CEPHIA

Consortium for the Evaluation and Performance of HIV Incidence Assays

SEDIA™ HIV-1 LAg-Avidity EIA

Evaluation Report

Blood Systems Research Institute

Public Health England

Bill & Melinda Gates foundation

UCSF University of California San Francisco advancing health worldwide

SACEMA

DST/NRF Centre of Excellence in Epidemiological Modelling and Analysis

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Evaluation of Sedia™ HIV-1 LAg-Avidity EIA

Single Well Avidity Enzyme Immunoassay for Detection of Recent Infection.

Cat. No. 1002

CEPHIA PROJECT TEAM

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Summary -SEDIA™ HIV-1 LAg-Avidity EIA

Background

Monitoring the prevalence of HIV provides a blunt tool for understanding both recent transmission rates and the impact of behavioural changes or public health interventions on these rates. Consequently, there has been increasing application of assays, which are able to distinguish between 'recently' acquired HIV-1 infections and 'long-standing' infections, in cross-sectional surveys, to estimate HIV incidence. A comparative analysis of these existing incidence assays is a logical and necessary next step to facilitate the introduction of HIV incidence assays into wide use.

Evaluation Panel

The 'evaluation panel' consists of 2,500 uniquely-labelled HIV+ve plasma specimens obtained from 928 distinct subjects, and was provided in 5 sets of 500 specimens each. 75 of these specimens represent 25 aliquots of each of 3 underlying specimens, and acted as (unmarked) controls. Laboratories were blinded to the specimen background information.

Data Analysis

The assay characteristics, namely the mean duration of recent infection (MDRI – average time 'recent' while infected for less than some time T) and false-recent rate (FRR – probability of a 'recent' result for an individual infected for longer than T), were estimated in a number of specimen sets. The MDRI (excluding treated subjects and identified elite controllers) is 188 days (95% CI: 165-211), for T=2 years and a Western blot HIV diagnostic test. The FRR in this specimen set is 1% (95% CI: 0-4%). High FRRs occur amongst treated subjects (>50%), elite controllers (>10%) and virally suppressed subjects (>45%).

Technical Appraisal

This assay is a commercially available assay developed specifically for the purpose of differentiating recent from long standing infections for use in studying cross-sectional studies. It is a manual EIA and requires apparatus available to most laboratories. There are two reagent packs one of which requires storage at -20^{o}C and the other at 4^{o}C. An EQA scheme and training in performance of the assay is available from CDC, Atlanta. Data management software for interpretation of the assay is available from the manufacturer. The assay is simple to perform following training.

Conclusions

This does not fulfil all components of the Target Product Profile (TPP) for use in cross sectional incidence assays. We do not recommend its use as a standalone assay but feel it may be useful as part of an Incidence assay algorithm.

Background

It has become recognized that monitoring the current burden or prevalence of HIV (the fraction of the population infected at a point in time) provides a blunt tool for understanding both recent transmission rates and the impact of behavioural changes or public health interventions on these rates. Consequently, there has been increasing application of tests, which are able to distinguish between 'recently' acquired HIV-1 infections and 'long-standing' infections, in cross-sectional surveys, to estimate HIV incidence (the rate of new infections). The term Recent Infection Test Algorithm (RITA) has been coined to describe assays, or combinations of assays and other (clinical) criteria, that are able to identify 'recent' HIV infection. A highly sensitive HIV diagnostic test is used to identify HIV-positive subjects in the survey, and then the RITA (which could make use of any of a number of assays or biomarkers) is applied to the specimens drawn from these HIV-positive subjects. Typically, the signal of the biomarkers that are measured by the RITA gradually increase over a period of several months following primary HIV infection, and infections are classified by reference to thresholds on the biomarker readings.

It has been recognized at various meetings of the World Health Organisation (WHO) Technical Working Group on Incidence Assays that a statistically sound comparative analysis of existing incidence assays is a logical and necessary next step to facilitate the introduction of HIV incidence assays into wide use. In 2011, The Bill & Melinda Gates Foundation funded a project called '*Development of specimen repository and evaluation of assays for identification of recent HIV infection and estimation of HIV incidence'* to help achieve this aim.

CEPHIA

The **Consortium for the Evaluation and Performance of HIV Incidence Assays** (CEPHIA) brings together world leaders in the development, performance evaluation and application of RITAs for identifying 'recent' HIV infection. CEPHIA's purpose is to successfully deliver the Bill & Melinda Gates Foundation funded project, to advance the understanding and performance of currently available assays, and to better describe the duration of time for which assays classify infections as 'recently' acquired and the rate at which they (mis)classify infections of long-infected subjects as 'recent'.

Specific project objectives are to evaluate and compare currently available incidence assays using a common set of specimens collected for this purpose; and to assess the ability of the assays, alone or in combination, to accurately and precisely estimate HIV incidence in populations.

An overview of CEPHIA, related documentation and updates are available at http://www.incidence-estimation.com/page/cephia (1).

Appendix 2 details CEPHIA group members.

Introduction

As part of the Bill & Melinda Gates Foundation funded project, 'Development of specimen repository and evaluation of assays for identification of recent HIV infection and estimation of HIV incidence', the CEPHIA group undertook evaluations of a number of available assays. This report details the results of the evaluation of the **Sedia™ HIV-1 LAg-Avidity EIA**.

A CEPHIA 'qualification panel' of specimens was used for preliminary assessment of the assay's potential for determining HIV recency, before a full assessment was undertaken using an 'evaluation panel' of specimens.
CDC were responsible for providing the kits and test method used for the evaluation. Testing was performed by four CEPHIA approved testing sites which all completed a CDC hosted training session in the use of the assay prior to undertaking the evaluation.

1. PHE – Public Health England, Microbiology Services, Colindale, London, UK
2. BSRI - Blood Systems Research Institute, San Francisco, California, USA
3. CDC - Centers for Disease Control, Atlanta, Georgia, USA
4. JHU – John Hopkins University, Baltimore, USA

The results of this analysis are discussed in 'The performance of candidate assays to detect recent HIV infection for cross-sectional incidence estimation: an independent, comparative evaluation'. Poster 1056, *20th Conference on Retroviruses and Opportunistic Infections* [2].

Full evaluation of the **Sedia™ HIV-1 LAg-Avidity EIA** was considered justified following review by the CEPHIA management team of data analysis from the Qualification Panel evaluation. Evaluation Panel testing was performed at Public Health England, Microbiology Services, Colindale, London.

The 2,500 HIV+ve plasma specimens used for the evaluation were sourced by the CEPHIA team at UCSF – University of California and comprised a wide range of suitable and challenging specimen types. Tables 3 - 5 summarises the specimen types used in the evaluation.

All evaluation data was analysed by the CEPHIA team at SACEMA - South African Centre for Epidemiological Modelling and Analysis, Stellenbosch, South Africa

This evaluation aims to advance the understanding and performance of currently available assays, and to better describe the duration of time for which assays classify infections as 'recently' acquired and the rate at which they (mis)classify infections of long-infected subjects as 'recent'. The reported analysis below focuses on estimation of the characteristics of the incidence assay, namely the mean duration of recent infection (average time spent 'recently' infected) and false-recent rate (proportion of long-infected subjects who are classified as 'recently' infected), for various subpopulations. Standard operating procedures for, and experiences in, the laboratory application of the incidence assay are also discussed.

SEDIA ™ HIV-1 LAg-Avidity EIA Information

Description of Assay

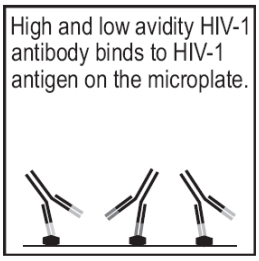
The SEDIA ™ HIV-1 LAg-Avidity EIA is an in vitro quantitative limiting antigen (LAg) avidity enzyme immunoassay for distinguishing recent HIV-1 infections from those which are long-term. Persons with recently acquired HIV-1 infections typically have lower avidity HIV IgG than those with long-term infections. The test measures HIV-1 antibody avidity in blood samples including plasma and serum [1, 40]. This assay kit is not for use with dried blood spot specimens. Users who which to test dried blood spot specimens must use SEDIA ™ HIV-1 LAg-Avidity EIA for dried Blood Spots (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 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. **(SEDIA ™ HIV-1 LAg-Avidity EIA Kit Insert LN 6039.05**). In preparation of this report the kit insert available at the time the assays were performed and initial analysis undertaken was used. Sedia have informed us that an updated kit insert is now available.

Summary and explanation of the test

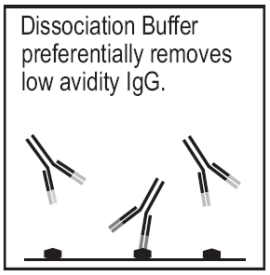
The Sedia™ HIV-1 LAg-Avidity EIA measures HIV-1 antibody avidity and determines the recent/long term HIV-1 status by referencing the EIA numerical result against that of an internal calibrator specimen. The principle of the test is based on the observation that in response to exposure to the HIV-1 virus, 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 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]. The effect of anti-retroviral 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 and have a mean duration of recent infection of 130 days (95% CI 118-142) [35]. **(Taken from kit insert LN 6039.05).** CEPHIA is grateful to Sedia for permission to reproduce parts of their kit insert in this report. Sedia have informed us that an updated kit insert is now available

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.

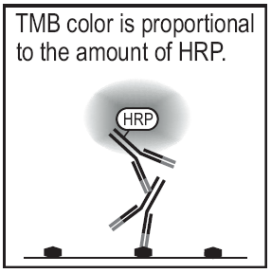


2. *Dissociation Buffer is added and incubated for 15minutes at 37°C to preferentially remove low avidity IgG from the antigen-coated plate.*



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 colour 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.*
   (Taken from kit insert LN 6039.05)

General Kit Information

The SEDIA ™ HIV-1 LAg-Avidity EIA is comprised of two component boxes of matching lot numbers that have separate storage requirements (frozen and refrigerated). The kit contains two 96-well plates with twelve (12) 1 x 8 removable strips and all the necessary reagents to run the assay. **(Taken from kit insert LN 6039.05)**

A summary of the characteristics of the SEDIA ™ HIV-1 LAg-Avidity EIA assay is given in Table 1. The table includes details relating to the kit such as product number, volumes required, completion times, antigens/antibodies used, and the controls/calibrators used. Table 2 quotes claims stated by the manufacturer in the provided kit insert regarding the performance of the assay and its limitations.

SEDIA™ HIV-1 LAg-Avidity EIA Kit Images

These images are taken from the assay used for evaluation. Assay packaging and labelling has been updated

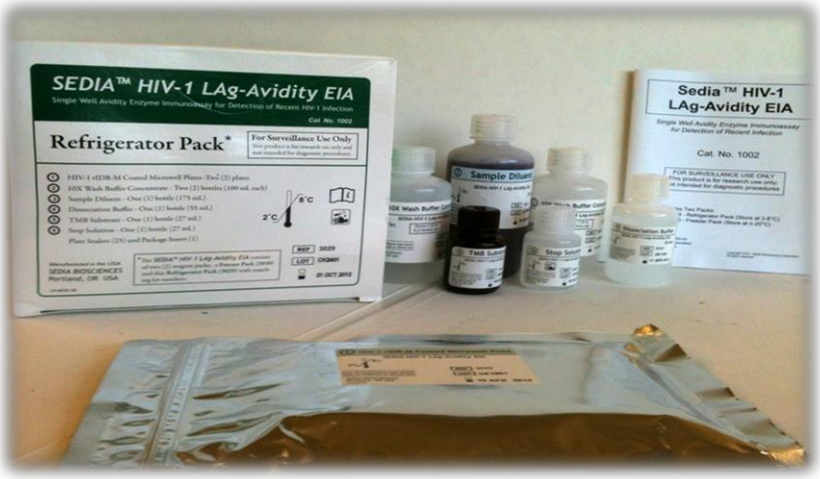


Table 1: Assay Information Summary

General	
Assay Name	**SEDIA ™ HIV-1 LAg-Avidity EIA**
Manufacturer	SEDIA Biosciences Corporation
Catalogue Number	1002
Number of Specimens can test/Kit – Screen mode	170 (Screen Mode), 56 (Confirmatory Mode)
Test Volume – Screen mode	5µl (Screen Mode), 5µl x 3 (Confirmatory Mode)

Presentation	
Assay type	In vitro quantitative limiting antigen (LAg) avidity enzyme immunoassay
Refrigeration Pack	Store at 2-8°C
Antigens (coated microwell plates)	Multi-subtype recombinant HIV-1 Ag (rIDR-M)
Dissociation Buffer	Contains dissociation agent in acidic buffer
10X wash buffer concentrate	Phosphate buffered saline, detergent and preservative
Substrate	Contains 3,3',5,5' tetramethyl-benzidine (TMB) in acidic buffer
Stop Solution	Contains dilute acid solution
Reading wavelength	450nm with reference wavelength 620-650nm
Freezer Pack	-25°C to -10°C
Conjugate	Goat Anti-Human IgG conjugated to horseradish peroxidase (HRP)
Calibrator (CAL)	Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
Low Positive control (LPC)	Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
High Positive control (HPC)	Inactivated human serum reactive to HIV-1 antigens. Non-reactive for HBsAg and antibodies to HCV. Contains preservative.
Negative control (NC)	Inactivated human serum non-reactive for HBsAg and antibodies to HCV and HIV. Contains preservative.

Stages	
Reagent Preparation time	60minutes to reach room temp
Specimen Pre-dilution set-up time	30 minutes
Sample/Dissociation/Conjugate/Substrate incubations	60mins/15mins/30mins/15mins
Total time to completion	3.5 hours

Additional Equipment Required	
Serological pipettes and tips	single (2-20ul, 10-100ul), multichannel (200ul)
Positive displacement pipette or microliter	Required to measure out conjugate concentrate

syringe capable of delivering 5-20µl	
Polypropylene tubes	1.2ml 'titertubes' 12-15ml with cap
Graduated cylinders	100ml, 1000ml
Reagent reservoirs	
Incubators	37oC (±2oC) and 25oC (±2oC)
Vortex Mixer	
Microwell Plate Washer	Either 96-well or strip
Spectrophotometer	450nm with reference filter 620-650nm
Household bleach and Biohazardous waste container	
Personal Protective Equipment (PPE)	Latex gloves, protective safety glasses, lab coat

Table 2: Manufacturer Claims for the assay and its limitations

Claims for the assay (from kit insert LN 6039.05)
Testing conducted by the U.S. Centers for Disease Control indicates that a cut-off 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. Test attributes, including reproducibility, inter-run and intra-run coefficient of variation (CV), and inter-operator 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 (from kit insert LN 6039.05)
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 cut-off). Therefore, the assay should not be used for individual assessment of recency of infection. This assay is based on a 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 thus less likely to be affected by disease states. Viral load or low CD4 counts as observed with other such assays [36]. 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.

Evaluation Panel and Method

The 'evaluation panel' consists of 2,500 uniquely-labelled HIV positive plasma specimens obtained from 928 distinct subjects, and was provided to laboratories in 5 sets of 500 specimens each. 75 of these specimens represent 25 aliquots of each of 3 underlying specimens, and acted as (unmarked) controls. Laboratory technicians were blinded to the specimen background information.

Evaluation panel testing is intended to provide the relevant data to estimate assay characteristics, assess and compare assay performance, and optimize the algorithms of assays and biomarkers used in RITAs, for purposes of estimating HIV incidence.

Tables 2 to 6, and Figure 1, describe the sources and characteristics of specimens included in the evaluation panel.

The CEPHIA 'evaluation panel' was tested by SEDIA ™ HIV-1 LAg-Avidity EIA following the procedure and validations detailed in the kit insert supplied with the Assay kits. The current kit insert is available from http://www.sediabio.com/products/lag-avidity-eia

Prior to beginning the evaluation the evaluator(s) attended a training workshop provided by the assay developer – U.S. Centers for Disease Control (CDC) to ensure evaluators were properly trained in the use of the kit and equipment.
The evaluation was conducted under the strict quality requirements as laid out in the CEPHIA Quality Management Strategy (Document 002). Refer to Appendix 1 – Evaluation Protocol for further details.

Two different Kit Lots of SEDIA ™ HIV-1 LAg-Avidity EIA were used during the evaluation. The kit insert supplied with each lot differed slightly but there were no major procedural differences.
KIT LOT: CK2401 supplied with Kit Insert LN 6039.02
KIT LOT: DE2501 supplied with Kit Insert LN 6039.03

Assay Kits were stored as requested; the Refrigerator pack was stored at 2-8°C and the Freezer Pack was stored at or below -20°C. This was the case until the manufacturers advised a change in storage conditions for the freezer pack from ≤-20°C, to -25°C to -10°C on 27th February 2013 (please note that this is after the testing of the Evaluation Panel was complete). The change in storage temperature was advised because "storage of temperatures below -25°C may result in freezing of the conjugate and repeated freeze/thawing of the Conjugate may result in reduced performance of the assay". All reagents (except conjugate concentrate) were allowed to reach room temperature before the test was run. The TMB was stored in a 25°C incubator until use.

All equipment (plate washers, pipettes, readers and incubators) had undergone proper installation, operation and performance/monitoring qualification prior to testing to minimise assay variability.

Table 2: Source of Specimens used in the Evaluation Set

Type of partner site	Site Name	Location of specimen draws
Seroconverter Cohorts		
US and Brazil cohorts enrol subjects diagnosed with acute HIV seroconversion. *IAVI Protocol C enrols subjects who seroconvert during participation in an HIV incidence cohort study.* *All cohorts follow subjects both prior to and after antiretroviral therapy.*	UCSF Options Project UCSD Acute HIV Study AMPLIAR IAVI Protocol C	San Francisco San Diego Brazil Kenya Rwanda Uganda South Africa Zambia
HIV positive cohorts		
SCOPE enrols HIV positive men and women both on and off ARV treatment, actively recruits elite controllers, and follows these subjects over time. *SFMHS enrolled both HIV negative and HIV Positive men from a population-based sample in SF and followed these subjects forward over time.*	SCOPE San Francisco Men's Health Study (SFMHS)	San Francisco
Blood Banks		
Blood banks identify repeat blood donors with a negative blood donation followed by a subsequent HIV positive donation.	American Red Cross Blood Centers of the Pacific South Africa National Blood Services (SANBS) Hemocentro do Sao Paulo	United States South Africa Brazil

Table 3: Demographic / infection characteristics of subjects contributing specimens to the evaluation panel

Subject/ specimen group	Number of subjects (% of subjects)	Number of specimens (% of specimens)
All subjects	928 (100)	2500 (100)
Gender		
Male	728 (78)	1872 (75)
Female	194 (21)	547 (22)
Country of specimen draws		
USA	523 (56)	1298 (52)
Zambia	166 (18)	508 (20)
Rwanda	65 (7)	281 (11)
Uganda	62 (7)	200 (8)
Brazil	18 (2)	85 (3)
South Africa	58 (6)	64 (3)
Kenya	36 (4)	63 (3)
Age at draw (years)		
<20	28 (3)	49 (2)
20-30	231 (25)	566 (23)
30-40	357 (38)	887 (35)
40-50	270 (29)	635 (25)
50-60	92 (10)	240 (10)
>60	21 (2)	45 (2)
HIV Subtype[1]		
B	525 (57)	1247 (50)
C	250 (27)	670 (27)
A1	92 (10)	290 (12)
D	42 (5)	157 (6)
Other	19 (2)	135 (5)

[1] 42% of subjects (capturing 52% of specimens) had their infection subtypes confirmed through laboratory testing, while the remainder of subtypes were based on the majority subtype in country of specimen draw.

Figure 1: Number of specimens drawn over time per subject, for specimens included on the evaluation panel

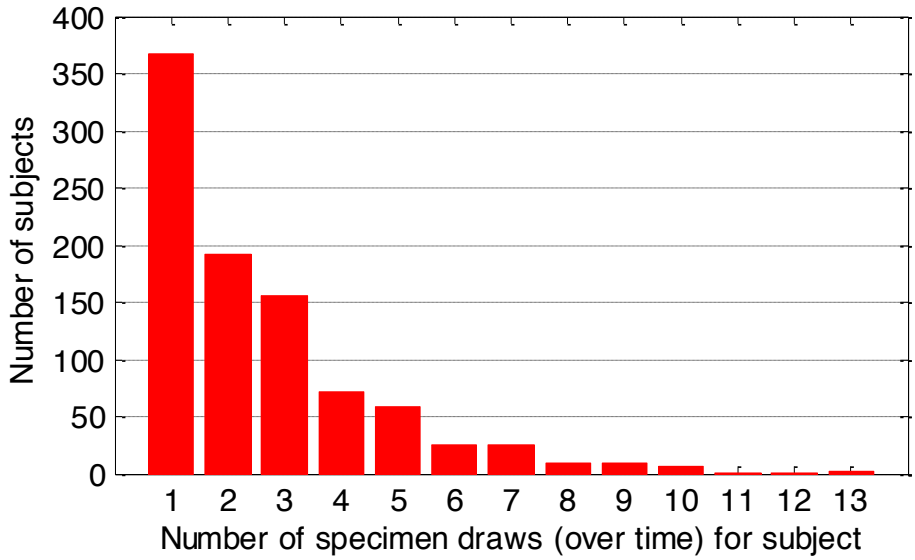


Table 4: Times from (estimated) infection to specimen draws for ARV-naïve subjects included in the evaluation panel (for estimation of the MDRI), stratified by subtype[1]

	All subtypes	Subtype B	Subtype C	Subtype A1	Subtype D
Subject/ specimen group	Number of subjects				
Subject has estimable infection date[2]	422	104	185	83	38
<1 year duration of infection (DOI) at specimen draw	283	59	145	39	33
1-2 years DOI	224	39	105	54	19
2-3 years DOI	125	22	56	34	11
3-4 years DOI	72	15	33	19	4
4+ years DOI	41	14	19	7	0
Subject/ specimen group	Number of specimens				
Subject has estimable infection date[2]	1386	344	588	263	149
<1 year duration of infection (DOI) at specimen draw	671	164	308	71	103
1-2 years DOI	346	70	146	93	27
2-3 years DOI	189	42	72	58	14
3-4 years DOI	104	28	40	29	4
4+ years DOI	66	34	20	11	0

[1] Elite controllers (defined in *Analysis of Assay Characteristics*) from SCOPE (see Table 2) are excluded as the study specifically recruited (untreated) subjects with sustained low HIV viral loads, and therefore data would otherwise be over-enriched with elite controllers.

[2] Infection refers to the time of positivity of Western blot. See *Analysis of Assay Characteristics* for the approach used for estimating infection times, and for identifying subjects with estimable infected times, for this particular analysis.

Table 5: Description of specimens from ARV-naïve long-infected subjects included in the evaluation panel (for estimation of the FRR), stratified by subtype[1]

	All subtypes	Subtype B	Subtype C	Subtype A1	Subtype D
Subject/ specimen group	Number of subjects				
Subject infected for greater than 1 year[2]	456	243	121	62	21
Subject infected for greater than 2 years[2]	316	190	75	37	11
Subject infected for greater than 3 years[2]	224	156	42	20	4
Subject infected for greater than 4 years[2]	161	137	18	6	0
Subject infected for greater than 5 years[2]	111	111	0	0	0
Subject/ specimen group	Number of specimens				
Subject infected for greater than 1 year[2]	1112	538	297	210	47
Subject infected for greater than 2 years[2]	665	388	144	106	18
Subject infected for greater than 3 years[2]	416	301	63	43	4
Subject infected for greater than 4 years[2]	285	256	19	10	0
Subject infected for greater than 5 years[2]	192	192	0	0	0

[1] Elite controllers (defined in *Analysis of Assay Characteristics*) from SCOPE (see Table 2) are excluded as the study specifically recruited (untreated) subjects with sustained low HIV viral loads, and therefore data would otherwise be over-enriched with elite controllers.

[2] Specimen drawn at least 1, 2, 3, 4 or 5 years (see row labels) after a first recorded HIV-positive diagnosis.

Table 6: Description of *challenge* specimens drawn from subjects infected for greater than 2 years included in the evaluation panel (for estimation of the FRR)[1]

	All subtypes[2]	
Subject/ specimen group	**Number of subjects**	**Number of specimens**
SCOPE elite controllers[2]	31	89
CD4 cell count < 200 at draw	124	214
Treated subjects[3]	113	185
Treatment initiated within 6 months of infection	53	90
Treatment initiated 6-24 months after infection	17	28
Treatment initiated >24 months after infection	33	54
Viral load < 75 copies/ml	154	273

[1] 98% of subjects (or specimens) represent subtype B infections.
[2] Subjects were identified as elite controllers by SCOPE (classification rules are outlined in defined in *Analysis of Assay Characteristics*).
[3] Treated for at least 3 months and without interruption.

Analysis of Assay Characteristics

The methods and results outlined below are reported in the following journal article: 'Kassanjee R, Pilcher CD, Keating SM, Facente SN, McKinney E, Price MA, Martin JN, Little S, Hecht FM, Kallas EG, Welte A, Busch MP, Murphy G, on behalf of the *Consortium for the Evaluation and Performance of HIV Incidence Assays* (CEPHIA); Independent assessment of candidate HIV incidence assays on specimens in the CEPHIA repository [3].

Definitions of Assay Characteristics

In 1995, Brookmeyer and Quinn [4] introduced the concept of cross-sectional HIV incidence estimation: incidence can be measured from a single survey conducted a point in time using both (i) observed survey counts of HIV-negative, 'recently' infected and 'non-recently' infected subjects, and (ii) knowledge about the dynamics of the test for recent infection. However, the state of 'recent' infection demonstrated in their work (namely, detectability of p24 antigens in the absence of detectable HIV antibodies) occurs for only a few weeks after infection, resulting in unrealistically large surveys being required for precise incidence estimation. Subsequently, various tests, with more enduring states of 'recent' infection, have been proposed. However, the behaviour of currently available tests has been imperfect – due to inter-subject variability, a substantial proportion of long-infected individuals nevertheless return 'recent' results.

As the methodology has matured, a general theoretical framework has been derived, which consistently accounts for these 'false-recent' results [5]. Two test characteristics that summarise test dynamics emerge as required for purposes of incidence surveillance:

- the mean duration of recent infection (MDRI), Ω_T, which is the average time spent alive and 'recently' infected, while infected for less than some time cut-off T, and
- the false-recent rate (FRR), β_T, which is the probability that an individual who is infected for longer than T will return a 'recent' result.

This general framework was developed by introducing a post-infection time cut-off, T, to separate 'true-recent' from 'false-recent' results. In a cross-sectional survey, the estimate of incidence would be

$$\hat{I}_T = \frac{n_R - \hat{\beta}_T n_+}{n_S \cdot \left(\widehat{\Omega}_T - \hat{\beta}_T T\right)},$$

where n_+ and $n_S = n - n_+$ are the counts of HIV-positive and HIV-negative (or susceptible) subjects in the survey, n_R is the number of 'recently' infected subjects in the survey, and $\widehat{\Omega}_T$ and $\hat{\beta}_T$ are the estimated MDRI and FRR for the test for recent infection respectively.

This analysis focuses on estimation of the MDRI and FRR. As the characteristics of incidence assays may vary across subpopulations, the characteristics are explored using various specimen sets.

Data Analysis Methods

Software. All data captured within CEPHIA are stored in a MySQL relational database. Database queries linked assay results to the background information on subjects and specimens for data analysis, which was then performed in Matlab (R2013b, the MathWorks Inc.).

Interpretation of assay results. The LAg results were interpreted according to developer's guidelines (see standard operating procedures on the CEPHIA project website [1]. In particular, a 'recent'/'non-recent' threshold of 1.5 was used to discriminate between 'recent' and 'non-recent' infection, with a measured normalised optical density (ODn) of 1.5 or less interpreted as indicating 'recent' infection.

Stratification of data. Assay characteristics were estimated using specimen sets defined by stratifying on treatment history, viral load, CD4+ T cell count, time from infection to specimen draw, and HIV subtype (which was based on country of draw, for the 48% of specimens which lack explicit laboratory subtype confirmation). The characteristics of assays in 'elite controllers' (ECs), broadly defined as subjects who maintain undetectable or very low HIV viral loads without antiretroviral therapy (ART), is of particular interest. As the SCOPE study purposefully recruited ECs, this data was analysed separately. These subjects were ART-naïve (or without ART for at least 6 months), with all off-treatment viral load measurements (HIV-1 RNA) below 200 copies/ml and at least 50% of these measurements below 75 copies/ml.

Time cut-off T. The definitions of the MDRI and FRR rely on the previously mentioned construct of a post-infection time cut-off, T. If T is chosen to be too short, this limits the possible MDRI and typically raises the FRR. If T is chosen to be too long, it becomes difficult to obtain sufficient data to analyse the test dynamics with sufficient precision over this time after infection, and the MDRI will also develop variation by time and place (properties inevitable for the FRR) rather than capture stable biological properties of the test. A cut-off of $T = 2$ years is used throughout this work. The value of T was increased from 1 year, as used in preliminary analyses [2], to 2 years in this analysis, to better capture the tails of persisting 'recent' results and thus reduce FRRs.

Definition and estimation of infection times. In practice, the notion of 'infection' implicit in the assay characteristic definitions refers to 'detectable infection' – which depends on the particular HIV diagnostic test used in the incidence study. In this analysis, 'detectable infection' was defined as the time of seroconversion on an HIV viral lysate-based Western Blot assay. Based on the methodology summarised below, infection times were estimated for 56% of subjects.

The estimation of a subject's infection time relies on both data describing the subject's testing history and knowledge of the sensitivities of the various diagnostics tests used on the subject, where sensitivity captures the probability of detecting HIV in a (truly infected) subject as a function of time since detectability on the reference diagnostic test that is to be used in the incidence study (Western Blot in this case). In general, the formal likelihood of observing a subject's testing history can be directly generated as a function of time since

HIV infection (through vertical and horizontal inversion of the various diagnostic tests' sensitivity functions). Under prior assumptions about infection times, this likelihood function can then be used to produce an inferred posterior density function for possible infection times, which can then be used in analyses. Depending on available data, various simplifications of this estimation procedure could be considered.

For this analysis, infection times were estimated for subjects with available first HIV-positive test dates, Fiebig staging [6] information for first HIV-positive tests, and, at times, last HIV-negative test dates (within 120 days of first HIV-positive dates). The likelihood function for the infection time (corresponding to the time of entering Fiebig stage 5) of a subject was then calculated using the average durations of Fiebig stages presented in Lee et al [7] (neglecting inter-subject variability, assuming independence between diagnostic results for a subject, and making some assumptions about the types of diagnostics tests used at last HIV-negative test dates). A uniform prior for infection times was combined with the likelihood function, and the mean of the resulting posterior distribution for infection times provided a subject's estimated infection time, which was used in all subsequent analyses.

Efforts are currently being made to capture more detailed information on cohort-level diagnostic testing protocols and more complete testing histories of individual subjects, thus providing the required data to refine estimation of infection times for later analyses of assay results.

For subjects with unambiguous acute retroviral syndrome (ARS) symptoms onset dates between last HIV-negative and first HIV-positive test dates, infection was estimated to occur 17 days after ARS onset (based on the observation that the incubation period of ARS symptoms is about 14 days [8-11] and that the time from exposure to Western blot seroconversion averages 31 days [6,7]).

Estimation of MDRI. A number of methods can reasonably be used to estimate the MDRI, each with its own accuracy, precision and complexity – as explored in a separate, detailed benchmarking exercise (manuscript in preparation, by a working group operating on behalf of the HIV Modelling Consortium [12]). In this analysis, linear binomial regression, an approach found to be robust across a number of scenarios in this benchmarking project, and previously used for this purpose [13], has been applied. The model form is $g(P_R(t)) = f(t)$ where $P_R(t)$ is the probability of testing 'recent' at time t after infection, g is the chosen link function and $f(t)$ is a linear function of the model parameters, which are estimated by a maximum likelihood approach. Results from a 4-parameter model form are presented, where g is the logit link, and $f(t)$ is a cubic polynomial in t (Model A). Data points more than $1.1 \times T$ post infection were discarded before model fitting (Data Exclusion Rule I), with the aim of achieving the best fit of the model over $[0, T]$ post-infection, while avoiding diluting the data around the boundary at T. Sensitivity of results when increasing the data exclusion cut-off to $2 \times T$ (Data Exclusion Rule II) was also considered. Variation in results was explored when fitting two other model forms, namely (i) a more restrictive 2-parameter model where g is the log-log link and $f(t)$ is a linear function of $ln(t)$ (Model B), and (ii) a flexible 7-parameter model where g is the logit link and $f(t)$ is a linear function of the natural cubic spline basis functions with interior knots occurring every 3 months after

infection, between 0 and T after infection (Model C). In all cases, the MDRI, expressed mathematically as $\int_0^T P_R(t)\mathrm{d}t$, was estimated using the fitted $P_R(t) = g^{-1}(f(t))$.

To correctly account for the structure of the data, in the absence of explicit subject-level clustering in the fitted models, bootstrapping was performed by sampling subjects (not observations) with replacement. The 2.5th and 97.5th percentiles of 10 000 MDRI estimate replicates provided 95% confidence interval (CI) limits [14].

Estimation of FRR. A population-level FRR is inherently dependent on the epidemiological and demographic history of a study population, and so a set of specimens, such as in the CEPHIA repository, can only be used to estimate the FRR in well-defined subpopulations. Therefore, specimens from long-infected subjects were identified (specimens drawn at least T after the subject's first recorded HIV-positive test time – adjusted to capture Western blot positivity), and the proportion of 'recently' infected subjects estimated in each of the specimen sets described above. To capture subject-level clustering, when a subject provided more than one result to any FRR estimate, the most frequent classification was used. When a subject had equal numbers of 'recent' and 'non-recent' classifications, the subject contributed 0.5 to the count of subjects who have a majority 'recent' classification. Exact Clopper-Pearson 95% CIs [15] are provided.

Reproducibility statistics. The sample mean, standard deviation and coefficient of variation of the multiple assay measurements obtained for each of the unique reproducibility specimens, as well as each of the labelled quality controls, were also calculated.

Results

The incidence assay dynamics, excluding treated subjects and SCOPE elite controllers, are shown in Figures 2 to 4. The evolution of assay measurements by time since infection is shown in Figure 2 and Figure 3. In Figure 4, the proportion of 'recent' results (assay measurements below the 'recent'/'non-recent' threshold) is plotted by time since infection, also stratified by HIV subtype (B, C, D, and A1). The figures show that there is natural variability in biomarker maturation, leading to a significant number of subjects reaching the standard 'recent'/'non-recent' threshold more than one year after infection.

The distribution of assay measurements for specimens drawn more than $T = 2$ years after infection is shown in Figure 5, for various specimen sets.

Figure 2: Spaghetti plot of subjects' assay measurements as a function of (estimated) time since infection (years), excluding treated subjects and SCOPE elite controllers

The figure represents 1376 data points from 418 subjects. The 'recent'/'non-recent' threshold is shown by a horizontal solid line.

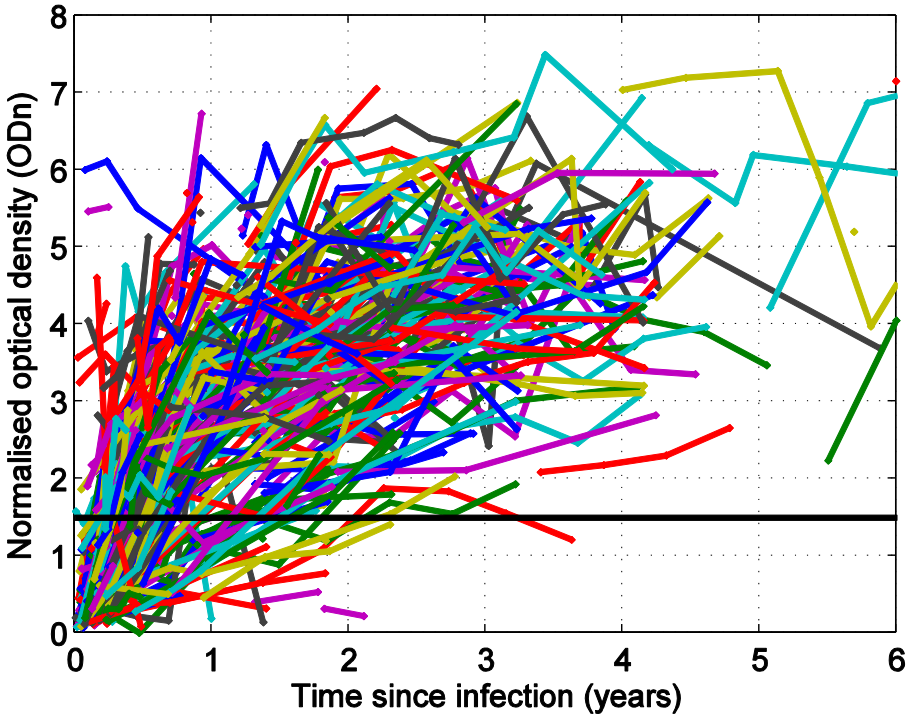


Figure 3: Box-and-whisker plots of assay measurements as a function of (estimated) time since infection (years), excluding treated subjects and SCOPE elite controllers

The plot indicates percentiles of measurements in 6-monthly intervals of time after infection. The central 50% and median of measurements are captured by the box and dividing line respectively, and whiskers and markers capture remaining measurements and outliers respectively. There are 40-450 data points per group. The 'recent'/'non-recent' threshold is shown by the horizontal solid line.

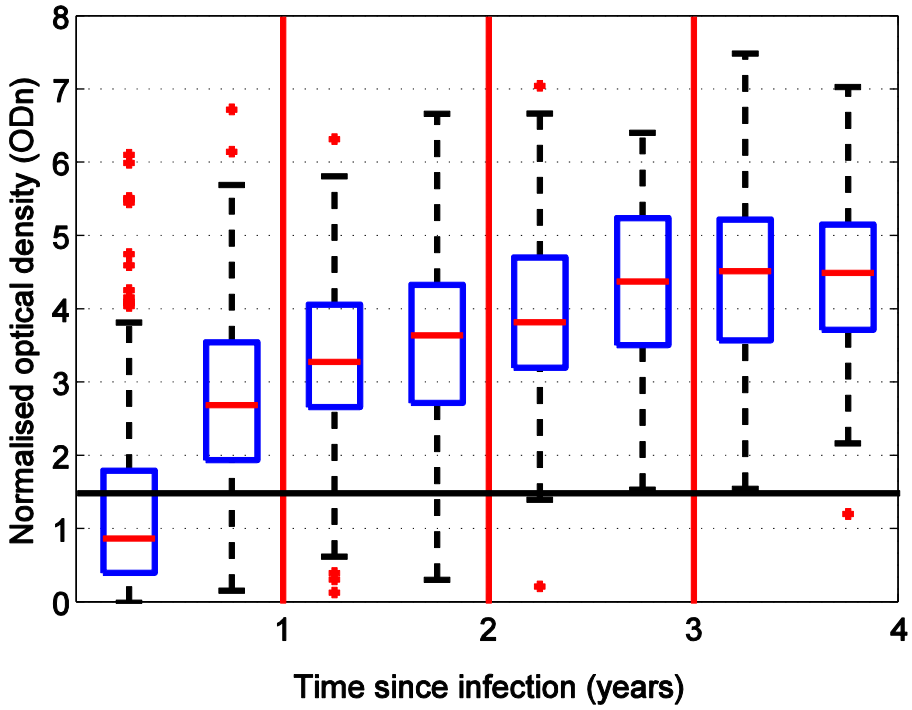
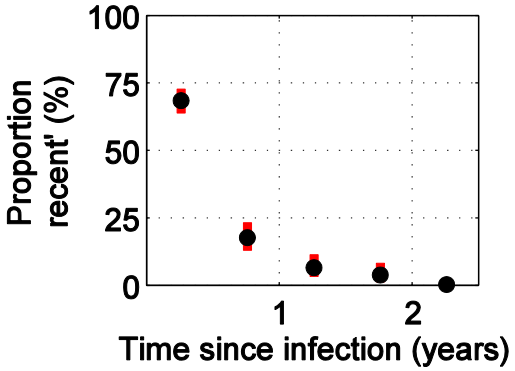


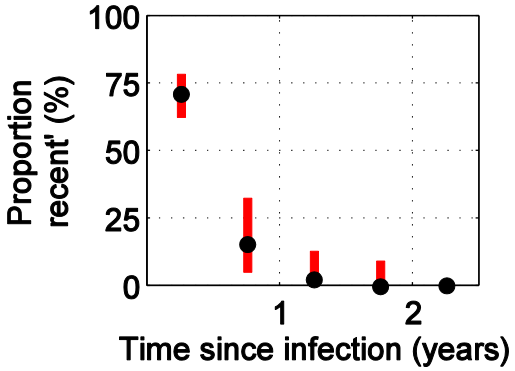
Figure 4: The proportion of 'recent' results (%) as a function of (estimated) time since infection (years), excluding treated subjects and SCOPE elite controllers and stratifying by HIV subtype (B, C, D and A1)

Circles and lines show observed proportions and 95% confidence intervals respectively. Specimens are grouped into 6-monthly intervals of time since infection until 2 years, after which all specimens are grouped together. There are 25 to 665 data points per group, except for subtype D which has fewer than 20 points 1-2 years after infection.

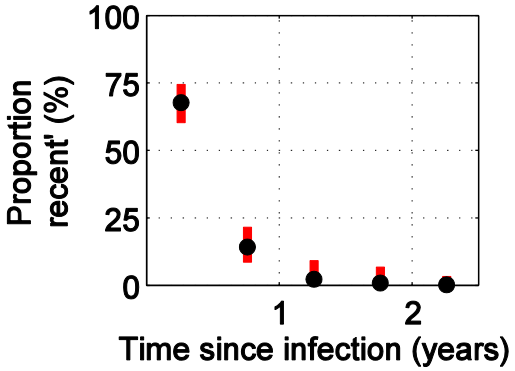
A. All subtypes



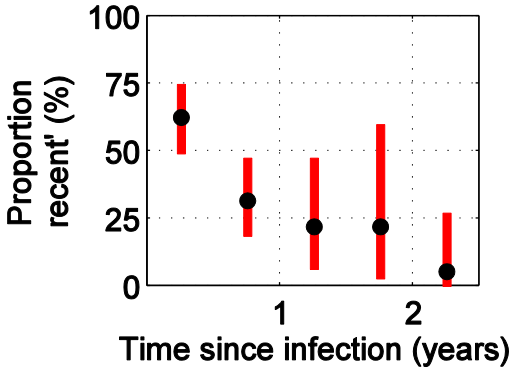
B. Subtype B



C. Subtype C



D. Subtype D



E. Subtype A1

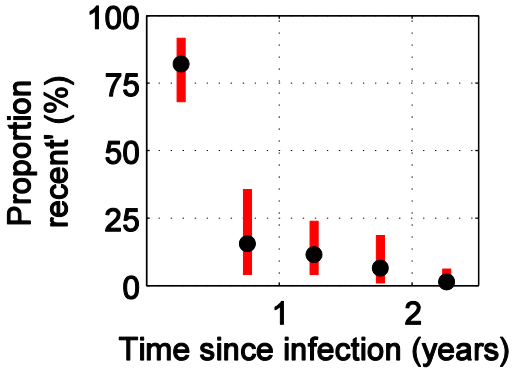
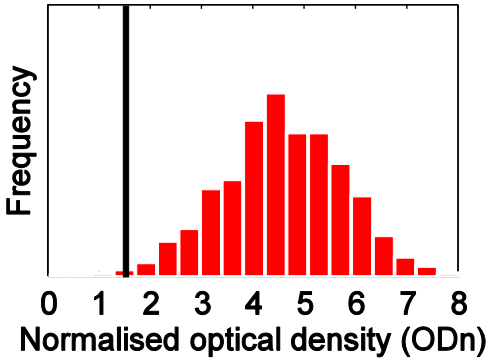


Figure 5: Empirical distribution of assay measurements for specimens drawn greater than $T = 2$ years after (estimated) infection time, by specimen set

'Recent'/'non-recent' thresholds are shown by vertical solid lines.

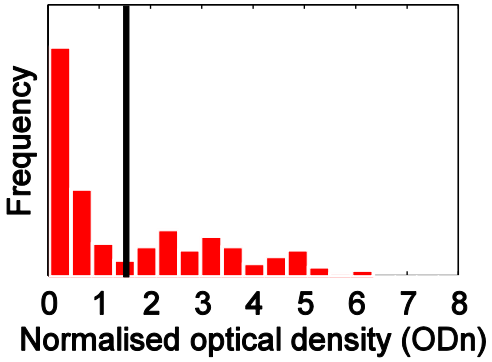
A. Excluding treated subjects and SCOPE elite controllers

665 data points from 316 subjects.



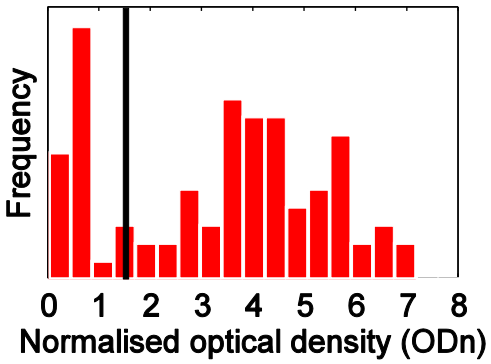
B. Treated subjects

Treated for at least 3 months without interruption. 185 data points from 113 subjects.



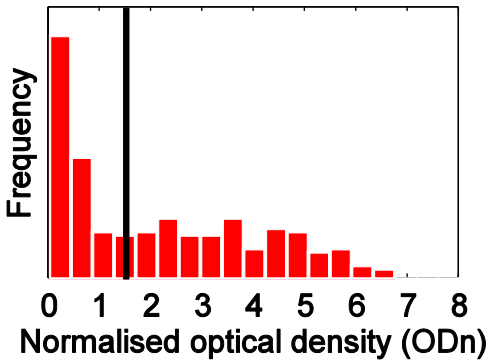
C. SCOPE elite controllers

89 data points from 31 subjects.



D. Low viral load

Viral load < 75 copies/ml at draw. 273 data points from 154 subjects.



E. Low CD4+ T cell count

CD4 cell count < 200 cells/µl at draw. 214 data points from 124 subjects.

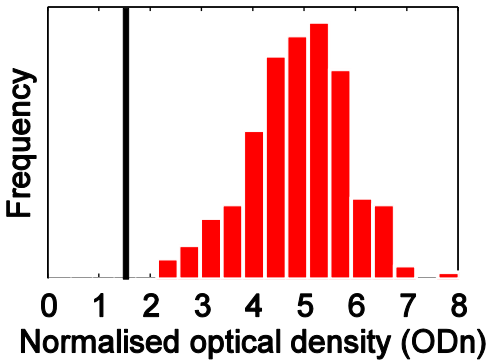


Table 7 provides estimated assay characteristics for various specimen sets. The estimated MDRI, excluding treated subjects and SCOPE elite controllers from the analysis, is 188 days (95% CI: 165-211). The result was insensitive (less than 2% change in result) to whether ARS symptoms onset dates were used to adjust estimated infection dates, a change to Data Exclusion Rule II, and the use of alternative Model C. The MDRI estimate increased by 3% when changing to Model B (1% increase in estimate when changing to Data Exclusion Rule II within Model B).

While characteristics have been estimated here on the standardized basis of a Western blot being used to identify HIV-positive subjects, other diagnostic screening tests are likely to be used in incidence studies, and the time between HIV exposure and reactivity on these tests can differ by several weeks [6,7,16]. Therefore, for application to incidence studies, the base case MDRI reported here would need to be increased or decreased – depending on the particular screening test or algorithm used in the study to classify a specimen as HIV-positive, and hence eligible for 'recent' infection testing.

The estimated FRRs provided in Table 7 are also plotted in Figure 6. Excluding treated subjects and SCOPE elite controllers, and analysing all remaining specimens drawn more than $T = 2$ years after infection, the measured FRR is 1.3% (95% CI: 0.3%-3.2%). When stratifying by time since infection, some persistence of 'recent' classifications is evident up to 4 years after infection.

The FRR amongst elite controller specimens is high at 13% (95 CI: 4%-30%). The FRR amongst treated subjects is even higher, at 59% (95% CI: 49%-68%). Further stratifying treated subjects by time from infection to treatment initiation, the FRR decreases as the time to treatment initiation increases: for early treatment initiation (within 6 months of infection) the FRR is 85% (95% CI: 72%-93%), while for later treatment initiation (more than 6 months after infection) it is 27% (95% CI: 16%-41%).

The FRR for subjects with low viral loads, here defined as below 75 copies/ml, is high, at 47% (95% CI: 39%-55%). This is consistent with results above, as 92% of this specimen set is made up of specimens from the identified elite controllers and treated subjects (and 94% of specimens from SCOPE elite controllers and treated subjects have a low viral load).

The FRR amongst subjects with low CD4+ T cell counts, namely less than 200 cells/µl and acting as a proxy for AIDS identification, was low at 0% (95% CI: 0%-3%).

Table 8 lists MDRI and FRR by subtype. A small p-value for pairwise subtype difference in the MDRI occurs for subtype B versus D, and there are at times large differences between MDRI and FRR point estimates when comparing subtypes. While these initial results highlight potential subtype differences and support further exploration of this topic, a more definitive analysis (beyond the present scope) should be performed – based on a large number of subtype D and A1 specimens and using estimation procedures specifically adapted to this stratification.

Table 7: Estimated assay characteristics (and 95% confidence intervals), for various specimen sets

Assay characteristics are estimated for $T = 2$ years and a context in which an HIV viral lysate-based Western blot assay is used to identify HIV-positive subjects in the incidence study.

	Number of subjects (number of data points)	Estimated assay characteristics (95% CI)
MDRI in days		
All specimens, excluding treated subjects and SCOPE elite controllers	400 (1032)	188 (165-211)
FRR as %		
All specimens, excluding treated subjects and SCOPE elite controllers	316 (665)	1.3 (0.3-3.2)
By time since infection (years), excluding treated subjects and SCOPE elite controllers		
(2,3]	140 (208)	2.5 (0.6-6.6)
(3,4]	77 (110)	0.6 (0.0-5.9)
(4,5]	35 (45)	0.0 (0.0-8.2)
>5	112 (193)	0.0 (0.0-2.6)
Elite controllers (identified by SCOPE cohort)	31 (89)	12.9 (3.6-29.8)
Treated subjects (no previous treatment interruption and treated for at least 3 months)	113 (185)	58.8 (49.2-68)
By time from infection to treatment (years)		
[0,0.5)	53 (90)	84.9 (72.4-93.3)
≥0.5	53 (88)	27.4 (16.0-41.3)
Low viral load (<75 copies/ml at draw)	154 (273)	47.1 (39.0-55.3)
Low CD4+ T cell count (<200 cells/µl at draw)	124 (214)	0.0 (0.0-2.4)

Figure 6: Estimated proportions of 'recent' results in various sets of specimens drawn from subjects infected for greater than *T=2* years

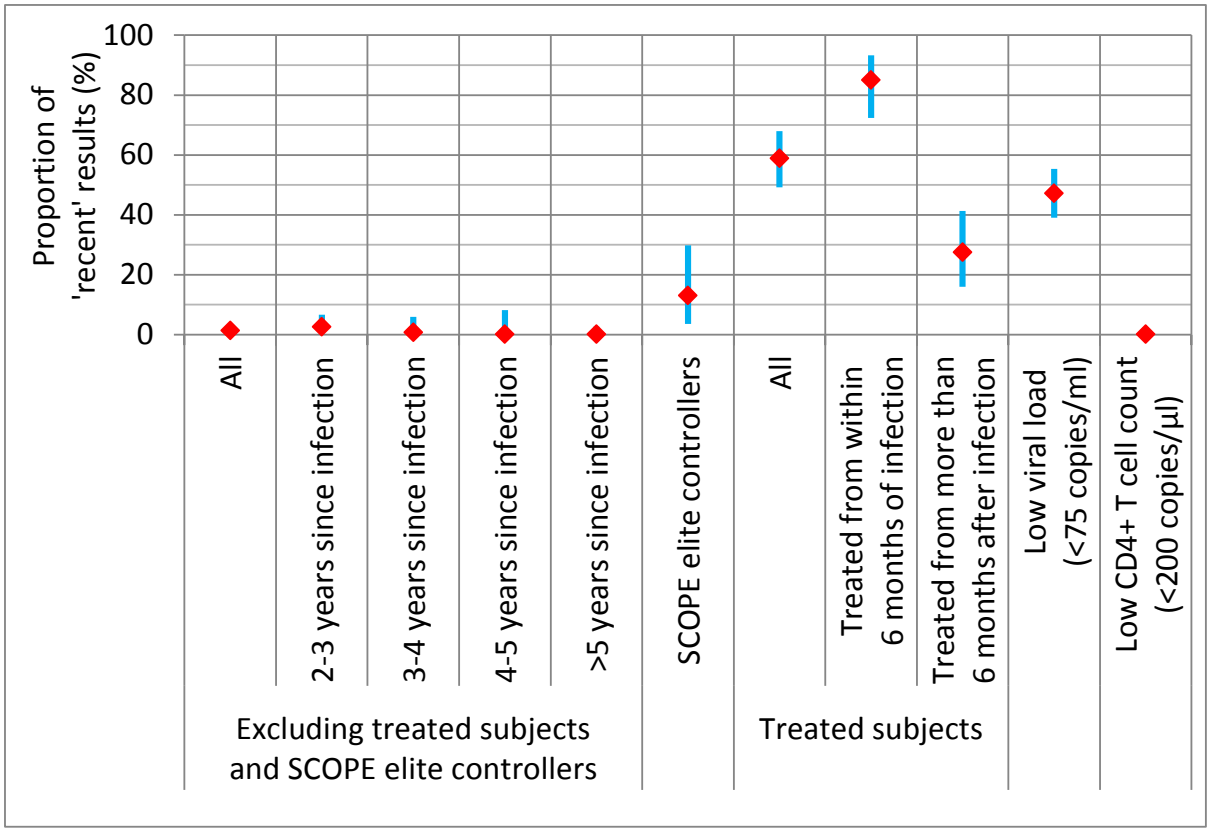


Table 8: Estimated assay characteristics (and 95% confidence intervals), for ARV-naïve subjects and excluding SCOPE elite controllers, by subtype

Assay characteristics are estimated for $T = 2$ years and a context in which an HIV viral lysate-based Western blot assay is used to identify HIV-positive subjects in the incidence study.

	Number of subjects (number of data points)	Estimated assay characteristics (95% CI)
MDRI in days[1]		
All specimens	400 (1032)	188 (165-211)
Subtype B	90 (246)	153 (117-196)
Subtype C	181 (454)	177 (150-206)
Subtype D	38 (131)	273 (170-387)
Subtype A1	80 (166)	211 (156-275)
FRR as %[2]		
All specimens	316 (665)	1.3 (0.3-3.2)
Subtype B	190 (388)	0.5 (0.0-2.9)
Subtype C	75 (144)	1.3 (0.0-7.2)
Subtype D	11 (18)	9.1 (0.2-41.3)
Subtype A1	37 (106)	2.7 (0.1-14.2)

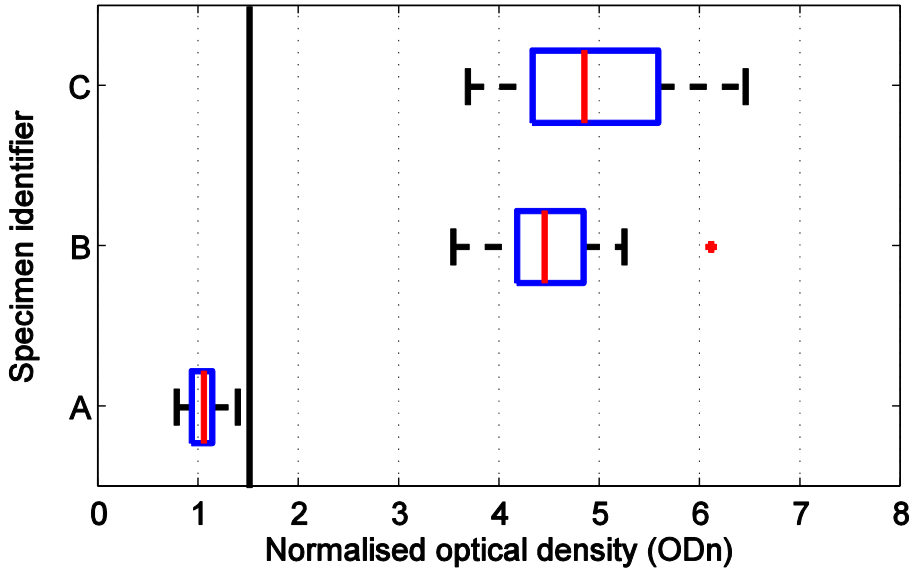
[1] In a test for pairwise differences in MDRIs by subtype, using a z-test, the following pairs provided p-values below 0.05: B&D. Estimated standard deviations of the MDRI estimators are used as proxies for true values, and therefore tests are anticonservative (particularly when sample sizes are small).

[2] In a test for pairwise differences in FRRs by subtype, using the Fisher-Boschloo test [17], no pairs provided p-values below 0.05.

Lastly, Figures 7 and 8 summarise the assay measurements for the reproducibility of CEPHIA control specimens included in the evaluation. 75 of the uniquely-labelled 2 500 specimens on the evaluation panel represent 25 aliquots of each of three underlying specimens. The reproducibility of measurements for these 3 'blinded' controls is described in Figure 7. Five labelled internal quality controls were also tested regularly during evaluation panel testing, for confirmation of stability of the assay. The results for these controls are summarised in Figure 8.

Figure 7: Reproducibility of CEPHIA *unlabelled* controls

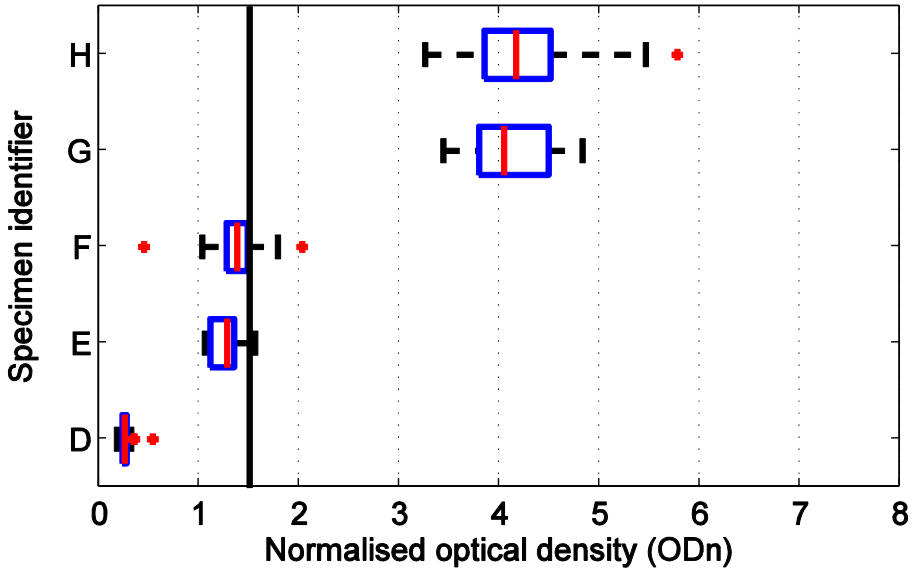
The box-and-whisker plots (top) provide percentiles of the 25 measurements for each of the three blinded reproducibility specimens (labelled A, B and C in the figure only). The central 50% and median of measurements are captured by the box and dividing line respectively, and whiskers and markers capture remaining measurements and outliers respectively. The 'recent'/'non-recent' threshold is shown by the vertical solid line. The observed reproducibility statistics (mean, standard deviation and coefficient of variation) of measurements are also tabulated (bottom).



	Summary of measurements			
Specimen identifier	Number of measurements	Mean (ODn)	Standard deviation (ODn)	Coefficient of variation (%)
A	25	1.0	0.2	16
B	25	4.5	0.6	13
C	25	4.9	0.7	15

Figure 8: Reproducibility of CEPHIA *labelled* controls

The box-and-whisker plots (top) provide percentiles of the 6-95 measurements for each of the five labelled quality control specimens (labelled D, E, F, G and H in the figure only). The central 50% and median of measurements are captured by the box and dividing line respectively, and whiskers and markers capture remaining measurements and outliers respectively. The 'recent'/'non-recent' threshold is shown by the vertical solid line. The observed reproducibility statistics (mean, standard deviation and coefficient of variation) of measurements are also tabulated (bottom).



	Summary of measurements			
Specimen identifier	Number of measurements	Mean (ODn)	Standard deviation (ODn)	Coefficient of variation (%)
D	80	0.3	0.0	18
E	6	1.3	0.2	14
F	95	1.4	0.2	14
G	30	4.1	0.4	9
H	95	4.2	0.5	12

Conclusion/Recommendations –

SEDIA™ HIV-1 LAg-Avidity EIA

1) **This assay does not reach all of the criteria of the Target Product Profile and therefore cannot be recommended for use on its own for use in cross sectional incidence assays.**

2) **The performance of the assay when known confounders of assay performance are removed from the study population suggest that this assay may be usable as part of a testing algorithm in combination with clinical and other supporting information and potentially other incidence assays.**

3) **The CEPHIA group identified, in consultation with CDC, that performance of the assay can be improved by modifying the thresholds and this has been incorporated into the kit inserts by Sedia. As more data become available the CEPHIA group believe that a further review of the appropriate cut-offs be undertaken.**

4) **Following the change in cut-off used during this evaluation the CEPHIA group recommend that groups review their results and reanalyse their results using the new agreed cut-offs.**

5) **CEPHIA recognise and commend the work performed by CDC in strengthening the quality control around the worksheets used to calculate results and that these improvements have been adopted by SEDIA**

Technical Appraisal

Assay Kits and Reagents

The SEDIA™ HIV-1 LAg-Avidity EIA is comprised of two component boxes that have **separate** temperature requirements. This means both a fridge and freezer are required at the test site to correctly store this assay. The Freezer Pack (3029) and the Refrigerator Pack (3030) have matching lot numbers and it is **critical** that only matching lot numbers are used together during testing.

The 10X Wash Buffer Concentrate is lot number independent and so can be used with other kit lots and with the SEDIA™ HIV-1 BED Incidence EIA (Cat No. 1000). This is helpful as the 1X Wash Buffer can be stored at 2-30°C for up to one month in which time kit lots may have changed.

The kit contains two 96-well plates with twelve (12 1 x 8 removable strips) which may be broken down further into individual wells to be inserted back into the plate frame so that only exact number of wells need to be used. This reduces waste of wells and hence cost, however this may cause problems for the plate washer to be used if it cannot be programmed on an individual basis. In this case empty spaces in the plate frame must be filled. Each test plate requires 11 wells be allocated for the controls and calibrators thus in the initial screening mode up to 85 specimens can be tested and in the confirmatory mode up to 28 specimens can be tested in triplicate. (See Appendix 3 for recommended plate configurations)

Each kit contains all the necessary reagents to run the assay. The bottles are well labelled and easily identifiable.

Equipment

Most of the equipment and materials required but not provided are standard laboratory pieces and so should not pose a problem for a testing laboratory. Further details on some items are described below.

Plate washing:

Test plates are washed 4 times (rotating the plate after the first 2 washes) with 1x wash Buffer using a 96-well or strip washer. Set to dispense 300µl with a 10-second soak (if a plate washer is used). This is a standard wash programme and should not pose a problem for testing laboratories, however, during this evaluation there were a high number of invalid test plates which through a process of elimination may be attributable to washer use.

During the CEPHIA Evaluation 120 LAg Avidity test plates were run. Of these 27% (32/120) failed the validity criteria for OD values set by the manufacturer SEDIA at the time.

CEPHIA believed the invalidity of control values may be mostly attributable to the washer used during testing. However it should be noted the same washer and wash programme was used for successful SEDIA HIV-1 BED Assay testing which suggests LAg may be more sensitive to washer variations or that the ranges were too narrow.

When CEPHIA data was re-evaluated using the new OD Acceptable Ranges the number of failed plates reduced to 10% (12/120 plates). However, there were 8 more plates that failed

the **ODn** criteria only, which again highlights the fact that even if ODs are within acceptable ranges and are deemed VALID by the Data management file, the subsequent ODn value may fall outside of the acceptable range, and this must be manually identified by the user.
As the changes to ranges occurred after the CEPHIA evaluation had taken place none of the data from these plates was included in the data analysis.

Positive displacement pipette or microliter syringe:
SEDIA stated that the preparation of the Conjugate Working Solution as critical in **version 3** of the Kit insert stating "small inaccuracies may significantly impact absolute OD values". The insert suggest the use of a Positive displacement pipette or microliter syringe during this stage. CEPHIA did not find the use of this pipette improved the failure rate of test plates.

25°C Incubator:
SEDIA require the use of a second incubator during the TMB substrate incubation. CEPHIA has observed that some laboratories do not have a 'room temperature' incubator and suggest that Sedia emphasise the importance of having such a spice of equipment.

Associated documentation

The availability, usability and reliability of accompanying documents e.g spreadsheets, worksheets, data files, kit inserts, QC charts, are vital to the performance of the assay. The user is reliant on the Developer/Manufacturer to ensure formatting, formulas and information is correct and available in any such documents.
There are two associated documents with the SEDIA ™ HIV-1 LAg-Avidity EIA:

1. **Kit Insert**

Printed Kit inserts are supplied with each Assay Kit detailing the test procedure, run validation and calculation and interpretation of results. Kit inserts are clearly presented and contain all relevant detail to perform the assay correctly. However, it is important that kit inserts match the criteria supplied in the Data file to ensure information consistency.

2. **LAg-Avidity EIA Data Management Worksheet**

Customers are alerted to a customised spreadsheet from CDC, by the SEDIA ™ HIV-1 LAg-Avidity EIA kit insert, to validate the run and calculate ODn. Two versions of the LAg-Avidity EIA Data Management Worksheet are provided by the manufacturer and are available on their website for use by any laboratory using the LAg Assay following links via http://www.sediabio.com/products/lag-avidity-eia

This spreadsheet was used throughout the CEPHIA evaluation to validate test runs and compile data. However, a number of errors were discovered with the spreadsheet during the evaluation which resulted in incorrect data, confusion for users and the retesting of some specimens. **These errors are described in detail below:**

Documentation Issues

ISSUE 1: Formulae error (November, 2012)

The *Initial Results* tab contained incorrect formulae in 8 different cells. The reference formulae for the calculation of ODn on Plate 7, Wells 7A – 7H are incorrect. i.e Cells U632 – U639 mistakenly reference the wrong cells (off by one row). This results in **8 specimen screen ODn results being incorrectly calculated** and hence possibly the wrong Result given. (See table below). This error was only discovered due to the CEPHIA data analysis system set-up by SACEMA. They identified inconsistent results for the 8 specimens involved and reported the error to PHE. PHE confirmed that the error arose from incorrect formulae supplied in the SEDIA Data Management File.

COPIED FROM ONLINE DATA MANAGEMENT FILE:

7	7A	='Specs and Initial PMs'!C562	=H73	**=S633/$T$586**	**=IF(U632>2,"LT","CONFIRM")**
7	7B	='Specs and Initial PMs'!C563	=H74	**=S634/$T$586**	**=IF(U633>2,"LT","CONFIRM")**
7	7C	='Specs and Initial PMs'!C564	=H75	**=S635/$T$586**	**=IF(U634>2,"LT","CONFIRM")**
7	7D	='Specs and Initial PMs'!C565	=H76	**=S636/$T$586**	**=IF(U635>2,"LT","CONFIRM")**
7	7E	='Specs and Initial PMs'!C566	=H77	**=S637/$T$586**	**=IF(U636>2,"LT","CONFIRM")**
7	7F	='Specs and Initial PMs'!C567	=H78	**=S638/$T$586**	**=IF(U637>2,"LT","CONFIRM")**
7	7G	='Specs and Initial PMs'!C568	=H79	**=S639/$T$586**	**=IF(U638>2,"LT","CONFIRM")**
7	7H	='Specs and Initial PMs'!C569	=H80	**=S640/$T$586**	**=IF(U639>2,"LT","CONFIRM")**

Effect on CEPHIA Data: Corrected data shown in Red

Well-ID	Spec ID	OD	Median	ODn	Result	OD	Median	ODn	Result
7A	7683-01	3.319		3.197	LT	3.319		4.217	LT
7B	7692-01	2.516		4.174	LT	2.516		3.197	LT
7C	7711-01	3.285		3.404	LT	3.285		4.174	LT
7D	7728-01	2.679		4.079	LT	2.679		3.404	LT
7E	7775-01	3.21		1.751	CONFIRM	3.21		4.079	LT

7F	7782-01	1.378		1.374	CONFIRM	1.378		1.751	CONFIRM
7G	7789-01	1.081		3.933	LT	1.081		1.374	CONFIRM
7H	7802-01	3.095		3.996	LT	3.095		3.933	LT

(July, 2012) The *Confirmatory Results* tab, contains incorrect formulae in 9 different cells. The reference formula for the OD on PLATE 7, Well 6H should be **G80**, but it has been incorrectly filled as **H73**. Nine subsequent cells are incorrectly filled because of this error (see table below). This means that the Median OD and the **Final result for 3 of the samples on Plate 7 are calculated incorrectly in the confirmatory assay**.

		COPIED FROM ONLINE DATA MANAGEMENT FILE:	SHOULD BE:	
7	6A		=G73	
7	6B	='Confirm PMs'!B193	=G74	
7	6C		=G75	
7	6D		=G76	
7	6E	='Confirm PMs'!B194	=G77	
7	6F		=G78	
7	6G		=G79	
7	6H	='Confirm PMs'!B195	=H73	G80
7	7A		=H74	H73
7	7B		=H75	H74
7	7C	='Confirm PMs'!B196	=H76	H75
7	7D		=H77	H76
7	7E		=H78	H77
7	7F	='Confirm PMs'!B197	=H79	H78
7	7G		=H80	H79
7	7H		=H81	H80
7	8A	='Confirm PMs'!B198	=I73	
7	8B		=I74	
7	8C		=I75	

ISSUE 2: Negative OD values

The Kit insert states that both individual OD values for the Negative Control must be within the stated range for the assay to pass. However, the original data management spreadsheet only based run validity on the median of the Negative OD values. The spreadsheet should include the full assay criteria as users may not manually verify both negative values when a VALID result appears on the spreadsheet.

This issue was rectified by the manufacturer and customers were made aware on 27th February 2013 (please note that this is after the testing of the Evaluation Panel was complete).

ISSUE 3: Control ODn values

The Data Management File spreadsheet does not take into account the validity of the kit Control **ODn** values. The kit insert gives acceptable ranges for both OD and ODn values.

However, the spreadsheet bases run validity on OD values only. This can be misleading when a VALID result appears on the spreadsheet, as users may not also manually evaluate the control ODn values to ensure they are within acceptable ranges.

The OD values being in the acceptable range does not necessarily mean the ODn values are within range. If both OD and ODn are to be within acceptable ranges the spreadsheet should reflect this in its VALID/INVALID interpretation.
This issue was rectified by the manufacturer and customers were made aware on 27th February 2013 (please note that this is after the testing of the Evaluation Panel was complete).

ISSUE 4: Acceptable ranges for assay controls

There are slight differences in the Acceptable ranges for assay controls listed on the Data Management File spreadsheet as to those listed in Kit inserts. This makes it difficult for users to be consistent in the verification of their assay Runs. Whilst understandable that Acceptable ranges change over time due to the gathering of additional data, it is imperative that the Spreadsheet Acceptable Ranges consistently match the current Kit Insert version.
From the Acceptable ranges observed in Kit inserts so far, none of the ranges match those listed on the Data Management File (see tables below). This indicates a lack of maintenance of the spreadsheet and a risk of inconsistency of Run validity between users.
At time of evaluation the Data Management File does not have version numbers attached and there is no official notification of when a new version may be added which may include updates or corrections. This leaves users unsure and unaware of changes.

ACCEPTABLE OD RANGES FROM L-Ag DATA MANAGEMENT FILE SPREADSHEET (SEDIA WEBSITE)

NC	CAL	LPC	HPC
0	0.5	0.275	1.0
0.16	0.9	0.500	1.8

FROM L-Ag KIT INSERT (VERSION 1):

	NC	CAL	LPC	HPC
Minimum	0.000	0.500	0.200	1.000
Maximum	0.250	0.900	0.500	1.800

FROM L-Ag KIT INSERT (VERSION 2):

	NC	CAL	LPC	HPC
Minimum	0.000	0.500	0.275	1.000
Maximum	0.160	0.950	0.525	1.900

FROM L-Ag KIT INSERT (VERSION 3):

	NC	CAL	LPC	HPC
Minimum	0.000	0.500	0.275	1.000
Maximum	0.160	0.950	0.525	1.900

FROM L-Ag DATA SHEET SENT FROM SEDIA (09/11/2012):

	NC	CAL	LPC	HPC
Minimum	0.000	0.500	0.250	1.000
Maximum	0.160	0.950	0.500	1.800

Interpretation of Specimen Results

During the CEPHIA evaluation a cut-off value of 1.0, as recommended by the manufacturer, was used ie. If ODn > 1.0 specimen classified as 'Long-term seroconversion', If ODn <1.0 specimen classified as 'Recent seroconversion'.
Testing conducted by the U.S. Centers for Disease Control indicates that a cutoff for ODn values of 1.0 represents a mean duration of recent infection of 141 days [35].

Following analysis of the CEPHIA data, the manufacturer (CDC) and CEPHIA agreed that 1.5 would be a more suitable cut-off value.
Kit inserts now state: *Testing conducted by the U.S. Centers for Disease Control indicates that a cutoff for ODn values of 1.5 represents a mean duration of recent infection of 130 days [35].*

Technical Conclusions

The manufacturer was informed of the issues with the **LAg-Avidity EIA Data Management Worksheet** via a CEPHIA report on 29/11/2012. CEPHIA was informed that the spreadsheet was being updated and would be available soon. Up until this time the original spreadsheet with errors was still supplied on the website for use by kit users. From a user perspective the continued supply of incorrect documentation and hence the low quality data that results from this is disappointing and frustrating. From an evaluation perspective it also raises concerns that other users are unaware of the errors within the spreadsheet and as such will have incorrect data.

The website was updated with a new spreadsheet (Version LN-6081) in February 2013, which had a number of amendments:

- Requirement for laboratory Plate reader Limit of Detection was added
- Initial and Confirmatory Results tabs have updated formulae. CEPHIA assumes that formulae errors discussed above have been rectified although the spreadsheet has not been extensively evaluated for errors.
- The Valid/Invalid criteria remains the same i.e Run validity based solely on OD values and users must manually check if ODn's are within acceptable ranges

- The most significant amendment is the extension of the Acceptable OD ranges for Kit Controls.

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

The Current Kit insert (**LN 6039.06** page 17) has been updated by the manufacturer with the new cut-off value of ODn 1.5.

CEPHIA appreciates that as assays develop there will be changes to ranges and documentation but recommends that a comprehensive method of informing users of changes should be employed as well as vigorous checking of spreadsheets containing formulae.

The current version of the SEDIA ™ HIV-1 LAg-Avidity EIA (Labelling Number 6039 version 7) can be downloaded from www.sediabio.com/products/lag-avidity-eia

Target Product Profile performance

Specification	Acceptable Performance	Ideal Performance	How does Lag fit?
Intended Use	*Population-based incidence estimate*	*Population-based incidence estimate, prevention-trial planning, community-level prevention intervention studies*	
Target Population	*Specific to clade*	*All clades*	*Possibly some clade variation –performance may be 'acceptable'*
False Recent Rate (FRR)	*Confidently measured to be less than 2% in different populations (with different clades, epidemic phases, treatment coverage etc)*	*0% in all population (No evidence of false-recent classifications).*	*On this sample set – 1 % FRR acceptable - however evidence that it is context dependant*
Mean Duration	*4 months (95% CI, +/- 0.2)*	*1 year (95% CI, +/- 0.2)*	**188 days (95% CI: 165-211)** *Acceptable*
Algorithm	*Included in a RITA*	*None required*	*Eevidence that ART and low VL affect assay thus algorithm is likely to be required*
Analyte	*Any*	*Any*	*Acceptable*

Sample Type	***Frozen serum, frozen plasma***	***Frozen serum or plasma , dried blood spots (or other easily obtained and stored sample)***	***Acceptable – DBS control soon to be available***
Sample Volume	***1 mL***	***10 uL or fingerstick***	***Acceptable***
Infrastructure requirements	***Centralized laboratory facility (clean water and electricity available)***	***None (all reagents and necessary materials to run assay are in self-contained kit)***	***Acceptable***
Storage/Shipping Conditions	***4-25 °C***	***Ambient temperature***	***Failed - Frozen storage for some reagents***
Incubation Temperature	***4-25 °C***	***Ambient temperature***	***Acceptable***
Shelf Life	***9 months***	***>18 months***	***Ideal***
Training	***Laboratory technician can be proficient with one week's training based on proficiency testing***	***Minimal training would allow any health worker to conduct the assay***	***Acceptable***
Regulatory Pathway	***GMP or ISO 13485 or equivalent, and/or approval by national governing body***	***FDA and equivalents***	***Assay produced in GMP facilities and approved by CDC.***

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Appendix

1. Evaluation Protocol

Background

The SEDIA ™ HIV-1 LAg-Avidity EIA is to be evaluated by the CEPHIA group as part of The Bill & Melinda Gates Foundation funded project, 'Development of specimen repository and evaluation of assays for identification of recent HIV infection and estimation of HIV incidence'. The group will also undertake evaluations of a number of other available assays for HIV recency. Each of these evaluations will have their own CEPHIA Book Report available.

Evaluation Purpose

To advance the understanding of currently available assays that can identify recent HIV infection; to better describe the duration of the infection state in which they identify recent infection; and to determine the rate at which they misclassify specimens as from recent infections

Conduct of the evaluation

All CEPHIA evaluations are conducted following the **CEPHIA Quality Management Strategy** (Document 002) which details the quality planning, quality control and quality assurance in place at Public Health England (PHE), Microbiology Services (MS), Colindale and the collaborating organisations of Blood Systems Research Institute (BSRI), San Francisco, University of California, San Francisco (UCSF) and South African Centre for Epidemiological Modelling and Analysis (SACEMA), University of Stellenbosch, South Africa, to ensure the delivery of the Bill & Melinda Gates Funded (BMGF) Project.

The main objectives of the quality strategy are: to define the quality requirements, how they are to be met, who is responsible for meeting requirements, and helping to align quality strategies between the multiple sites involved in the overall project.

The CEPHIA Quality Management Strategy details the quality procedures in place for all CEPHIA evaluations with regards to Project Organisation – roles, responsibilities and personnel, Facilities, Equipment, Standard Operating procedures (SOP), Worksheets, Plans, Sub-contracting, Conduct of project, Computer systems, Safety and risk, Method validations, Results , Reporting process and templates, Repeat analysis, Retention of data/specimens, Confidentiality. The quality strategy will be based on UK CPA standards and also MHRA Good Clinical Practise 'Guidance on the maintenance of regulatory compliance in laboratories that perform the analysis or evaluation of clinical trial samples', it will also refer to local site regulations and standards.

- **The assay under evaluation will be tested in exactly the manner laid down in the manufacturer/developers instructions.**
- **Evaluator(s) will strictly adhere to the quality requirements laid out in the CEPHIA Quality Management Strategy.**

- **Prior to beginning the evaluation, the manufacturer/developer will be invited, if they so wish, to provide training to the evaluator in the use of the assay kit and equipment and to satisfy themselves that the evaluator(s) is trained sufficiently.**

Specimen Handling

A main objective of this project is to compile large-volume, standardized sample sets appropriate for comparative evaluation of tests for recent HIV infection in an accessible central repository.

These serum samples will be sourced by the CEPHIA team at University of California San Francisco (UCSF), blinded so evaluator(s) will not know the expected results, then aliquotted at the central repository (Blood Systems Research Institute, San Francisco) and shipped to the relevant test site.

Documentation

The CEPHIA group have compiled a folder of documents relating to the plans, procedures and protocols required for the high quality performance and completion of the Bill & Melinda Gates Foundation funded project. These documents are securely stored in a management-only access folder until the project end. Some relevant documents are available for public reading on the CEPHIA website at http://www.incidence-estimation.com/archivesuploads/index/NAME/11

Other aspects of the evaluation

Technical appraisal of the procedure, assay kit and equipment required for the performance of the assay. This may include ease of use, reliability, packaging, clarity, health and safety considerations.

Discordant results

A discrepancy may arise at the test site and should be investigated by an appropriately trained person prior to data being verified and reported for analysis. If a discrepancy is identified at the analysis site (SACEMA), a report detailing the error will be sent to the test site for further investigation.

Analysis of results and evaluation report

The raw laboratory data is compiled and verified at the test site. It is stored electronically in a Data Table formatted as described in the *CEPHIA Data Processing Protocol: Data Flow, Recording and Standard Formats.*

Verified and formatted data is e-mailed to the analysis site (SACEMA). The analysis site will run data through checks and generate a report prior to using the data for analysis. Data analysis will be reported in the CEPHIA Book. The manufacturer/developer of the assay concerned will be given the opportunity to comment on results prior to any publishing of data.

2. CEPHIA Project Management Contact Details

1. PHE – Public Health England, Microbiology Services, Colindale, London, UK
2. BSRI - Blood Systems Research Institute, San Francisco
3. UCSF – University of California, San Francisco
4. SACEMA - South African Centre for Epidemiological Modelling and Analysis

Project Management Team:				
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3. Recommended Test plate configurations

Screening plate diagram

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

Confirmatory plate diagram

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*

Contact details for manufacturer

Sedia Biosciences Corporation
Portland, Oregan USA
Phone: 1-(503)-459-4159
E-mail: customerservice@sedbio.com
Web: www.sediabio.com

The current version of the kit insert is available from the manufacturer.

Manufacturer's comments

An early draft copy of this report was provided to Sedia. Following their review the CEPHIA management group reviewed the report and a number of changes were made. These mainly related to factual inaccuracies and clarifications of a number of points. Following this review a further copy of the report was provided to Sedia and their comments on this report are shown below. The CEPHIA management group wish to acknowledge, with thanks, the efforts Sedia have made in their comprehensive review of our report. AS alerted to Sedia a small number of corrections were made to the version that Sedia received as part of a final proof read.

Sedia Biosciences' Manufacturer's Response to CEPHIA Evaluation Report on Sedia™ HIV-1 LAg-Avidity EIA.

Version Number 2: Report Dated 30 January 2015; Received by Sedia 03 February 2015

Manufacturer's Overview:

CEPHIA has graciously provided its Evaluation Report on the Sedia™ HIV-1 LAg-Avidity EIA for Sedia Biosciences' ("Sedia") comments which are included below. Sedia understands and appreciates the challenges in evaluating HIV Incidence Assays in a fair and unbiased way. From an industry standpoint, development of such assays present their own unique challenges. The market size for such products is currently very small, the evaluation of the developed assay, both during development and as final validation of the assay, require specimens not readily available to the developer except at considerable cost, and limited funding either within or outside of industry is available for the pursuit of such assays.

Sedia is concerned that the structure of these reports appears to be a binary recommend/not recommend conclusion, without adequate consideration of other options that are practically available today. We base this concern on our reading of this report, additional published data analyzing other HIV incidence assays by CEPHIA (3) and our familiarity with the HIV incidence test market and customers. From a potential user's perspective, CEPHIA's evaluation of all currently available incidence assays concluding that none can be recommended is of limited benefit to the user who does not have the resources to conduct a longitudinal cohort study but must rely on "the best assay" for the intended use that the user requires. A conclusion that an assay cannot be recommended suggests that the assay is without value, a position we believe many of our customers would not agree with. The development of dedicated HIV incidence assays is a fairly recent journey undertaken by a very small number of developers and manufacturers. Providing guidance by recommendation of assays most optimal for this intended application, even if conditional, would facilitate potential users' decision making process in developing improved incidence studies.

The format of the evaluation also does not appear to take into account the effect of using an unmodified assay whose manufactured intent is for use as an incidence assay, versus using an assay manufactured and intended for diagnostic use but modified by a research lab or third party for use as an incidence assay. As both an incidence assay and a diagnostic assay manufacturer, Sedia appreciates that incidence assays are developed and qualified, manufacturing and raw materials changes made, by considering the impact to the assay's use for estimating incidence, as part of the manufacture of such an assay under GMPs.

Similarly, a diagnostic assay is developed, qualified, manufacturing and raw materials changes made, by considering the impact to the assay's use as a diagnostic assay, not as an incidence assay. The end user of a diagnostic assay modified to be used for incidence estimation has no knowledge if the assay has been changed since initial evaluations have been performed to establish MDRI and FRR values, and thus has no idea as to whether these critical values apply to the lot of product they are modifying and using. Sedia's experience is that incidence assays are more challenging to manufacture and qualify than diagnostic assays, and for good reason. Incidence assays require more controls and data analysis to determine the performance of an incidence assay to be qualified for product release. Specific issues about the report provided by CEPHIA are listed below:

Summary:

Technical Appraisal

The storage temperatures cited throughout the document are over-generalized ("4°C" instead of "2-8°C", "<-20°C" instead of "-25°C to -10°C"). The Sedia™ HIV-1 LAg-Avidity EIA Refrigerator Pack is labeled for storage at 2-8°C and the Freezer Pack is labeled for storage at -25 to -10°C.

Conclusions

The CEPHIA report has determined that the product does not reach "all components of the Target Product Profile (TPP) for use in cross sectional incidence assays" and "do not recommend its use as a standalone assay, but feel it may be useful as part of an incidence assay algorithm". No explanation for this conclusion is offered other than the failure to reach the TPP criteria. Sedia is unaware of any reported or published studies in which there is any HIV-1 incidence assay that reaches the TPP. The LAg-Avidity EIA is one of only two types of assays developed specifically for estimation of HIV-1 incidence and which do not use an adaptation of an assay which is designed for diagnostic use. Such assays are not controlled, regulated or validated relative to HIV-1 incidence measurement, a relatively small market, given the greater market demands typical for diagnostic use. Sedia believes that the Sedia™ HIV-1 LAg-Avidity EIA represents the current state-of-the-art in laboratory based assays for estimation of incidence, despite the fact that it may not meet all criteria of the TPP. We are unaware of any assays that CEPHIA has suggested meet the TPP criteria, and respectfully challenge CEPHIA to provide more practical guidance to researchers based on what is currently available, than what will hopefully be developed in the future. Identification of the incidence assays which are most effective at assessing incidence provides more practical relevance to researchers who need some tool, even though less than ideal, to conduct incidence surveys, without resorting to more burdensome methods such as. longitudinal cohort studies.

Introduction

The authors state that "Full evaluation of the Sedia™ HIV-1 LAg-Avidity EIA was considered justified based on the data analysis from the Qualification Panel evaluation and Evaluation Panel testing performed at Public Health England, London." However, the basis for inclusion or exclusion of assays based on these evaluations and data analysis is not described in the report, nor is any complete listing of assays excluded and included for "full evaluation" provided.

SEDIA™ HIV-1 LAg-Avidity EIA Information

The assay kit(s) evaluated were those manufactured in 2011 and 2012, and expired in 2013. Ongoing continuous product improvement is conducted to improve product consistency, manufacturability and performance in general, although internal target specifications (typically more extensive and constraining than the validity ranges followed by the end user) have changed minimally to ensure the product performs consistently with previous lots specifically for HIV incidence testing. These improvements continue today.
The kit insert referenced for the information in this section is version LN-6039.05. This insert was implemented in June 2013 and been updated twice since October 2013. Sedia recommends that users use the current version of the insert on the Sedia website (www.sediabio.com) which is LN-6039.07. It should also be noted that the photograph of the kit in the report is of an outdated kit, as it contains "For Surveillance Use Only" labeling, which is no longer valid, and includes temperature ranges reflecting older versions of the kit.
It should be noted that the LAg-Avidity EIA is a commercial assay that was developed and designed specifically for estimating HIV-1 incidence, as opposed to adapting an assay manufactured, tested and released for the purposes solely of HIV diagnosis. As such, each lot of the product manufactured is tested and verified specifically for performance as an incidence assay, both by Sedia and the CDC. Sedia feels this is a significant advantage over other "adapted diagnostic assays" which may undergo changes or deviations by the manufacturer without verify the impact an unknowing end-user may experience when applying the assay to incidence estimation.

Table 2: Manufacturer Claims for the assay and its limitations

The table is excerpted from an outdated (6/2013 – 10/2013) kit insert. Minor wording changes have since been incorporated. Sedia recommends the authors reference the current kit insert.

Evaluation Panel and Method

Table 3: Demographic / infection characteristics of subjects contributing specimens to the evaluation panel.

The subtype distribution in this report is heavily skewed towards subtype B and C subjects with certain key subtypes (e.g. CRF's including AE and AG dominant in SE Asia and common in west Africa, respectively, Subtype G subjects common in east Africa, etc) are missing. Sedia does not at this time have access to the distribution of specimens used by the Centers for Disease Control (CDC) which was used by the CDC to generate the mean duration of recent infection (MDRI) and false positive rates (FRR) cited in the Sedia product insert. Sedia understands that a publication documenting this information is forthcoming. In addition, only 42% of the subjects in the CEPHIA evaluation have confirmed subtypes, with the rest presumed to be the most common serotype of the country of the subject's origin, which may or may not be the case. This may potentially impact the data related to assay performance relative to subtype if these are included in subtype analysis and therefore the authors' conclusions about subtype specific performance of the assay. It is unclear whether subjects with unverified subtypes were excluded from the subtype-related data analysis.

Analysis of Assay Characteristics

Results – Figures 2 and 3

The data in Figure 2 is of limited value as the assay is not intended to estimate time of infection, but to simply differentiate recent versus long-term infections relative to a single

point, the MDRI (ω=130 days [95% CL 118-142] as determined by the CDC). To this end, it would be more valuable to assess time since infection relative to the cutoff (ODn=1.5) in a scatter plot with quadrants demarcated by a vertical line at the MDRI (130 days) and a horizontal line at the cutoff (ODn=1.5), and assessing the relative distribution in each quadrant of data points. The lines on the present figure obfuscate this information that correlates to the intended use of the assay. Similarly, data in Figure 3 reported out 1 or 2 years or more is of limited value except to the extent that data points are reported as false recent (i.e. falling into the lower right quadrant). This is more of an issue, however, with Figure 2, where the distribution of individual values cannot be visualized due to the connecting lines.

Table 7

The calculated MDRI is longer in this report (188 days, 95% CI: 165-211) than that reported by the CDC for the LAg-Avidity EIA and noted in Sedia's product insert (130 days, 95% CI: 118-142). Sedia has been told by CDC that this value is based on calculations performed on CDC data collected from populations tested by CDC and co-analyzed by CDC and CEPHIA member SACEMA. As a result, Sedia would expect the method of determining the MDRI to be the same as that used for this CEPHIA report. Obviously the population tested is different. Sedia does not have sufficient information to establish the basis for the different values. However, since CEPHIA (through SACEMA) was involved in the CDC data analysis, it may be useful to provide a view as to the differences since SACEMA is the sole party with access to both sets of data. There could be multiple factors causing this difference. Sedia understands that the CDC data is based on exclusion of individuals likely to be misclassified including recipients of antiretroviral therapy and elite controllers (excluded by the authors in their calculations) but also persons with diagnosis of AIDS or low CD4+ T cell counts, which the kit insert also advises should be excluded (see Limitations of the Assay). It's not clear if these latter individuals were also excluded from the analysis conducted by the authors. The effect due to differences in subtype distribution from the CDC population and that of the CEPHIA panels potentially has an impact. A longer MDRI calculated by the CEPHIA authors compared to that calculated by the CDC also may increase the calculated FRR (false recency rate) which appears to be slightly higher, but may not be significantly different.

Conclusion/Recommendations

Specific conclusions:

1) The first conclusion states that the Sedia™ HIV-1 LAg-Avidity EIA cannot be recommended for use on its own for use in cross sectional incidence assays since it does not reach all criteria of the Target Product Profile. This conclusion either implies there are other assays which do meet all of the criteria of the TPP, or alternatively the reader or researcher seeking a laboratory based assay to assess incidence is left with no direction of where to go for the best possible assay to use. Sedia does not have access to all the assays evaluated but it does not believe there are currently no other assays that meet all of the TPP but notes that CEPHIA member labs have continued to purchase and use the Sedia™ HIV-1 LAg-Avidity EIA subsequent to this evaluation for their own studies. By CEPHIA's own published studies (3), the Sedia™ HIV-1 LAg-Avidity EIA gave lower FRR's overall, for each

subtype, and for all confounding conditions against all or most tests. Sedia believes this suggests that the Sedia™ HIV-1 LAg-Avidity EIA, while perhaps not a perfect option relative to the TPP, is the optimal test for those researchers pragmatically needing a solution today to a laboratory based assay for estimation of incidence. Sedia believes that the binary recommend/not recommend approach taken by the authors provides a disservice to individuals and institutions attempting to seek guidance as to what assays to use.

2) The authors seem to recommend against using any assay by itself to estimate incidence, but suggest that the Sedia™ HIV-1 LAg-Avidity EIA may be usable as part of a testing algorithm in combination with clinical and other supporting information. The report does not state whether such a testing algorithm would also include another assay. If the report is intended to state that the algorithm that "may be usable" simply includes incorporation of "a combination of clinical and other supporting information", and this does not represent a standalone assay, then virtually no assay should be considered suitable as a standalone assay. Sedia believes that as a matter of practice that virtually any assay of this type should be and currently is used in combination with clinical and other supporting information, for example, not used on persons who are determined to be HIV-1 negative.
3) The authors state that CEPHIA has identified, in consultation with the CDC, that the performance of the assay can be improved by modifying the thresholds. Although this statement is in "present tense", we are only aware of a modification of the thresholds that CEPHIA and CDC have proposed previously and that were implemented June 2013 (cutoff of ODn=1.5 and MDRI of 130 days). The tense and content of the authors' statement suggest that additional improvements to the existing performance are possible. While Sedia certainly welcomes improvements that can be contributed to the assay, such proposed changes have not yet been shared with Sedia.
4) CEPHIA has recommended that "groups review their results and reanalyze their results using the new agreed cut-offs". Sedia agrees and has advised known customers and users of the Sedia™ HIV-1 LAg-Avidity EIA to follow these instructions when it announced the changes in the assay procedure in 2013.

Technical Appraisal

Assay Kits and Reagents

Sedia disputes the characterization that the feature of removable strips and individual wells "may cause problems if the plate washer in use cannot be programmed on an individual well/strip basis." This is not a problem given the simple solution offered in the insert to use blank wells or strips in unused portions of the plates, an approach that both other customers and Sedia scientists have successfully used. Obviously there are many procedures in any assay that are problems if there are no solutions or directions on how to address them. Sedia feels this feature of the assay is a significant benefit to users in saving cost and reducing waste, not a problem that needs to be resolved.

Equipment: Plate Washing

The authors have reported that "during the CEPHIA Evaluation 120 LAg Avidity test plates were run. Of these 27% (32/120) failed the validity criteria for OD values set by the manufacturer SEDIA at the time." Product that CEPHIA received contained Revisions 2 and 3 of the Sedia™ HIV-1 LAg-Avidity EIA product insert. Changes to the validity ranges were implemented with Revision 4 which were implemented in February 2013. The authors indicate that "the data was re-evaluated using the new OD Acceptable Ranges" and imply the new ODn Acceptable Ranges were considered as well, but still obtained a 10% invalid rate. The authors suggest that "LAg may be more sensitive to washer variations or that the ranges were too narrow." Sedia's experience has been that the Sedia™ HIV-1 LAg-Avidity EIA is, in fact, more sensitive to variations in equipment as well as raw materials used to manufacture the material than most diagnostic ELISAs or even the BED EIA. This is in part because the assay is a limiting assay, and therefore by definition does not have saturated solid phase binding ligand that many assays have. It is also in part because the assay is designed to have <u>both</u> consistent correlation (R^2) and concordance (slope and cutoff) to previous lots within the range of critical interest (ODn's of <3.0) to minimize the risk of incorrectly assigning specimens to the wrong recency status or providing results that vary from lot to lot. Sedia releases product based on significantly narrower validity ranges than those the customer uses, and CDC approves the individual product lot confirming validity of the assays. As a result, Sedia has over time encountered a number of factors which can contribute to results that are "invalid" using its own narrower internal limits. Assisting customers in troubleshooting their invalid runs (including CDC's own experience in training labs to perform the LAg-Avidity EIA) has also helped identify the source of problems in labs. These issues have most often included equipment issues (improper, inadequate or no calibration of equipment, use of wrong equipment or equipment at wrong or uncontrolled temperature, improper setup of equipment, use of equipment outside of manufacturer's stated specifications), or failure to follow assay instructions (failure to use incubators for incubation steps, failure to warm reagents to appropriate temperature, improper storage of reagents and materials, cross-contamination due to insufficient care pipeting, mis-entry of data into spreadsheet and/or miscalculation of data, failure to perform confirmatory testing, etc. It should be noted that these types of errors have not been found only in inexperienced labs in developing countries but identified in very sophisticated labs, including the CDC and some of the labs of associated with CEPHIA. Sedia itself is not immune to such errors in our own labs, and the fact that experienced labs make such mistakes speaks not only to the human nature impact of performing these assays, but also to the sensitivity of the assay to individual or multiple errors. As Sedia has gained experience in identifying the root cause of invalid results, we have tried to incorporate more detailed instructions and emphasized where potential variation may be reduced through careful but unburdensome means. In conclusion, as with any assay (but perhaps more so with this assay), it is important to <u>perform the assay as instructed</u>, <u>using equipment that is properly set up, calibrated and used as instructed by the manufacturer</u>. Plate washers can be a contributing factor, when not properly set up according to the manufacturer's instructions (all manufacturers we are aware of have a procedure for optimizing their settings depending on the assay plates being used.)

<u>Equipment: Positive displacement pipette or microliter syringe</u>

The report correctly states that use of a positive displacement pipette or microliter syringe is required, for the Sedia™ HIV-1 LAg-Avidity EIA to measure out conjugate. This does not mean that valid results cannot be obtained with an air-displacement pipette, but we have accumulated data that indicates that the variability of volume of conjugate dispensed with properly calibrated and maintained air-displacement pipettes is significant enough that it increases the risk of invalid results. Incorporation of this requirement was justified based on the sensitivity of the assay to other potential errors, such as those cited in the section immediately above. Given the cost of a microliter syringe (<USD 100) relative to the investment most labs make in laboratory equipment and the assays themselves, we feel it is prudent to use this tool to reduce variability in the assay, which along with other factors, could result in an invalid assay. Most manufacturers of air-displacement pipettes recommend the use of positive displacement pipettes (microliter syringes are an inexpensive alternative since they work by positive displacement) for ~50% glycerol solutions (the kit's concentrated conjugate contains a minimum of 50% glycerol) and laboratories that may be having difficulty with precision or meeting the validity criteria of assays may find these devices improve assay performance in their hands. The fact that CEPHIA did not find that this contributed to frequency of valid assays may simply be a reflection of the fact that this pipetting step in conjunction was not in itself, a significant contributing factor to assay variability within their own labs.

25°C Incubator

The report correctly states that "Sedia requires the use of a second incubator during the TMB substrate incubation. CEPHIA has observed that some laboratories do not have a 'room temperature' incubator and suggest that Sedia emphasise the importance of having such a [piece] of equipment." It should be noted that the third warning in the product insert states "It is **critical** that all aspects of the procedure be strictly adhered to, particularly timing and temperatures." This requirement is typically one of the first issues verified when an inquiry arises about out of range values for the validity ranges. A fundamental principle of the assay is the enzymatic oxidation of the TMB substrate by the peroxidase labeled conjugate in the assay to generate a measurable chromagen. Enzyme activity is well known to be affected by temperature changes, and certainly, "room temperature" can vary considerably by season and locale. Failure to utilize a fixed 25°C temperature for the development of the TMB substrate can impact alone, or with other techniques such as those discussed above, to push control and Calibrator values outside of the validity range. This of course, comes back to the warning quoted at the beginning of this paragraph, that users strictly adhere to the procedure.

Associated documentation

2. LAg-Avidity EIA Data Management Worksheet

Considerable discussion is presented in the report regarding changes needed and made to the LAg-Avidity EIA Data Management Worksheet approximately 2 years ago. These issues should no longer apply to current users (who the report acknowledges were notified of the changes) or new users, who would access the Worksheet on Sedia's website. The Worksheet was originally developed by the CDC as an aid to users of the assay to help with data analysis. Sedia provided the Worksheet for download by customers, but Sedia did not have access to the Worksheet content which was locked by CDC. As noted in the report, CEPHIA advised Sedia of errors it had identified. Sedia

verified the errors and reported these to the CDC, which implemented corrections and provided Sedia with corrected Worksheets. At that time Sedia gained access to the Worksheet coding and could do its own internal software validation. Once this was completed, the software was uploaded onto the Sedia website and customers were notified of the updated Worksheet. Sedia appreciates CEPHIA's notification of the Worksheet issues and currently has the means to evaluate reported errors, correct and validate them in a timely manner. There have been no reports of errors since these corrections were implemented in February 2013.

Technical Conclusions

CEPHIA has recommended a comprehensive method of informing users of changes to Sedia Worksheets. Sedia's policy is to notify all customers of major substantive changes to the Worksheets or to the Product Inserts, and has done so to date. Major substantive changes are those likely to impact results or interpretation of data due to changes in procedures, calculations, validity ranges, cutoffs, MDRI, assay design or critical interpretations. Minor changes are typically those to update background scientific literature, provide clarification of wording, update company related information (new products, website links, etc) and notification is not routinely provided to customers for these. Users should note that Sedia Customer Service will notify the purchaser of the product of such changes. However, it should be noted that in many instances, the purchaser may not be the actual user. Users should ensure that the purchaser passes on any communications about the assay to them or contacts Sedia to get on Sedia's customer mailing list. Additionally, Sedia has a Facebook page and Twitter account which are intended to be used for such notifications as well. Users may link to those media (located on the top right corner of the Sedia website) to keep up to date on major changes in technical literature.

Target Product Profile

There are several minor issues with the Target Product Profile in terms of the Sedia™ HIV-1 LAg-Avidity EIA's fit. Sedia recommends CEPHIA use a consistent format for how this column is reported. For example, the Sedia™ HIV-1 LAg-Avidity EIA TPP table simply enters "Acceptable" for those items that comply rather than the actual value or result as is done with the Sedia™ BED HIV-1 Incidence EIA TPP as presented in CEPHIA's evaluation report on that product.

- Intended Use — Nothing stated for "fit". The Sedia™ HIV-1 LAg-Avidity EIA is intended for the same purposes as in the Ideal Performance column
- False Recent Rate (FRR) — The calculated FRR is stated as acceptable, but the value is not stated here, although it is stated elsewhere.
- Mean Duration — Stated as acceptable but value not stated here, although it is stated elsewhere. It does not address the differences between the CEPHIA calculated MDRI and that which CDC calculated in consultation with CEPHIA's partner SACEMA and which is incorporated into the product insert.
- Algorithm — The report states that an "algorithm is likely to be

	required", although this definitive conclusion is inconsistent with the more equivocal conclusion in the summary which states use of the assay "may be useful as part of an Incidence assay algorithm". It's not clear if the considered algorithm would be a second assay or simply a "combination of clinical and other supporting information." The assay, was only evaluated in the report as a solo test, not in any reported algorithm.
• Sample Type	Assay also can test fresh serum or plasma;
• Sample Volume	Actual volume not stated (5 µL per well)
• Shelf Life	Actual shelf life not stated. Shelf life is currently 24 months from date of manufacture.

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The Conclusions and recommendations here are those of the authors and not of their institutions. They do not constitute an endorsement of any product.