

Sedia[®] HIV-1

LAg-Avidity EIA

(for Serum or Plasma Specimens)

*Single Well Avidity Enzyme Immunoassay for
Detection of Recent HIV-1 Infection
Using Serum or Plasma Specimens Only*

Cat. No. 1002

FOR RESEARCH USE ONLY
Not for use in
diagnostic procedures

Includes Two Packs:

3029 - Refrigerator Pack (Store at 2 to 8°C)

3030 - Freezer Pack (Store at -25 to -10°C)

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NAME AND INTENDED USE

The Sedia® HIV-1 LAg-Avidity EIA is an in vitro quantitative limiting antigen (LAg) avidity enzyme immunoassay for distinguishing recent HIV-1 infections from those which are long-term using serum or plasma specimens. 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 serum and plasma [1, 40]. This assay kit is not for use with dried blood, dried serum or dried plasma spot specimens. Users who wish to test dried blood spot specimens must use the Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (Cat. No. 1003). The Sedia® HIV-1 LAg-Avidity EIA is solely intended for research use only such as estimating HIV-1 incidence in a population, monitoring and evaluating intervention programs, and identifying 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 laboratory-based 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, assessment 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-based assays based on antibody maturation as measured by antibody avidity [23-31]. These studies have demonstrated low false recency rates in U.S. populations [30-32, 44-46]. Several of these studies have suggested that further reduction of false recency rates may be possible by using a multi-assay algorithm incorporating both BED CEIA and avidity assays. However, little is known about the performance of these avidity assays across multiple clades. Avidity assays based on commercial diagnostic assays are based on a single antigenic subtype and may demonstrate the same subtype bias described above for desensitized assays. In addition, some of these algorithms are complex, being composed of up to 4 assays, and include CD4 measurements, the latter which limit applicability with surveys conducted with dried blood spot specimens. U.S. CDC has 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 (the "HIV-1 LAg-Avidity EIA") [1]. Testing with this assay performed on a number of well characterized samples by the U.S. CDC indicates that subtype bias is minimized by the use of the multi-subtype antigen. Furthermore, results of this HIV-1 LAg-Avidity EIA in Africa have demonstrated a false recency rate of less than 1%, suggesting improved accuracy over previous technologies [41]. Recently, a less complex algorithm incorporating LAg-Avidity EIA and viral load testing ("VL") has been recommended by the U.S. CDC and the Consortium for Evaluation and Performance of HIV Incidence Assays ("CEPHIA") which further reduces misclassification significantly over HIV-1 LAg-Avidity EIA alone [35,48]. False recent cases occur most frequently with elite controllers and subjects on anti-retroviral therapy ("ART"). However, these can be readily identified such that individuals initially classified as HIV-1 LAg-Avidity EIA recent, but VL < 1000 copies/mL would be reclassified as long term. This algorithm is now recommended by the U.S. CDC Global AIDS Program and is incorporated into the recommendations on page 21. The technology for this assay was licensed by the U.S. CDC to Sedia Biosciences and the HIV-1 LAg-Avidity EIA was first commercialized by Sedia with modifications to enhance stability and reproducibility without affecting fundamental assay characteristics or accuracy. The licensed product is available as the Sedia® HIV-1 LAg-Avidity EIA, for serum and plasma specimens (Catalog No. 1002) and Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (Catalog No. 1003).

SUMMARY AND EXPLANATION OF THE TEST

The Sedia® HIV-1 LAg-Avidity EIA 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 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]. Data from CEPHIA and the U.S. CDC suggest that some (not all) individuals who have been on ART and have suppressed viral load may be classified as recent infection. The extent of misclassification will vary depending on initiation and duration of treatment as well as efficacy of ART in suppressing viral load. Therefore, individuals on ART should be excluded from testing with this assay. If this information is not available, use of viral load in an algorithm can help identify those likely to have been misclassified (VL<1000 copies/mL). 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 and have a mean duration of recent infection of 130 days (95% CI 118-142) [35].

PRINCIPLES OF THE PROCEDURE

1. The Sedia® HIV-1 LAg-Avidity EIA 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.

High and low avidity HIV-1 antibody binds to HIV-1 antigen on the microplate.



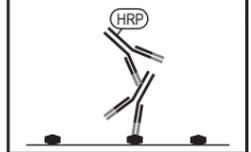
2. Dissociation Buffer is added and incubated for 15 minutes at 37°C to preferentially remove low avidity IgG from the antigen-coated plate.

Dissociation Buffer preferentially removes low avidity IgG.



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

Goat anti-human IgG-HRP binds to bound IgG.



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

TMB color is 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

- 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.
- The simple 1:101 sample dilution step can be accomplished in a single step.
- Simple and fast test procedure (approximately 2 hours) 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 compatible with liquid plasma and serum. If dried blood spot specimens are to be tested, the Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spots (Cat. No. 1003) should be used instead.
- Serum controls and Calibrator have 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 has gone through a stringent QC check before release to customers.

GENERAL KIT INFORMATION

The Sedia® HIV-1 LAg-Avidity EIA 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 (#3029): Store contents at 2-8°C.

- 1 **HIV-1 rIDR-M Coated Microwell Plates** - 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® BED HIV-1 Incidence EIA (Cat. No. 1000) and Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (Cat. No. 1003). The Wash Buffer from these kits may be used interchangeably regardless of lot number if two or more of these assays are being run by the lab.
- 3 **Sample Diluent** - One (1) bottle (175 mL). Contains phosphate buffered saline, detergent, blocking agents and preservative.
- 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.
- NA **Plate Sealers** - One (1) pack of 25.
Package Insert - One (1).

FREEZER PACK (#3030): Store contents at -25°C to -10°C.

- 7 **Negative Control (NC, blue cap)** - One (1) vial (100 µl). Inactivated human serum non-reactive for HBsAg and antibodies to HCV and HIV. Contains preservative.
- 8 **Calibrator (CAL, green cap)** - One (1) vial (100 µl). Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
- 9 **Low Positive Control (LPC, yellow cap)** - One (1) vial (100 µl). Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
- 10 **High Positive Control (HPC, red cap)** - One (1) vial (100 µl). Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
- 11 **Goat Anti-Human IgG-HRP conjugate (purple cap)** - 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 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 of universal precautions and good laboratory working practices are strongly recommended. Although the kit's serum controls and Calibrator have been inactivated, these reagents and any clinical specimens should be handled as if capable of transmitting infectious agents.
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 is comprised of two packs, a Freezer Pack (#3030) and a Refrigerator Pack (#3029) 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 Sedia® HIV-1 LAg-Avidity EIA kit lots (both Cat. No. 1002 and Cat. No. 1003), 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 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 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 (#3029) and the kit controls and Calibrator in the Freezer Pack (#3030) 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. 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.
4. 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.
5. 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.
6. Mix all reagents immediately before use. Refrigerator Pack (#3029) reagents can be mixed by gentle inversion 3-5 times. Freezer Pack (#3030) 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.
7. Preparation of 1:101 dilutions of specimens, the kit controls and Calibrator requires thorough mixing and accurate measurement. Carefully mix the Sample Diluent 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.
8. Avoid multiple freeze-thaws of Freezer Pack components. Short term storage of components of up to 48 hours at 2-8°C is acceptable if running assays over 2-3 days.
9. The kit and its components should be returned to their recommended storage conditions after use.

MATERIALS REQUIRED BUT NOT PROVIDED

- Polypropylene micro-dilution 1.2 mL tubes (“titer tubes”) in an 8x12 rack (e.g. Bio-Rad, +1-800-424-6723, www.biorad.com, catalog number 223-9390; E & K Scientific, +1-800-934-8114, www.eandkscientific.com, catalog number 604508-RC and RCS, or USA Scientific, +1-800-522-8477, www.usascientific.com, catalog number 1412-1400; or equivalent).
- Polypropylene tubes with cap, 12-15 mL.
- Graduated cylinders, beakers and flasks.
- Serological pipettes with pipette bulb (or equivalent).
- Positive displacement pipette or microliter syringe capable of delivering 5-20 μ L (e.g. Hamilton Co., +1-775-858-3000, www.hamiltoncompany.com, 25 μ L syringe catalog no. 7643-01, with needle, catalog no. 7770-02; Gilson Co., +1-608-836-1551, www.pipetman.com, 25 μ L 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 μ L) and multi-channel pipette (100-200 μ L). 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 (\pm 2°C) and 25°C (\pm 2°C).
- 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 or distilled water.
- Household bleach (5-8% hypochlorite).
- Personal protection equipment (PPE) (disposable gloves, safety glasses, etc.).
- Biohazardous waste container.

SPECIMEN COLLECTION AND PREPARATION

1. The Sedia® HIV-1 LAg Avidity EIA is intended for use with liquid serum or plasma specimens. If dried blood spot specimens are to be tested, the Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (Cat. No. 1003) should be used instead. This kit cannot be used with dried blood, serum or plasma spot specimens.
2. Specimens may be stored at 2-8°C for up to two weeks. Longer-term storage should be at frozen conditions (-10°C or lower).
3. Specimens diluted in Sample Diluent 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 specimens.
5. Test performance with grossly hemolyzed, lipemic or cloudy specimens has not been determined.
6. Specimens should be mixed well by inversion or vortex before testing. Particulate matter can be removed by appropriate centrifugation.
7. 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 (#3029) and the kit controls and Calibrator in the Freezer Pack (#3030) 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 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 ($\pm 2^\circ\text{C}$) 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 or distilled 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 the Sedia® BED HIV-1 Incidence EIA (Cat. No. 1000) or from the Sedia® HIV-1 LAg-Avidity EIA for Dried Blood Spot Specimens (Cat. No. 1003) may also be used in this assay. See pages 7 and 8 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 20). 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.

NC	HPC	6	14	22	30	38	46	54	62	70	78
NC	HPC	7	15	23	31	39	47	55	63	71	79
CAL	HPC	8	16	24	32	40	48	56	64	72	80
CAL	1	9	17	25	33	41	49	57	65	73	81
CAL	2	10	18	26	34	42	50	58	66	74	82
LPC	3	11	19	27	35	43	51	59	67	75	83
LPC	4	12	20	28	36	44	52	60	68	76	84
LPC	5	13	21	29	37	45	53	61	69	77	85

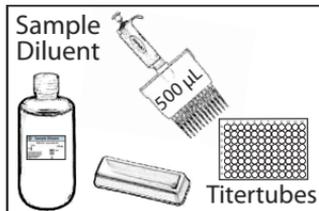
Note: The recommended plate configuration is compatible with the Excel spreadsheet available for downloading at <http://www.sediabio.com/products/lag-avidity-eia>.

Note: Confirmatory testing requires specimens tested in triplicate. See Section 5.0, page 19.

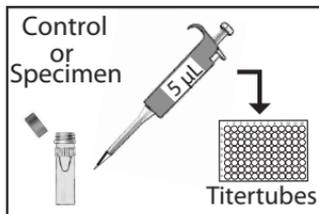
2.0 Test Procedure

Liquid serum or plasma specimens must be diluted 1:101 in polypropylene micro-dilution 1.2 mL tubes ("titer tubes") in an 8x12 rack consistent with the recommended plate map on page 13. Perform this dilution as follows:

- 2.1 Use a multichannel pipette to transfer 500 μ L Sample Diluent to each titer tube in the 8x12 rack.



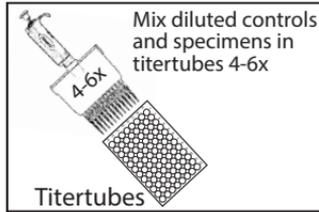
- 2.2 Use a pipette to transfer 5 μ L of each control, Calibrator, and sample to separate titer tubes containing 500 μ L Sample Diluent. Use a new pipette tip for each control, calibrator or sample to avoid cross-contamination. Sample addition must be consistent with the plate map. Liquid plasma and liquid serum specimens can be tested simultaneously.



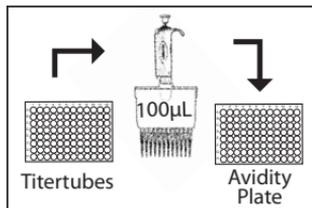
- 2.2.1 Prepare 2 separate replicate dilutions of the Negative Control (NC).
- 2.2.2 Prepare 3 separate replicate dilutions of the Calibrator (CAL).
- 2.2.3 Prepare 3 separate replicate dilutions of the Low Positive Control (LPC).
- 2.2.4 Prepare 3 separate replicate dilutions of the High Positive Control (HPC).
- 2.2.5 Prepare a single dilution of each specimen to be tested.

Note: Confirmatory testing requires specimens be tested in triplicate. See Section 5.0, page 19.

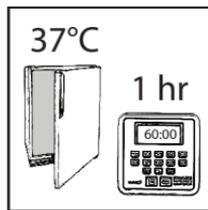
- 2.3 Carefully mix the controls and specimens in the titer tubes by gently withdrawing and expelling the fluid in the tips of a multichannel pipette 4-6 times. Avoid bubbles by proceeding slowly. Change pipette tips for each sample.



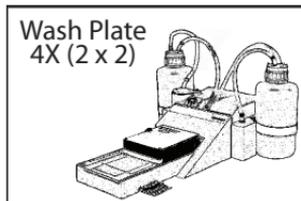
- 2.4 After mixing, use a multichannel pipette to transfer 100 μ L of each specimen or control from the titertubes to the Avidity Plate. Be sure to use a new pipet tip for each specimen or control. If the frame for the Avidity Plate contains positions that do not contain controls or specimens, it is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



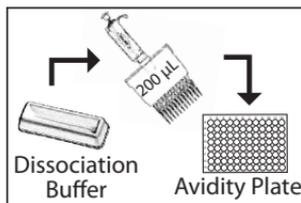
- 2.5 Apply a Plate Sealer to the plate and incubate for 1 hour at 37°C (\pm 2°C).



- 2.6 After the 1 hour sample incubation, wash the plate 4 times (rotating the plate after the first 2 washes) with 1X Wash Buffer (*see Reagent Preparation, page 12*) using a 96-well or strip plate washer. Set the washer to dispense 300 μ L per 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.

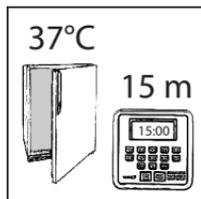


- 2.7 Use a multichannel pipette to transfer 200 μ L of Dissociation Buffer to each well.



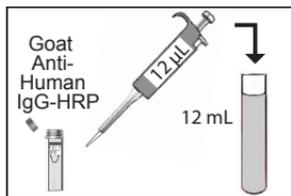
- 2.8 Apply a plate sealer to the plate with Dissociation Buffer and incubate for **EXACTLY** 15 minutes at 37°C (\pm 2°C).

Note: Immediately proceed to step 2.9 during the Dissociation Buffer incubation.



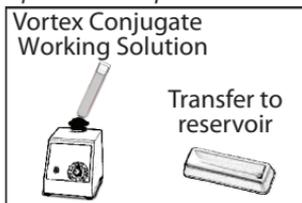
2.9 Retrieve the Goat Anti-Human IgG-HRP conjugate from -25 to -10°C storage. Warm conjugate in hand for a couple minutes to help conjugate reach room temperature before dispensing.

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



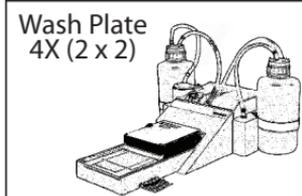
Note: For a partial plate, prepare 1 mL for each strip to be used plus an extra mL (e.g. transfer 7 µL into 7 mL for 6 strips). In any event, a minimum of 6 ml should be prepared to ensure accurate volume measurement.

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

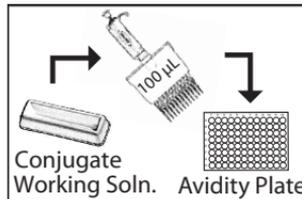


2.12 Transfer the Conjugate Working Solution to a reagent reservoir.

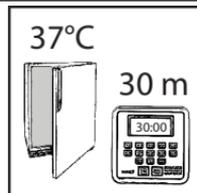
2.13 Immediately after the 15 minute Dissociation Buffer incubation, wash the plate 4 times with 1X Wash Buffer as described in Section 2.6, page 15.



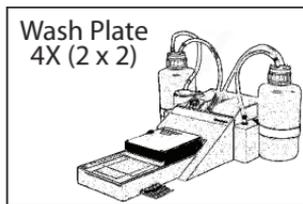
2.14 Transfer 100 µL per well of the Conjugate Working Solution from the reservoir to the washed Avidity Plate using a multichannel pipette.



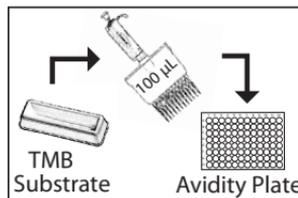
2.15 Apply a Plate Sealer to the Avidity Plate with Conjugate Working Solution and incubate for 30 minutes at 37°C (±2°C).



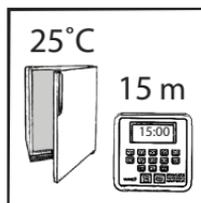
- 2.16 After the 30 minute incubation with the Conjugate Working Solution, wash the plate 4 times with 1X Wash Buffer as described in Section 2.6, page 15.



- 2.17 Take the TMB Substrate out of the 25°C incubator and transfer to a reagent reservoir. Use a multichannel pipette to transfer 100 μ L of TMB Substrate to each well.

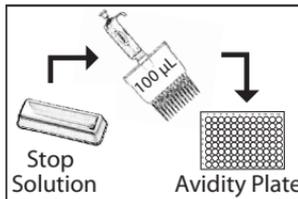


- 2.18 Incubate the Avidity Plate with TMB Substrate for **EXACTLY** 15 minutes at 25°C ($\pm 2^\circ$ C). Do not cover the plate with a Plate Sealer.

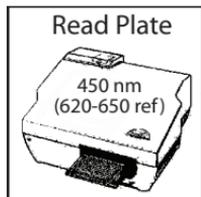


- 2.19 Following the 15 minute TMB Substrate incubation, stop the reaction by adding 100 μ L of Stop Solution per well using a multichannel pipette.

Note: Do not wash the plate before adding the Stop Solution.



- 2.20 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

	NC	CAL	LPC	HPC
Minimum	0.000	0.400	0.190	0.830
Maximum	0.175	0.950	0.520	1.820

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 (OD_n) for each Control, the Calibrator, and each specimen. The OD_n is calculated by dividing the OD value 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].

$$\text{OD}_n = \frac{(\text{OD of sample, Control, or Calibrator})}{(\text{median OD of Calibrator})}$$

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

Acceptable OD_n Ranges

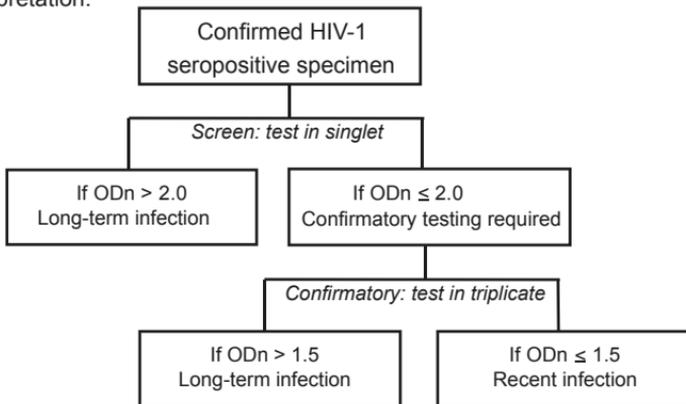
	NC	CAL	LPC	HPC
Minimum	0.000	1.000	0.370	1.500
Maximum	0.240	1.000	0.700	2.400

- 3.5 A customized spreadsheet is available from CDC and at <http://www.sediabio.com/products/lag-avidity-eia> to validate the run and calculate ODn.

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 ≤ 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.



- 4.2 If the ODn of a specimen obtained during screening or confirmatory mode is ≤ 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 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

NC	HPC	2	5	8	10	13	16	18	21	24	26
NC	HPC	3	5	8	11	13	16	19	21	24	27
CAL	HPC	3	6	8	11	14	16	19	22	24	27
CAL	1	3	6	9	11	14	17	19	22	25	27
CAL	1	4	6	9	12	14	17	20	22	25	28
LPC	1	4	7	9	12	15	17	20	23	25	28
LPC	2	4	7	10	12	15	18	20	23	26	28
LPC	2	5	7	10	13	15	18	21	23	26	BL*

*BL = Blank Well

5.2 Upon completion of confirming a set of specimens by the Sedia® HIV-1 LAg-Avidity EIA, 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 of the Sedia® HIV-1 LAg-Avidity EIA conducted by the U.S. CDC and CEPHIA indicate that a cutoff for ODn values of 1.5 represents a mean duration of recent infection of 130 days (95% CI 118-142) [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 U.S. 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

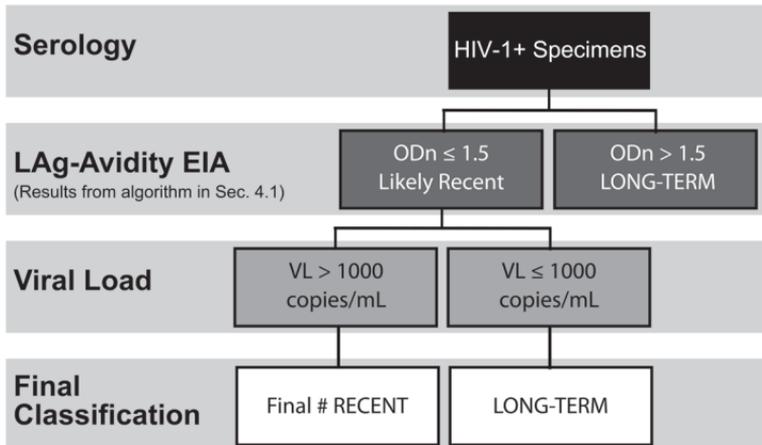
Classification of individuals by the Sedia® HIV-1 LAg-Avidity EIA as recent seroconverters or long-term infections is based on average development of higher avidity HIV-antibodies calculated from data using a large number of people [1, 35]. However, there are differences among individuals in terms of 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. Accordingly, this assay is less likely to be affected by disease states or low CD4 counts as observed with other such assays [36]. In fact, initial results suggest that the false recency rate (FRR) of the Sedia® HIV-1 LAg-Avidity EIA is significantly lower than other methods [41,43]. Persons with diagnosis of AIDS or low CD4+ T cell counts (below 200 cells per μ l), recipients of anti-retroviral therapy (“ART”) and known “elite controllers” (HIV-infected individuals with known low or undetectable viral loads) appear to contribute to the misclassification of long-term infections, albeit at a lower rate than other assays. Such individuals, if known, should be excluded from the study populations. Alternatively, use of the recommended algorithm below will reduce the impact of these individuals.

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 [47], U.S. CDC [35] and CEPHIA [48], 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 [48].

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:

$$I_F = \frac{R - (FRR * P)}{(1-FRR) \omega N} \times 100$$

- I_F = annual HIV incidence rate
- N = number of HIV-negative samples in the survey
- P = number of HIV-positive samples in the survey
- R = number of HIV-positive samples testing as recent on the test
- ω = mean duration of recency for the test specified in years
- FRR = false recent rate for the test

Estimates of both ω 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 includes: 1) a calculation of the local FRR within an acceptable level of precision 2) detection of individuals on ART 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 U.S. CDC, Division of Global HIV-AIDS. Contact Joyce 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>.

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SYMBOLS AND ABBREVIATIONS

The following symbols appear in Sedia® HIV-1 LAg Avidity EIA product labeling.



Use by (expiration date)



Corrosive



Consult instructions for use



Temperature limitation
(*temperature storage range*)



Part number



Temperature limitation
(*max. temperature storage*)



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 Dried Blood Spot Specimens (Cat. No. 1003)

Asante™ HIV-1 *Rapid Recency*® Assay (Cat. No. 1130)

Asante™ Rapid Test Strip Reader (Cat. No. 1200)

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