 96-well or strip plate washer. Set the washer to dispense 300 µL well with a 10-second soak. Soaking is not required if a strip washer is used. After the wash is complete, remove any residual buffer in the wells by wrapping the plate in absorbent paper and gently tapping it upside down.
2.17 Use a multichannel pipette to transfer 200 µL of Dissociation Buffer to each well.

2.18 Apply a Plate Sealer to the Avidity Plate with Dissociation Buffer and incubate for **EXACTLY** 15 minutes at 37°C (±2°C).

*Note: Immediately proceed to step 2.19 during the Dissociation Buffer incubation.*

2.19 Retrieve the Goat Anti-Human IgG-HRP Conjugate from -25 to -10°C storage. Warm conjugate in hand for a couple of minutes to help conjugate reach room temperature before dispensing.

2.20 To prepare the Conjugate Working Solution, briefly vortex the Goat Anti-Human IgG-HRP Conjugate vial and using a **positive displacement pipette or microliter syringe**, transfer 12 µL conjugate concentrate to a tube containing 12 mL Sample Diluent-DBS to prepare the 1:1001 dilution. Flick or spin down the tube to make sure the volume is at the bottom of the tube prior to transfer. (Conjugate concentrate is viscous. Pipette slowly to ensure that the proper volume is transferred)

*Note: For a partial Avidity Plate, prepare 1 mL for each strip to be used plus an extra mL (e.g. transfer 7 µL into 7 mL for 6 strips). In any event, a minimum of 6 ml should be prepared to ensure accurate volume measurement.*

2.21 Cap the tube and vortex or mix by gently inverting tube 6-8 times.

2.22 Transfer the Conjugate Working Solution to a reagent reservoir.
2.23 Immediately after the 15 minute Dissociation Buffer incubation, wash the plate 4 times with 1X Wash Buffer as described in Step 2.16, page 16.

2.24 Transfer 100 µL per well of the Conjugate Working Solution from the reservoir to the washed Avidity Plate using a multichannel pipette.

2.25 Apply a Plate Sealer to the Avidity Plate with Conjugate Working Solution and incubate for 30 minutes at 37°C (±2°C).

2.26 After the 30 minute incubation with the Conjugate Working Solution, wash the Avidity Plate 4 times with 1X Wash Buffer as described in Step 2.16, page 16.

2.27 Take the TMB Substrate out of the 25°C incubator and transfer to a reagent reservoir. Use a multichannel pipette to transfer 100 µL of TMB Substrate to each well.
2.28 Incubate the Avidity Plate with TMB Substrate for **EXACTLY** 15 minutes at 25°C (±2°C). Do not cover the plate with a Plate Sealer.

2.29 Following the 15 minute TMB Substrate incubation, stop the reaction by adding 100uL of Stop Solution per well using a multichannel pipette.

*Note:* *Do not wash the Avidity Plate before adding the Stop Solution.*

2.30 Immediately after the addition of the Stop Solution, use a spectrophotometer to read the Avidity Plate at a wavelength of 450 nm **using a reference filter wavelength of 620-650 nm.**

3.0 Run Validation and Calculation of Results

3.1 Calculate the median (not mean) optical density (OD) for each Control and the Calibrator. The median value of three OD values will be the “middle” value. For example, 0.790 would be the median of these three OD values: 0.790, 0.834, and 0.775. The median value of the two Negative Control OD values will be their average.

3.2 Determine if the median OD values fall within the acceptable range specified for each Control and the Calibrator.

### Acceptable OD Ranges

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<td>0.175</td>
<td>0.950</td>
<td>0.520</td>
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</table>
If the median OD value of any of the Controls or the Calibrator is not within the ranges above, the run is invalid and must be repeated using fresh specimen dilutions. Note: Both individual OD values for the Negative Control must be within the stated range.

3.3 Determine the normalized OD results (ODn) for each Control, the Calibrator, and each specimen. The ODn is calculated by dividing the median OD value of the specimen by the median OD of the Calibrator. Studies performed by the US CDC have demonstrated OD normalization by an internal calibrator decreases run-to-run variability and increases reproducibility [1, 21].

\[
\text{ODn} = \frac{\text{OD of sample, Control, or Calibrator}}{\text{median OD of Calibrator}}
\]

Note: For confirmatory testing, the “OD of the sample” is the median value of the three replicate-tested specimen OD values.

3.4 Determine if the ODn value for each Control and the Calibrator falls within the acceptable range listed below. If the ODn of any Control falls outside of its range, the run is invalid and must be repeated using fresh specimen dilutions.

### Acceptable ODn Ranges

<table>
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<tr>
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<tr>
<td>Maximum</td>
<td>0.240</td>
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</table>

3.5 A customized spreadsheet is available from CDC and at [http://www.sediabio.com/products/lag-avidity-eia](http://www.sediabio.com/products/lag-avidity-eia) to validate the run and calculate ODn. This spreadsheet may be used for either liquid plasma or serum or for dried blood spot specimens.
4.0 Interpretation of Assay Results

4.1 **During the screening mode**, the ODn value for each specimen (calculated in step 3.3) dictates whether the specimen is considered a “long-term” infection or whether it must be subjected to confirmatory testing. Specifically, if the ODn of a specimen is $> 2.0$ during the screening mode, no further testing is required and the specimen is considered a long-term infection. If the ODn of a specimen is $\leq 2.0$ during the screening mode, then the specimen must be subjected to confirmatory testing (Section 5.0).

**During confirmatory mode**, if the ODn of a specimen is $< 1.5$, then the specimen is considered a recent infection. If the ODn is $> 1.5$ then the specimen is considered a long-term infection. Below is the algorithm used for testing and interpretation.

![Algorithm Diagram]

4.2 If the ODn of a specimen obtained during screening or confirmatory mode is $\leq 0.4$, perform confirmatory serological diagnostic testing to ensure the specimen is HIV positive.

5.0 Confirmatory Testing

5.1 As presented in the previous section, if the ODn of a specimen is $< 2.0$ during the screening mode, then the specimen must be subjected to confirmatory testing. Confirmatory testing entails repeating the Sedia® HIV-1 LAg-Avidity EIA for DBS protocol exactly as specified in Sections 1.0 through 3.0 except that specimens are tested in triplicate rather than singly. The recommended plate configuration is presented on the following page. Note that if the entire plate is used, 28 specimens can be tested in the Confirmatory mode and that one well is intentionally left blank.
### Confirmatory Testing: Recommended Plate Configuration

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*BL = Blank Well

5.2 Upon completion of confirming a set of specimens by the Sedia® HIV-1 LAg-Avidity EIA for DBS, compare initial ODn with confirmatory ODn in an X-Y scatter plot with linear regression line. Good reproducibility should yield a correlation coefficient ($R^2$) close to 1.0, slope close to 1.0 and y-intercept close to 0.0. Outliers with different initial and confirmatory classification (recent vs. long-term) should be repeated after ruling out transcription error. However, it is not unusual for specimens to have changed classification between initial and confirmatory testing if the ODn is close to the cutoff of 1.5.

### INTERPRETATION OF SPECIMEN RESULTS

Testing and data analysis conducted by the U.S. Centers for Disease Control and CEPHIA indicates that a cutoff for ODn values of 1.5 represents a mean duration of recent infection of 130 days [35].

The predictive value of any assay depends on the prevalence of that condition in a population. Therefore, the predictive value of detecting recently infected individuals in low incidence populations would be lower than in higher incidence populations. [42]

Test attributes, including reproducibility, intra-run and inter-run coefficient of variation (CV), and interoperator variability have been studied by CDC scientists and the manufacturer. Preliminary studies suggest that the assay has high reproducibility with a CV of <10% in the dynamic range and a false recency rate of less than 1% [41].

### LIMITATIONS OF THE ASSAY

Classification of individuals by the Sedia® HIV-1 LAg-Avidity EIA for DBS as recent seroconverters or long-term infections is based on average development of higher avidity HIV-antibodies calculated from data using a large number of people [1, 35]. However, there are differences among individuals in terms of maturation of HIV-antibodies and the rates at which high avidity HIV-antibodies are made. Although
this assay is useful at the population level, its predictive value for individuals has not been determined (especially when ODn levels are close to the cutoff). Therefore, the assay should not be used for individual assessment of recency of infection. This assay is based on the functional property of maturation of developing HIV antibodies, i.e. maturing avidity or antibody binding strength, as opposed to other assays which measure a passive parameter such as increasing levels of HIV antibodies and are thus less likely to be affected by disease states, viral load or low CD4 counts as observed with other such assays [36]. In fact, early results suggest that the false recency rate (FRR) of the Sedia® HIV-1 LAg-Avidity EIA upon which the Sedia® HIV-1 LAg-Avidity EIA for DBS is based, is significantly lower than other methods [41]. However, until such time as additional studies on this assay are performed, persons with diagnosis of AIDS or low CD4+ T cell counts (below 200 cells per μl), recipients of anti-retroviral therapy and known “elite controllers” (HIV-infected individuals with known low or undetectable viral loads) should be excluded from the study populations to reduce the likelihood of misclassification of recency of infection.

**RECOMMENDED RECENT INFECTION TEST ALGORITHM**

It is recognized that the estimation of local FRR values, necessary for the estimation of incidence (see next section), can be challenging or even impractical in some locales. As a result, based on recommendations by UNAIDS/WHO [44], U.S. CDC [35] and CEPHIA [45], it is recommended that the user incorporate viral load testing into the test algorithm of population surveys analyzed by the Sedia® HIV-1 LAg-Avidity EIA as shown below to reduce and minimize the impact of false recent infections, primarily attributable to elite controllers and subjects on ART, on HIV incidence estimates.

**Recommended LAg-Avidity EIA and Viral Load Algorithm**
In a CEPHIA study, performing viral load testing as an adjunct to Sedia™ HIV-1 LAg-Avidity testing of specimens and reclassifying the LAg-Avidity EIA “recent” specimens that had VL <1000 copies/mL as long term infections resulted in the lowest FRR among 7 different HIV incidence assay methodologies under a broad range of population prevalence rates, incidence rates and treatment coverage [45].

Viral load testing is done only on LAg-Avidity EIA samples classified as “recent infections” (usually <10% of total positives in most populations).

CALCULATING INCIDENCE
HIV incidence is defined as the number of new HIV infections occurring in a population, usually expressed as a rate of infection per person per unit time (e.g. “infections per 100 person-years”) [37]. The incidence formula recommended by the U.S. Centers for Disease Control and Prevention, the Office of the Global AIDS Coordinator and the UNAIDS/WHO Working Group on Global HIV/AIDS and STI Surveillance for estimating population level incidence in cross-sectional samples [37, 38, 39] is described below:

\[
IF = \frac{R - (FRR \times P)}{(1-FRR) \omega N} \times 100
\]

Estimates of both \( \omega \) and FRR will carry a degree of uncertainty which will impact the final incidence estimate. Minimum criteria needed to apply this test for incidence estimation include: 1) a calculation of the local FRR within an acceptable level of precision; 2) detection of individuals on ARV in incidence surveys and their exclusion from the incidence analysis; and 3) appropriate sample sizes for incidence surveys and FRR estimation surveys.

Additional guidance and information on how to calculate FRR and sample sizes is available online from UNAIDS/WHO at http://www.who.int/hiv/pub/surveillance/sti_surveillance/en/ [37]. Additional information is available from the CDC, Division of Global HIV/AIDS. Contact Joyce J. Neal (jxn4@cdc.gov) for guidance around study design, implementation, and incidence estimation, including sample size calculations, and Bharat Parekh (BParekh@cdc.gov) for laboratory related issues. Additional data analysis tools for calculating incidence are available at http://www.sacema.com/page/assay-based-incidence-estimation.
BIBLIOGRAPHY


SYMBOLS AND ABBREVIATIONS
The following symbols appear in Sedia® HIV-1 LAg Avidity EIA for DBS product labeling.

- ![Symbol](image) Use by (expiration date)
- ![Symbol](image) Corrosive
- ![Symbol](image) Consult instructions for use
- ![Symbol](image) Temperature limitation (temperature storage range)
- ![Symbol](image) Part number
- ![Symbol](image) Temperature limitation (max. temperature storage)
- ![Symbol](image) Lot number (batch code)

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Related Products Available from Sedia Biosciences:
SEDIA® BED HIV-1 Incidence EIA (Cat. No. 1000)
SEDIA® BED HIV-1 Incidence EIA Dried Blood Spot Controls Pack (Cat. No. 1001)
SEDIA® HIV-1 LAg-Avidity EIA (for Liquid Serum or Plasma Specimens)(Cat. No. 1002)
Asante™ HIV-1 Rapid Recency® Assay (Cat. No. 1130)
Asante™ Rapid Test Strip Reader (Cat. No. 1200)

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