CEPHIA

Consortium for the Evaluation and Performance of HIV Incidence Assays

Asanté™ HIV-1 Rapid Recency® Assay

Evaluation Report

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Evaluation of the Asanté™ HIV-1 Rapid Recency® Assay

Lateral Flow Point of Collection Immunoassay for Detection of Recent Infection.

Cat. No. 1130

CEPHIA PROJECT TEAM

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Summary

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 that are able to distinguish between 'recently' acquired HIV-1 infections and 'long-standing' infections, to estimate HIV incidence within cross-sectional surveys. 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 CEPIHA Evaluation Panel consists of 2,499 uniquely-labelled HIV-1-positive plasma specimens[1] 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 critical assay/recent infection testing algorithm (RITA) characteristics for cross-sectional incidence estimation, 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 of the Asanté™ HIV-1 Rapid Recency® Assay by itself (excluding treated subjects and identified elite controllers in the CEPHIA evaluation panel) when recency discrimination is made visually was estimated at 105 days (95% confidence interval 86-125). When the electronic reader device is used at the standard threshold, the MDRI was 197 days (171-224). The FRR in the same specimen set is 1.6% and 3.6% respectively. High FRRs occur amongst treated subjects (53.5% and 58.1%) and elite controllers (11.5% and 16.0%).

Technical Appraisal

This assay is a commercially available assay developed specifically for the purpose of differentiating recent from longstanding infections in cross-sectional studies. It is a lateral flow point of collection immunoassay requiring minimal apparatus available to most laboratories. The assay comes as individual tests in a 20 or 100 batch format and is stored at 2-30°C. No EQA scheme is currently available. The assay is simple to perform following training.

Conclusions

This product does not fulfil all 'ideal' components of the Target Product Profile (TPP) for use in cross sectional incidence assays but does reach all acceptable criteria. We are in agreement with the company that this assay should not be used as a standalone assay but feel it may be useful as part of a recent infection testing algorithm.

[1] One HIV-negative specimen is included in the panel but excluded from analyses.

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 that 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 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, ultimately resulting in the formation of the **Consortium for the Evaluation and Performance of HIV Incidence Assays** (CEPHIA).

CEPHIA

CEPHIA brings together world leaders in the development, performance evaluation and application of RITAs for identifying 'recent' HIV infection. CEPHIA's purpose was to successfully deliver a 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 several available assays. Following the end of this project CEPHIA continues to perform evaluations funded either by grant awards from other agencies or through funding from assay manufacturers.

This report details the results of the evaluation of the **Asanté™ HIV-1 Rapid Recency® Assay.** This evaluation was funded by Sedia Biosciences through a grant awarded by the NIH (R44-AI114365-03). A project plan was agreed in advance and training provided by Sedia staff, but the evaluation was carried out independently with no input from Sedia.

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

All evaluation data was analysed by the CEPHIA team at Vitalant Research Institute.

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.

The Asanté™ HIV-1 Rapid Recency® Assay

Description of Assay

The Asanté™ is a single-use rapid in vitro immunoassay that distinguishes HIV-1 infections on the basis of recency of infection. The assay is intended for use with blood (both venous and finger-stick), serum or plasma specimens as either a laboratory or point-of-collection test to detect HIV antibodies and recency of HIV-1 infection at the same time. The Asanté™ HIV-1 Rapid Recency® Assay comprises a Blood Specimen Collection Loop, a capped tube containing 0.5 mL of Sample Buffer and a Test Strip. The Test Strip itself comprises several materials which in combination are capable of detecting HIV antibodies when a blood, serum or plasma sample containing HIV antibodies is added to the Sample Buffer Tube.

The manufacturer claims the assay may also be used to estimate the HIV-1 incidence rates in a population, to monitor and to evaluate intervention programs, and to identify high-incidence populations so that prevention research, vaccine trials, and resources are most appropriately utilized.

Figure 1: Asanté™ test kit

Summary and explanation of the test

This assay uses a lateral flow-type technology to differentiate recent from longstanding infection. Specimen mixed with a Protein A conjugate passes over 3 reagent lines (Control, Verification and LT/R) which each form part of the interpretative and assay validation algorithm.

The Control line contains goat antibodies reactive to human antibodies ("goat anti-human antibodies") which will bind human antibodies in the liquid regardless of whether those antibodies are HIV positive or negative. If an adequate sample has been collected and the test is both performing correctly and run correctly, antibodies will be present in the specimen, and will have bound to the conjugate and be captured on the Control Line, giving a visible reddish-purple Control Line indicating that a valid test has been performed.

The Verification line is required to be present for a valid LT/R Line result. The Verification line is made up of HIV antigens that will bind HIV antibodies in the sample being tested. The appearance of a Verification line serves to verify that only specimens that have been determined to be HIV-positive are being tested. If no Verification line appears from a previously diagnosed HIV specimen, the test should be rerun with a freshly prepared sample and assay and the specimen's diagnostic status be reconfirmed. The Verification line is not intended to determine the diagnostic status of the individual but only to verify that the test is suitable for use on the sample tested.

The LT/R line is used to determine whether the infection is likely to be a recent or long-term infection. A reaction with this line, above a defined threshold, indicates a long-term infection. No reaction or a reaction below the defined threshold, in the presence of reactive verification and control lines, indicates a recent infection.

Principles of the procedure

1. *The **Asanté™ HIV-1 Rapid Recency® Assay** is a lateral flow point of collection immunoassay.*
2. *Sample is mixed with a sample buffer and agitated to release the blood/serum/plasma into the buffer.*
3. *The test is then initiated by placing the Test Strip into the Sample Buffer Tube containing the sample. The sample/Sample Buffer mixture is absorbed into the absorbent pad at the end of the Test Strip and travels the length of the strip over a 20-minute period.*
4. *The mixture passes across two invisible lines of HIV antigens and one line of goat anti-human antibodies. Reaction with the various lines of antigens and antibodies indicates whether the test is valid and whether the sample is likely a recent or longstanding HIV infection.*
5. *Low avidity antibody will not bind in sufficient amounts to be detected by the LT/R line but will be detected by the verification line thus indication a recent infection. Anti-human antibody will confirm that the specimen contains human antibody and therefore verify the test. Specimens must be confirmed as anti-HIV-1 positive before using this test.*

General Kit Information

The **Asanté™ HIV-1 Rapid Recency® Assay** is comprised of a Blood Specimen Collection Loop, a capped tube containing 0.5 mL of Sample Buffer and a Test Strip. The **Asanté™ HIV-1 Rapid Recency® Assay** is available in 20 Test Packs (Cat. No. 1130-20) and 100 Test Packs (Cat. No. 1130-100).

ASSAY EVALUATION

A summary of the characteristics of the **Asanté™ HIV-1 Rapid Recency® 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.

Table 1: Assay Information Summary

General	
Assay Name	**Asanté™ HIV-1 Rapid Recency® Assay**
Manufacturer	Sedia Biosciences Corporation
Catalogue Number	1130
Number of Specimens can test/Kit	Test available in 20 strips (Cat. No. 1130-020) and 100 strips (Cat. No. 1130-100) batches. Each strip can test one specimen
Test Volume	Approximately 5µl serum, plasma or whole blood (collected using a supplied collection loop)

Presentation	
Assay type	Lateral flow type, single use point of collection immunoassay
Storage	Store at 2-30°C unopened – open immediately prior to use
Antigens (attached to test strip)	Multi-subtype recombinant HIV-1 Ag (rIDR-M) for LT/R line P24, gp41 and p36 for verification line Goat anti-human antibodies for control line
Sample buffer	0.5ml individual use vial. Buffer contains blocking agents and sample conditioning agents.
Conjugate	Protein A colloidal gold
Asanté™ Rapid test strip reader	If not reading visually

Stages	
Reagent Preparation time	None if stored at 15-30°C, 60 minutes to reach room temperature if stored at lower temperatures
Specimen dilution set-up time	2-5 minutes
Assay run time	20 minutes
Total time to completion	25 minutes

Additional Equipment Required	
Serological pipettes and tips	single (2-20ul) if not using blood collection loop
READER – Asanté™ Rapid Test Strip Reader Cat. No. 1200	Battery powered, connection to a PC computer is via a USB cable. It should be noted that the RDS-1500 Software has been modified with settings proprietary to Sedia.
Black cartridge adapter	Holds the test strip for insertion into the Reader
White calibration cartridge	Needed to verify and update a unit's calibration status at the beginning of each testing day
Stylus	For use with screen on the automated Reader
Lancet for fingerprick collection	
Timer	Capable to accurately measure 20 minutes
Household bleach and Biohazardous waste container	10% bleach solution
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 (Adapted from kit insert LN-6122.05)

The US CDC has reported that the Verification Line of the Asanté™ HIV-1 Rapid Recency® Assay detected 575/580 HIV positive specimens correctly resulting in a sensitivity of 99.1% (95% CI 98.0-99.6%) while specificity of the Verification Line was 98.7% (903/920)(95% CI 97.7-99.3%) There was a high correlation (Spearman rank correlation r=0.785) between ODn of LAg-Avidity EIA and the LT/R Line intensity of the Asanté™ HIV-1 Rapid Recency® Assay for 570 HIV-1 specimens with cutoff of 3.0 matching with LAg ODn of 2.0 corresponding to a Mean Duration of Recent Infection of about 180 days 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.

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%.

Limitations of the assay (from kit insert LN-6122.05)

1. Failure to perform the assay according to the instruction for use may lead to inaccurate results.
2. The assay must be read in the defined time frame as inaccurate timing may lead to inaccurate results.
3. The Asanté™ HIV-1 Rapid Recency® Assay is for Research Use Only. It is not intended for use in diagnostic procedures or for use with other sample types than those described in the Instructions for Use.
4. The Mean Duration of Recent Infection (MDRI) or “window period” of recent infection for this assay has not yet been definitively determined. The False Recency Rate (FRR) of this assay has been estimated to be similar to the Sedia® HIV-1 LAg-Avidity EIA or about 1% excluding treated patients and elite controllers.
5. 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.
6. This assay should be used when possible as part of an algorithm with HIV viral load.
7. Individuals with HIV-2 infection are likely to misclassify as recent due to the lack of HIV-2 antigens in the LT/R line however they may be detected as present in the verification line.

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 **Asanté™ HIV-1 Rapid Recency® Assay** 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/asante-rapid-hiv-1-recency-assay.

Prior to beginning the evaluation the two evaluators were trained in performance of the assay by Sedia over a two-day period. Training was performed using a company-provided training panel and both staff were deemed competent by Sedia at the end of the process.

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 **Asanté™ HIV-1 Rapid Recency® Assay** were used during the evaluation. The kit insert supplied with each lot was the same.

KIT LOT: KC2703 supplied with Kit Insert LN-6122.03
KIT LOT: KC2704 supplied with Kit Insert LN-6122.03

Assay Kits were stored at room temperature until use. Room temperature in the testing laboratory was between 19.5 ^{o}C and 24.7^{o}C over the course of the testing.

The electronic reader was supplied by Sedia. All other equipment (pipettes etc) had undergone proper installation, operation and performance/monitoring qualification prior to testing to minimise assay variability as per the evaluating laboratories accreditation standard.

The specimens were tested as per the kit insert and following the testing algorithm shown below. Specimen were tested in batches of 40 over a period of 134 days. Visual readings were taken within 5 mins of the end of the run and readings via the supplied reader within 5 minutes. Visual readings were then completed by a separate technician.

4 specimens had to be repeated due to assay failure. Assay failure was determined as a failure of the control line.

44 specimens were retested at the end of the study after producing unverified/unconfirmed negative results despite being confirmed HIV-1-positive using other diagnostics. These specimens were then classified as either repeat non-reactive on the Verification line or interpreted according to standard procedures (including being subjected to confirmatory testing where indicated).

Visual reading and electronic reading were treated as separate experiments, although reading was conducted in parallel. Upon importing testing results into the CEPHIA database, an algorithm was applied separately to visual and electronic results, with final results assigned according to the testing procedure for each.

For visual reading, the procedure was:

1. If an invalid result is obtained at any stage, discard and repeat.
2. If a valid result is obtained and the Verification line is absent but the specimen is confirmed HIV-1-positive, repeat the test. If the Verification line is present, assign a recent/long-term classification.
3. If upon repeat testing a specimen where the initial result was unverified, the Verification line is again absent: classify as repeat non-reactive on Verification line. If it is present, assign a recent/long-term classification.

For electronic reading, the procedure is shown in the flow diagram below (Figure 2).

Overall, using the visual algorithm, 54 specimens were initially classified as unverified; upon repeat testing 5 were re-classified as verified infection, and 49 specimens were classified as repeat non-reactive on the Verification line. Using the electronic reading algorithm, 103 specimens were initially unverified, but upon repeat testing 2 were re-classified as verified infection, 45 specimens were assigned a final status of unverified infection after confirmatory testing (i.e. initial results were in the “grey zone” as indicated in the flow diagram below), and 56 specimens were classified as repeat non-reactive after being tested with the Asanté™ a second time. Thus, a total of 49 and 101 (out of 2,499) HIV-1-positive specimens were not verified and not eligible for a recency classification on the visual and electronic reading algorithms respectively. The HIV-1-negative specimen was correctly classified as unconfirmed negative. It appears that visual reading of the Verification line is substantially more sensitive than electronic reading, although the relative specificity cannot be determined from these data.

Figure 2: Electronic reader testing and interpretation algorithm

SEDIA BIOSCIENCES CORPORATION

ASANTÉ HIV-1 RAPID RECENCY® ASSAY
WORK-FLOW INSTRUCTIONS:
Algorithm Flowchart for Reader Results

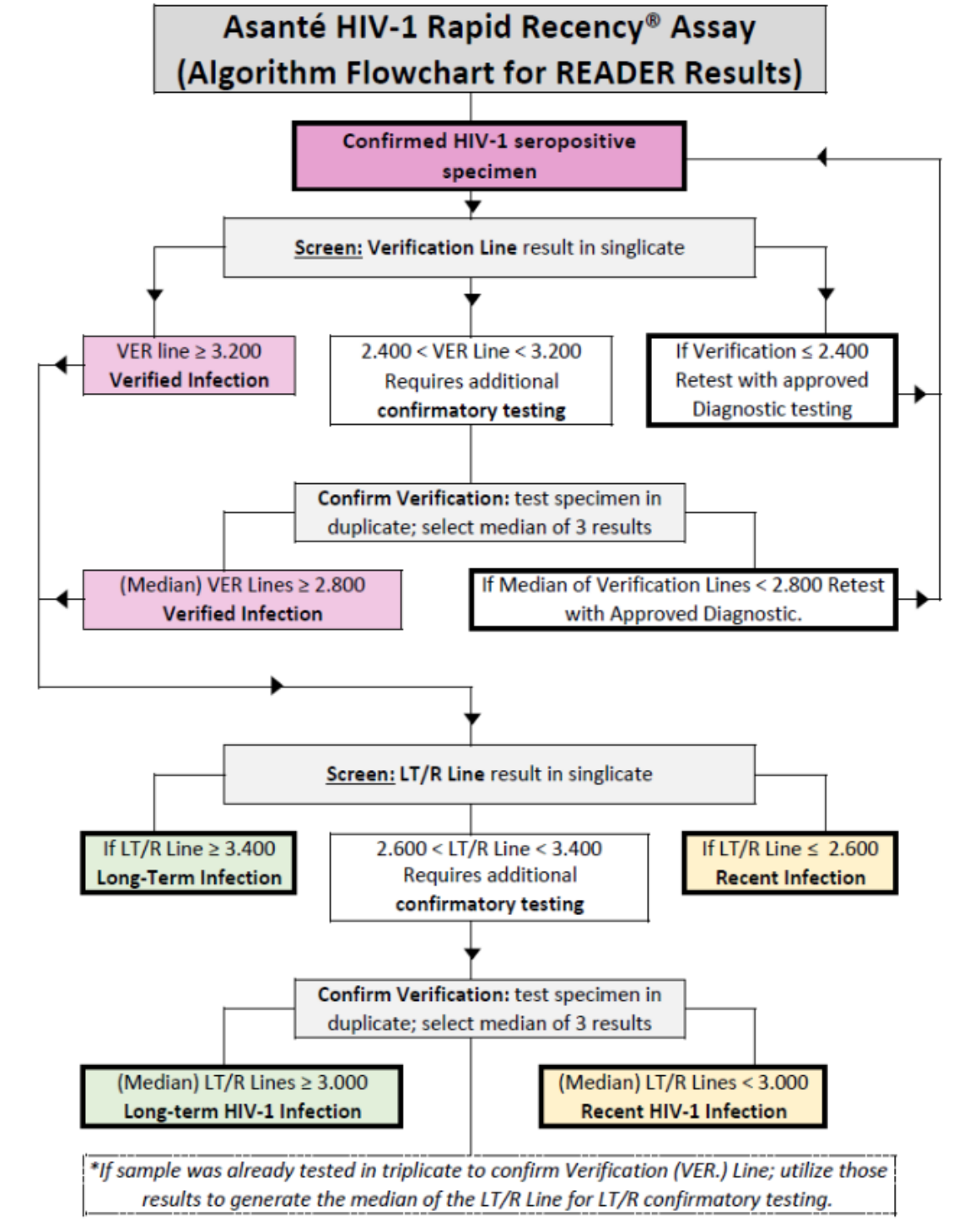

Table 3: Source of Specimens used in the Evaluation Set

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+ cohorts		
SCOPE enrols HIV+ men and women both on and off ARV treatment, actively recruits elite controllers, and follows these subjects over time. *SFMHS enrolled both HIV- and HIV+ men from a population-based sample in San Francisco 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 4: 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 3: Number of specimens drawn over time per subject, for specimens included on the evaluation panel

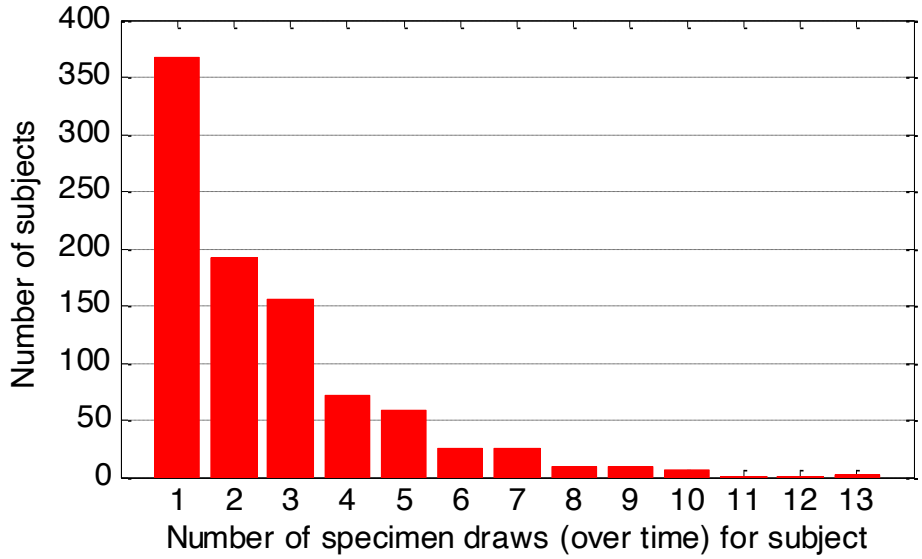

Table 5: Times from (estimated) infection to specimen draws for ARV-naïve subjects included in the evaluation panel (for estimation of the MDRI), stratified by subtype[1]

	All subtypes	Subtype B	Subtype C	Subtype A1	Subtype D
Duration of infection	Number of subjects				
Estimable duration of infection[2]	873	233	373	169	74
0–1 year duration	315	85	142	41	37
1–2 years duration	266	57	114	64	23
2–3 years duration	148	36	62	37	10
3–4 years duration	87	25	36	20	4
>4 years duration	57	30	19	7	0
	Number of specimens				
Estimable duration of infection[2]	1,658	549	608	290	156
0–1 year duration	756	250	298	70	103
1–2 years duration	418	98	164	109	36
2–3 years duration	242	77	81	67	13
3–4 years duration	129	45	45	31	4
>4 years duration	113	79	20	13	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 first detectability on a viral load assay with a limit of detection of 1 RNA copy/mL. See *Analysis of Assay Characteristics* for the approach used for estimating infection times.

Table 6: 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
Duration of infection[2]	Number of subjects				
1–2 years	294	91	112	61	22
2–3 years	167	54	62	38	10
3–4 years	101	39	36	20	4
4–5 years	76	49	19	7	0
>5 years	102	102	0	0	0
Duration of infection[2]	Number of specimens				
1–2 years	446	143	155	103	34
2–3 years	260	98	80	65	13
3–4 years	144	60	45	31	4
4–5 years	96	63	20	12	0
>5 years	184	184	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] For FRR estimation, duration of infection is defined as time since Last Plausible Date of Detectable Infection, i.e. subject is infected at least the period specified.

Table 7: Description of *challenge* specimens drawn from subjects infected for greater than 2 years included in the evaluation panel (for estimation of the FRR)

	All subtypes[2]	
Subject/ specimen group	**Number of subjects**	**Number of specimens**
SCOPE elite controllers[1]	31	89
CD4 cell count < 200 at draw	124	214
Treated subjects[2]	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] Subjects were identified as elite controllers by SCOPE (classification rules are outlined in defined in *Analysis of Assay Characteristics*).

[2] Treated for at least 3 months and without interruption.

Analysis of Assay Characteristics

The methods outlined below follow previous CEPHIA evaluations as reported in several journal articles (1-3) and are well-benchmarked (4).

Definitions of Assay Characteristics

In 1995, Brookmeyer and Quinn (5) 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) persists for only a few weeks after infection, resulting in very low population level prevalence of 'recent infection' and hence 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 suboptimal – due to the extremes of inter-subject variability, a substantial proportion of long-infected individuals return 'recent' results.

As the methodology has matured, a general theoretical framework has been derived, which consistently accounts for these 'false-recent' results (6). 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.

Note the critical use of 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:

$$\hat{I} = \frac{n_R - \beta_T n_+}{n_S \cdot (\Omega_T - \beta_T T)}$$

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 Ω_T and β_T are the estimated MDRI and FRR for the test for recent infection respectively. The formula can be generalised to use estimates of prevalence of HIV and prevalence of recent infection amongst HIV-infected individuals (together with the variance-covariance structure of these) in order to accommodate data from surveys with complex sampling frames:

$$\hat{I} = \frac{P_+ \cdot (P_{R|+} - \beta_T)}{(1 - P_+) \cdot (\Omega_T - \beta_T \mathrm{T})}$$

where P_+ is the prevalence of HIV infection and $P_{R|+}$ is the prevalence of recent infection in the HIV-infected population. T is an arbitrary cutoff time post-infection beyond which "recent" cases are defined as falsely recent.

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 the R statistical computing environment version 3.6.0 using the R package *inctools* version 1.0.14 (7).

Interpretation of assay results. The Asanté™ results were interpreted according to developer's guidelines (see standard operating procedures on the CEPHIA project website (8). In particular, a threshold of 1.0 (presence) on the LT/R line read visually, and 3.0 on the LT/R band intensity read using the electronic reader, were used to discriminate between 'recent' and 'non-recent' infection. Recency classifications were only applied to specimens with positive Verification line readings (present on visual reading and ≥ 2.8 when confirmatory testing was conducted or ≥ 3.2 when tested in singlet). For visual readings intensities on an ordinal scale of 0, 1, 2 ,3, 4, plus half units in between, were assigned by technicians subjectively. However, in analysis, only the absence or presence of the line were utilized, according to manufacturer's recommended interpretation.

Stratification of data. Assay characteristics were estimated using specimen sets defined by stratifying on treatment history, viral load, time from infection to specimen draw, and HIV subtype (which was based on country of draw, for the 50% of specimens (57% of subjects) that 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, these data were 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.

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 this analysis, we used detailed diagnostic testing histories to estimate the date of earliest 'detectable infection' on a hypothetical viral load assay with a threshold of detection of one copy per ml of plasma. These "Estimated Dates of Detectable Infection" (EDDIs) were estimated for 67% of subjects in the Evaluation Panel. Each subject is further assigned an interval (earliest plausible and latest plausible dates of

detectable infection, EP-DDI and LP-DDI) representing the period during which the first detectability is likely to have occurred.

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. 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 a 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.

Estimation of MDRI. A number of methods can reasonably be used to estimate the MDRI, each with its own accuracy, precision and complexity (4). 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 (9), 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 as primary results, where g is the logit link, and $f(t)$ is a cubic polynomial in t. The MDRI, expressed mathematically as $\int_0^T P_R(t)\mathrm{d}t$, was estimated using the fitted $P_R(t) = g^{-1}(f(t))$. We also estimated MDRI using an alternative 2-parameter model form, using a complementary log-log link function with simply the natural logarithm of t as the predictor. Estimates are usually insensitive to model choice. These methods are documented in the *inctools* R package (7).

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 (10).

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) should in the first instance be used to estimate the FRR in well-defined subpopulations. Specimens from long-infected subjects were identified (specimens drawn at least T after the subject's LP-DDI), and the proportion of 'recently' infected subjects estimated in various subsets. 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 (11) are provided.

Performance in Surveillance. While the performance of recent infection tests is ultimately encoded in the MDRI and FRR, these properties interact with (and indeed depend on) contextual factors, to produce what is ultimately important – namely the variance or standard

error of an incidence estimate which can be obtained in a given surveillance application (12). The MDRI mainly varies according to the sensitivity of the diagnostic testing algorithm used to identify HIV positive cases, as this determines how soon after HIV exposure cases become detectable, at which point in time the proverbial clock on duration of recent infection starts. There may also be subtype variability of MDRI resulting from differences in primary subtypes between contexts. The FRR is inevitably prone to more complex context-dependencies, as FRR varies sharply between groups such as treated or untreated individuals, elite controllers, and also by subtype (see Results).

In order to estimate performance, a hypothetical epidemiological surveillance scenario was constructed:

- HIV prevalence of 30%.
- Incidence of 2 cases per 100 person-years.
- Treatment coverage of 50% (defined as proportion of HIV infected individuals who are on ART, which is assumed to lead to viral suppression).
- Sample size of 10 000 individuals (simple, non-stratified, non-hierarchical sampling frame).
- HIV case definition based on highly sensitive Viral Load Assay with a threshold of detection of 30 copies/ml. This crucially differs from most previous analyses (such as (2)) which have tended to use Western blot confirmation as the critical HIV positivity confirmation assay. It also leads to a shortening of MDRI by 5 days relative to that reported in the primary results tables, which uses a hypothetical viral load detection threshold of 1 copy/ml as the definition of detectable infection.

To estimate the FRR in untreated individuals, the function $P_R(t)$ was fit using data from all times post-infection, and weighted according to the probability density function for times since infection in the untreated population, which was parameterised as a Weibull survival function, with the shape and scale parameters chosen to produce the desired treatment coverage in a population with the specified incidence and prevalence. The FRR in treated subjects is simply the binomially estimated proportion of treated subjects infected for longer than T that produce a recent result.[2]

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.

[2] It should be noted that, in the CEPHIA Evaluation Panel, all treated subjects are virally suppressed, resulting in an estimate of $FRR = 0$ in among treated subjects in all cases where a supplemental viral load threshold is applied. In real-world populations, it is likely that some (currently unknown, but probably very small) proportion of treated subjects would be virally unsuppressed and that the FRR in treated subjects would therefore be non-zero.

Results

The evolution of LT/R reactivity (as measured by the electronic test strip reader) by time since infection, excluding treated subjects and SCOPE ECs, is shown in Figures 4 and 5. Quantitative results are not shown for visual reading, as the visual read is considered qualitative and is dichotomous. The figures show that there is natural variability in biomarker maturation, leading to a significant number of subjects reaching the standard recent/long-term threshold (3.0) more than one year after infection.

Table 8 provides estimated assay characteristics for various specimen sets. The estimated MDRI, excluding treated subjects and SCOPE ECs from the analysis, is 105 days (95% CI: 86–124) when recency classifications were assigned visually, and 197 days (171–224) when using the standard threshold applied to electronically-read band intensities. Using the alternative model form, MDRI estimates were 115 days (98–134) and 205 days (179–231) for visual and electronic reading, respectively. MDRI estimates were unusually sensitive to model choice, probably related to an unusual feature of the fitted $P_R(t)$ curves: the estimated probability of obtaining a recent result at very early times post-infection is substantially below 1. Since the verification procedure resulted in many specimens drawn early in infection that were not eligible for a recency interpretation, it is likely that the regression models suffered from sparse data at very low values of t. The possibility of interpreting Asanté™-negative/unverified specimens that are confirmed HIV-1-positive using other diagnostic assays will be explored in a future publication.

If a recent infection testing algorithm relies on verification by the Asanté™, the MDRI would likely be reduced substantially from the MDRIs reported here, which are estimated relative to a highly sensitive NAT assay with a limit of detection of 1 RNA copy/mL. Based on the average diagnostic delay of third-generation IgM-sensitive antibody rapid tests, a reasonable expectation of MDRI for Asanté™ used in an algorithm with an IgM-sensitive rapid antibody test as the screening test would be a reduction of 22 days. This would yield an MDRI for visual interpretation of 83 days and for electronic reader interpretation of 175 days. The "diagnostic delay" of the Asanté™ verification line is the subject of a future study by the authors of this report and their collaborators.

We estimated the difference between visual and electronic MDRIs and approximated a 95% CI on the MDRI difference by bootstrapping the dataset at subject level and computing the MDRI difference on each iteration. The difference was estimated at 92 days (95% CI: 75–110), which excluded zero and was therefore considered statistically significant. *The dramatic difference in visual and electronic MDRIs may indicate that electronic reading should be preferred for population-level incidence estimation.* The latter procedure further allows the use of alternative recent/longstanding thresholds in order to optimize the precision of incidence estimates.

Excluding treated subjects and SCOPE ECs, and analysing all remaining specimens drawn more than $T = 2$ years after infection, the measured FRRs are 1.6% (95% CI: 0.5%–3.7%) for the visual interpretation and 3.6% (1.8%–6.3%) for electronic reading. The FRRs amongst elite controller specimens are very high at 11.5% (2.5%–30.2%) and 16.0% (4.5%–36.1%), respectively and even higher amongst treated subjects at 53.5% (43.2%–63.6%) and 58.1% (47.0%–68.7%) respectively. Further stratifying treated subjects by time from infection to

treatment initiation, the FRR decreases as the time to treatment initiation increases (see Table 8). The FRRs for subjects with low viral loads – here defined as equal to or below 75 copies/ml, and which overlaps significantly with the EC and treated subjects – are 73.9% (65.6%–81.1%) and 52.1% (42.8%–61.2%) for visual and electronic, respectively.

Table 9 lists MDRI and FRR by subtype. In a test for pairwise differences in visual MDRIs by subtype, using a z-test, no pairs provided *p*-values <0.05, although B vs. D and B vs. A1 provided *p*-values between 0.05 and 0.1. For electronic MDRIs, no pairs provided *p*-values <0.05, although C vs. D provided a *p*-value between 0.05 and 0.1.

In a test for pairwise differences in FRRs by subtype, using Boschloo's exact test for binomial proportions (13), no pairs provided *p*-values <0.05 on either the visual or electronic algorithms.

Figure 6 summarises 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 are included in Table 10. Coefficients of variation on electronic LT/R band intensity measurements across the 25 replicates of each control specimen varied between 4.5% and 8%.

Figure 4: Scatter and spaghetti plots of subjects' assay measurements as a function of estimated time since detectable infection, excluding treated subjects and SCOPE elite controllers (electronic)

The figure represents 1431 data points from 431 subjects. The 'recent'/'non-recent' threshold is indicated by a horizontal line.

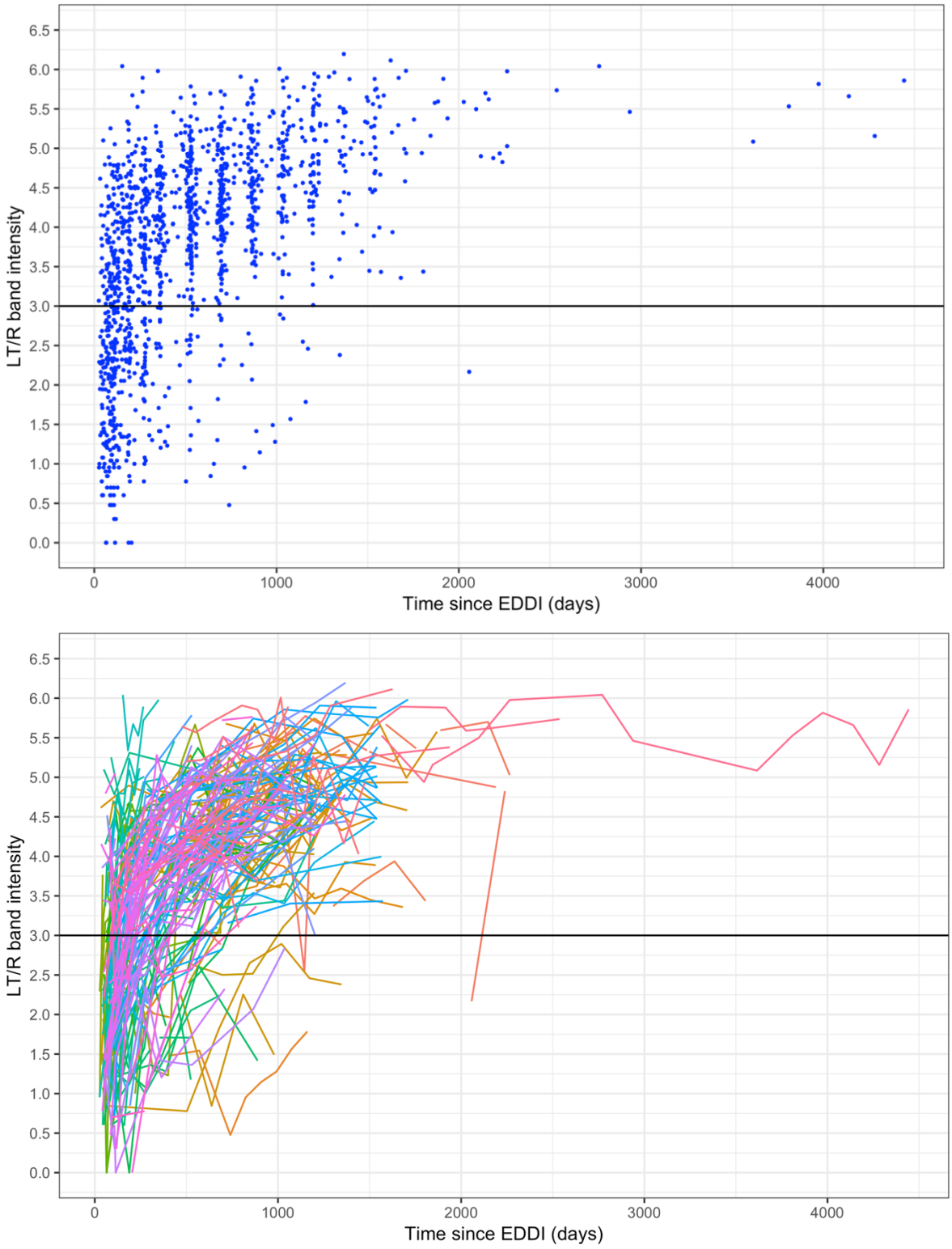

Figure 5: Box-and-whisker plots of assay measurements as a function of estimated time since detectable infection, excluding treated subjects and SCOPE elite controllers (electronic)

The plot indicates measurements in 2-month intervals after infection. The central 50% and median of measurements in each bin are captured by the box and dividing line respectively. Whiskers and markers capture remaining measurements and outliers respectively. The number of data points per bin range from 2 to 210. The recent/non-recent threshold is indicated by the horizontal line.

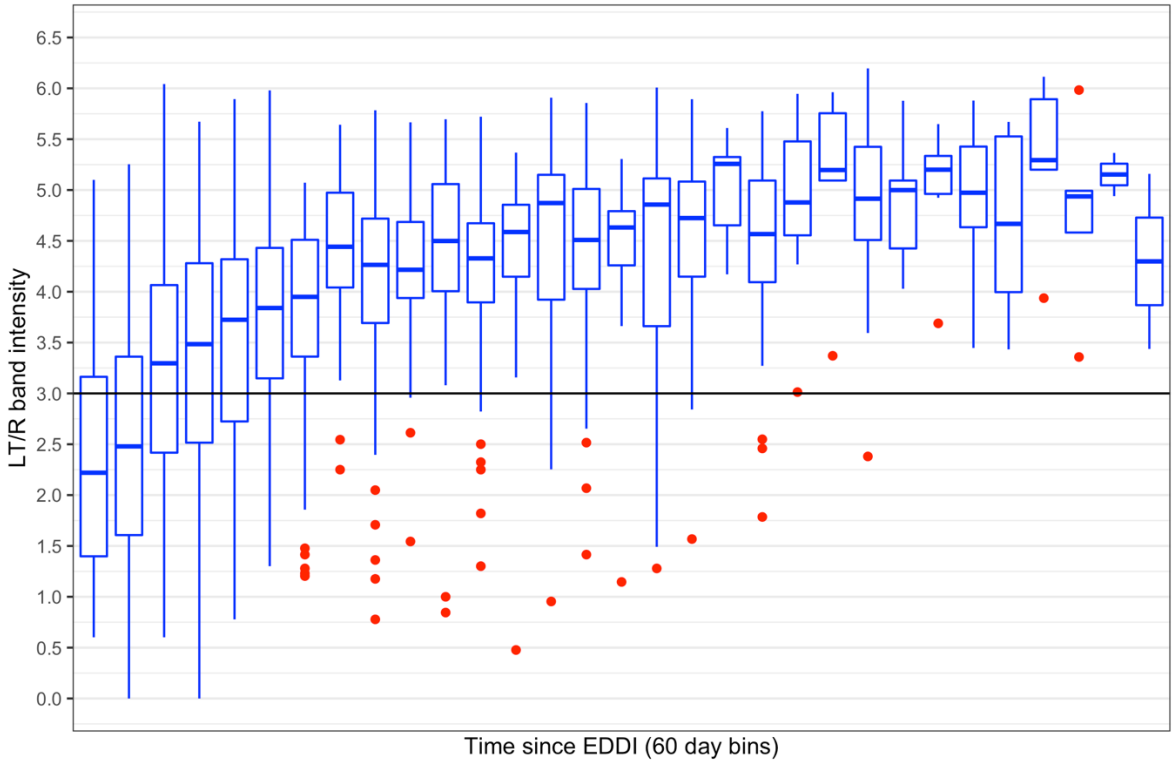

Table 8: Estimated assay characteristics (and 95% confidence intervals), for various specimen sets

Assay characteristics are estimated for $T = 2$ years and detectable infection defined as positivity on a hypothetical viral load assay with a detection threshold of 1 copy/ml.

	Visual		Electronic	
	Number of subjects (number of data points)*	**Estimated assay characteristics (95% CI)**	**Number of subjects (number of data points)***	**Estimated assay characteristics (95% CI)**
MDRI in days				
ARV-naïve subjects, excluding SCOPE elite controllers	440 (1111)	105** (86–124)	436 (1098)	197** (171–224)
FRR as %				
ARV-naïve subjects, excluding SCOPE elite controllers	309 (683)	1.6 (0.5–3.7)	309 (679)	3.6 (1.8–6.3)
Elite controllers (identified by SCOPE cohort)	26 (66)	11.5 (2.5–30.2)	25 (64)	16.0 (4.5–36.1)
Treated subjects (no previous treatment interruption and treated for at least 90 days)	99 (160)	53.5 (43.2–63.6)	86 (139)	58.1 (47.0–68.7)
Early treated subjects (time from infection to treatment initiation ≤ 6 months and treated for at least 90 days)	41 (67)	95.1 (83.5–99.4)	31 (49)	96.8 (93.3–99.9)
Late treated subjects (time from infection to treatment initiation > 6 months and treated for at least 90 days)	56 (91)	26.8 (15.8–40.3)	53 (88)	35.9 (23.1–50.2)
Low viral load (≤75 copies/ml at draw)	134 (209)	73.9 (65.6–81.1)	121 (184)	52.1 (42.8–61.2)

**Valid and verified results. Unverified/"unconfirmed negative" specimens excluded.*

***Note that these MDRI estimates are valid for an HIV-infected case definition of positive on a NAT assay with a limit of detection of 1 RNA copy/mL. If, as per manufacturer's instructions for use, only Asanté™-verified specimens are interpreted as recent, the MDRI would be substantially reduced – likely to approximately 83 and 175 days for visual and electronic reading, respectively.*

Table 9: 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 detectable infection is defined as HIV-positive on a hypothetical viral load assay with a detection threshold of 1 copy/ml. Specimens are considered falsely recent if Avidity Index < 80 and time since LP-DDI > 2 years.

	Visual		Electronic	
	Number of subjects (number of data points)*	**Estimated assay characteristics (95% CI)**	**Number of subjects (number of data points)***	**Estimated assay characteristics (95% CI)**
MDRI in days				
All specimens	440 (1111)	105 (86–124)	436 (1098)	197 (171–224)
Subtype B	113 (303)	82 (45–124)	111 (300)	182 (129–241)
Subtype C	187 (457)	105 (86–127)	187 (455)	183 (146–219)
Subtype D	40 (135)	154 (89–229)	39 (131)	283 (183–396)
Subtype A1	87 (174)	143 (90–197)	86 (170)	230 (169–295)
FRR as %				
All specimens	309 (683)	1.6 (0.5–3.7)	309 (679)	3.6 (1.8–6.3)
Subtype B	183 (404)	1.6 (0.3–4.7)	183 (400)	3.8 (1.6–7.7)
Subtype C	75 (145)	1.3 (0.0–7.2)	75 (145)	1.3 (0.0–7.2)
Subtype D	10 (17)	10.0 (0.3–44.5)	10 (17)	10.0 (0.3–44.5)
Subtype A1	38 (108)	2.6 (0.0–13.8)	38 (108)	5.3 (0.6–17.8)

**Valid and verified results. Unverified/"unconfirmed negative" specimens excluded.*

Figure 6: Reproducibility of CEPHIA *unlabelled* controls (electronic)

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). 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 horizontal solid line. The observed reproducibility statistics (mean, standard deviation and coefficient of variation) of measurements are also tabulated in Table 3.

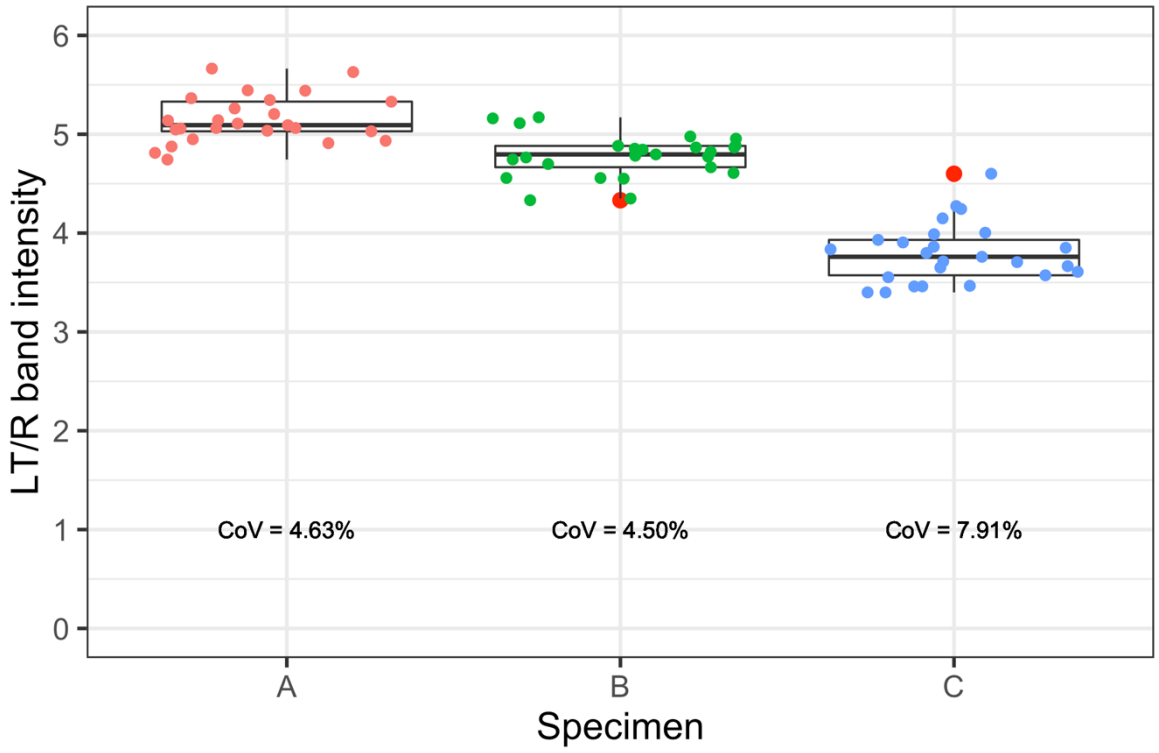

Table 10: Reproducibility of CEPHIA unlabelled controls (visual and electronic)

Specimen	Number of measurements	Visual		Electronic		
		N recent (N tested)	Proportion recent (%)	Mean (LT/R band intensity)	SD (LT/R band intensity)	CoV (%)
A	25	0 (25)	0%	5.15	0.238	4.63
B	25	0 (25)	0%	4.78	0.215	4.50
C	25	0 (25)	0%	3.79	0.300	7.91

Field performance in a hypothetical application scenario

While characteristics, most notably the MDRI, have been reported here on the basis of detectable infection defined as detection on a hypothetical viral load assay with a detection threshold of 1 copy/mL, 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 (14-16). Therefore, for application to incidence studies, the base case MDRI reported here would need to be 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 hypothetical application scenario used to evaluate field performance was summarised under Methods.

Table 11 shows the estimated MDRI (adjusted for individual NAT screening), context-specific FRR, the relative standard error on the incidence estimator and an indicative 95% confidence interval on the incidence estimate that would be produced under the scenario described above. Results are reported for the test developers' recommended assay threshold, with no supplemental viral load testing and with viral load thresholds of 100 and 1 000 copies/ml (LT/R below threshold and viral load above threshold means 'recently infected'). If no supplemental viral load testing is performed, the high context-specific FRR of 30.5% for visual reading and 33.1% for electronic reading renders incidence estimates uninformative (point estimate of 2.00 and 95% CIs of 0.00-19.42 and 0.00–48.61 new cases per 100 person years, respectively). With a supplementary viral load threshold of 100 copies/ml, FRR is reduced markedly to 0.3% and 0.4% respectively and with a viral load threshold of 1 000 copies/ml to 0.3% on both visual and electronic reader algorithms. The relative standard error on incidence estimates are reasonable at between 20 and 30% on the visual algorithms with viral load, and under 20% when using the electronic reader in conjunction with viral load.

Table 11: Estimated assay performance in hypothetical application scenario

Assay threshold	Viral load threshold[1]	MDRI[2]	FRR[3] (%)	RSE on incidence estimate (%)	95% CI on incidence estimate (cases/100PY)
1 (visual)	N/A	100	30.5%	400.4%	0.00–19.42
1 (visual)	100	86	0.3%	22.9%	1.10–2.90
1 (visual)	1000	65	0.3%	27.2%	0.93–3.07
3 (electronic)	N/A	192	33.1%	1188.9%	0.00–48.61
3 (electronic)	100	177	0.4%	15.8%	1.38–2.62
3 (electronic)	1000	146	0.3%	16.9%	1.34–2.66

[1] Viral load greater than threshold means recent.

[2] Adjusted for sensitivity of HIV screening assay (4.8 days shorter).

[3] Context-specific FRR for the specified epidemiological scenario.

Conclusions and Recommendations

1) The performance of the assay when known confounders of assay performance are removed from the study population suggest that this assay is usable as part of a recent infection testing algorithm. The manufacturer recommends use in an algorithm including viral load testing to reduce false recency.

2) This assay performs well but does not reach all of the ideal criteria of the Target Product Profile and therefore cannot be recommended for use on its own in cross-sectional surveys for population-level incidence estimation.

3) Its ease of use makes it very transferable to use in the field. However, use of the Digital Reader should be encouraged to reduce subjective variability and, in population-level incidence estimation, to improve the precision of incidence estimates that can be obtained (thanks to longer MDRI and tuneable threshold).

4) The assay would benefit from being part of an independent EQA scheme. In the absence of a company-supplied control specimen it is recommended that testing sites should ensure each new batch of assays is working as expected in the field by use of an internal quality control specimen.

5) Our results suggest that alternative cut-off values for the LT/R line could be used to increase the MDRI of the assay depending on the use case. Further work to define these should be undertaken.

6) CEPHIA recommends that the manufacturer-suggested testing algorithm be changed to recommend that confirmed anti-HIV-1 positive specimens that test as "unconfirmed negative"/unverified be considered indicative of 'recent' infection, rather than being excluded from further analyses. Especially in the case of visual interpretation, the requirement to verify specimens using the IgM antibody-sensitive verification line results in a very short MDRI. However, this should be done taking into account available diagnostic tests and the potential availability of PCR to confirm infection.

Technical Appraisal

Assay Kits and Reagents

The **Asanté™ HIV-1 Rapid Recency® Assay** is comprised of a single component box that has simple storage conditions. The reagents are lot-specific and therefore reagents cannot be used between lots. The test strips are individually pouched, which is extremely efficient and means a technician need only to use the required number of strips each time, with no wastage. The specimen diluent comes as individually supplied single use vials of 0.5ml and should be used immediately when opened.

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 in the *Technical Conclusions* section.

Associated documentation

The availability, usability and reliability of accompanying documents, e.g kit inserts, are vital to the performance of the assay.

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.

Technical Conclusions

This assay is a point of care immunoassay, but this evaluation was carried out under laboratory conditions, using the blood collection loop in all cases. The presence of a blood collection loop does make the assay more usable in non-laboratory settings.

The assay is quick to perform, and the instructions are clear. Although training was provided by the company for technicians in this evaluation, it is unlikely that specialist training would be required for all individuals performing the assay. However, for visual reading Sedia does recommend that training be provided, by an experienced user, to improve the new operator's visual acuity. This may be even more important for this test than for most diagnostic lateral flow assays, given the frequency of borderline results on the LT/R line – especially in populations where recent infections are common. Staff should demonstrate they are competent in the performance of the assay through use of a set of known specimens prior to using patient specimens. The development and distribution of training and proficiency testing panels that include borderline HIV-positive and borderline recent/long-term specimens is therefore critical in any programme utilizing the assay.

In our evaluation we felt it was reasonable to be able to perform 120 individual tests in a day (conducted in 3 batches of 40), but the numbers that can be performed outside of these controlled conditions may vary.

The assay can be read quantitatively using the Sedia-supplied reader. This reader is easy to use; however, failure to insert the strip correctly into the reader can lead to some erroneous reading and therefore care should be taken to ensure that the strip sits properly in the reader. Visual reading was undertaken in this evaluation and it was clear that weak banding may lead to some ambiguous results and discordant reads from different operators. The calculated MDRI is significantly shorter when using visual reads.

The kit insert does not detail the lighting conditions to be used when reading the assay. If being used in non-standardised areas (i.e. not in a laboratory) the presence of variable lighting and conditions should be considered if performing visual reads. There is also no discussion of the effect of prevailing conditions on the performance of the test, e.g. whether direct sunlight impacts on assay performance or ability to interpret the test, if performed outside.

Although the assay is marketed as being able to be read visually or with a supplied electronic reader, we would recommend that only the reader be used to reduce the potential for subjective error.

The testing algorithm is clear and simple to follow. However, there remains the possibility to miss samples that are recently infected if the Verification line is below a certain threshold, as the sensitivity of the Verification line may differ when compared with the antibody sensitivity of other common diagnostic assays. In this evaluation, sensitivity of the Verification line was very good with specimens that were strongly reactive in other antibody assays, but was reduced with those closer to the cut-off. Interpretative algorithms should be developed to support correct inclusion in incidence estimates of specimens where the Verification line does not reach positivity despite being known to be anti-HIV-1 positive. As noted earlier, visual reading produced repeat-nonreactive results on the verification line for 44 specimens and electronic reading 56 specimens. However, amongst specimens confirmed reactive on the Geenius assay, the vast majority were verified on the Asanté™.

The MDRI and FRR described in the kit insert are likely to be further refined as more studies on this assay are performed. It will be important for users to regularly review updated estimates, and they potentially may need to reinterpret historical data based on these new estimates.

The assay is marketed as research use only, but several potential uses of the assay have been identified. These may require different application and interpretation of resulting data and this should be considered carefully, especially if this impacts on how the assay is interpreted. Any variation from the calculated MDRI or FRR should be described and be a specific rather than a generalised time frame (e.g. less than one year).

This assay has been licensed from CDC Atlanta on a non-exclusive basis therefore alternative versions of the assay may become available. These may have different performance characteristics; thus it is imperative that the exact title and manufacturer of the assay be used when describing results.

ASSAY EVALUATION

We have not been able to perform any batch-to-batch evaluations of this assay to determine whether there is significant variation between batches in terms of performance or in associated MDRI or FRR.

The current version of the **Asanté™ HIV-1 Rapid Recency® Assay** kit insert can be downloaded from www.sediabio.com.

Target Product Profile performance: Asanté™ HIV-1 Rapid Recency® Assay

Specification	*Acceptable Performance*	*Ideal Performance*	*How does Asanté™ fit?*
Intended Use	*Population-based incidence estimate*	*Population-based incidence estimate, prevention-trial planning, community-level prevention intervention studies*	*Acceptable*
Target Population	*Specific to clade*	*All clades*	*Acceptable –some clade variation in MDRI and FRR*
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).*	*Acceptable*
Mean Duration	*4 months (95% CI, +/- 0.2)*	*1 year (95% CI, +/- 0.2)*	*Acceptable*
Algorithm	*Included in a RITA*	*None required*	*Acceptable*
Sample Type	*Frozen serum, frozen plasma*	*Frozen serum or plasma , dried blood spots (or other easily obtained and stored sample)*	*Acceptable – Not suitable for DBS*
Sample Volume	*1 mL*	*10 uL or fingerstick*	*Ideal*
Infrastructure requirements	*Centralized laboratory facility (clean water and electricity available)*	*None (all reagents and necessary materials to run assay are in self-contained kit)*	*Ideal*
Storage/Shipping Conditions	*4-25 ℃*	*Ambient temperature*	*Ideal*
Incubation Temperature	*4-25 ℃*	*Ambient temperature*	*ideal*
Shelf Life	*9 months*	*>18 months*	*Acceptable*
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*	*Ideal*
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.*

Acknowledgements

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Appendix: Evaluation Protocol

Background

The **Asanté™ HIV-1 Rapid Recency® Assay** is to be evaluated by the CEPHIA group as part of NIH grant to Sedia Biosciences Corporation (R44-AI114365-03).

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,** which details the quality planning, quality control and quality assurance in place at Public Health England (PHE), National Infection Service, London, United Kingdom and the collaborating organisations of Vitalant Research Institute (formerly Blood Systems Research Institute), San Francisco, University of California, San Francisco (UCSF) and Stellenbosch University, Stellenbosch, South Africa, to ensure the consistent delivery of evaluations as part of this program.

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/developer's 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 aliquoted at the central repository (Vitalant 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 project. 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 are 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 in that case will 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, 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 evaluated at the analysis site. 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 Evaluation Report. The manufacturer/developer of the assay concerned will be given the opportunity to comment on results prior to any publishing of data. Raw data will be made available to the manufacturer for potential regulatory uses.

Contact details for CEPHIA

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Manufacturer's comments

Sedia BioSciences Corporation received a draft copy of this report on which they were invited to make comments. Their comments were reviewed by the CEPHIA management team and a final draft of the report prepared. Sedia then reviewed the changes and provided additional comments. Sedia's comments and CEPHIA responses in *italics* are shown below.

Sedia Comments on CEPHIA Draft Evaluation Report of Asanté™ HIV-1 Rapid Recency® Assay (Jul 8 2019)

General

Sedia and *Rapid Recency* are registered trademarks of Sedia Biosciences Corporation in the U.S. and several other countries and should be notated as ® as appropriate. *Asanté* is pending registration and should be notated as ™ as appropriate.

Following this company feedback we have incorporated these suggestions

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Conclusions (page 5)

This section states that “This does not fulfill all components of the Target Product Profile (TPP) for use in cross sectional incidence assays.” Yet, the Target Product Profile Table on page 36, under column “How does RR fit?” indicates that all Specifications meet either the “Acceptable Performance” or the “Ideal Performance”. Furthermore, the Conclusions state “We do not recommend its use as a standalone assay, but feel it may be useful as part of an incidence assay algorithm”. Sedia also does not recommend, and in fact specifically recommends using in an algorithm incorporating Viral Load testing to significantly reduce the False Recent Rate. Given that the specifications are all met by CEPHIA’s own indication, albeit sometimes only satisfactorily but not ideally, and the primary reservation is that the assay is not recommended for use as a standalone assay, but makes no recommendation for its use, one wonders what CEPHIA would require for a recommendation, perhaps only a test that meets all ideal specifications? Is there another assay that CEPHIA would recommend that meets all of the specifications in the TPP?

Based on this feedback the conclusions were adjusted to take into account the company statement of use

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Principles of the procedure (page 9)

Paragraph 5 states that “Low titre and low avidity antibody will not bind in sufficient amounts to be detected by the LT/R line but will be detected by the verification line thus indication [sic] a recent infection.” In fact, low titre antibody can bind to the LT/R line if the antibody is high affinity, although obviously there will be a point when the titre is low enough that LT/R won’t detect very low levels of high titer antibody. For this reason, most persons that have been on extended antiretroviral therapy, which typically have high affinity antibody but low titers of antibody, will give a reactive result on the LT/R, but because the titer is so low in a significant minority of these subjects they don’t bind antibody, causing an elevated False Recent Rate when these subjects are included in incidence estimations. Suggest deleting “Low titre and” from the beginning of the first sentence.

Following this feedback the sentence was adjusted

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Table 1, (page 11)

Test volume – Suggest specifying that “Approximately 5 µL of plasma, serum, or whole blood…” be stated. Serum may also be use and clearly the loop is not a precision instrument with intrinsic variability (which we have determined has no significant impact on the assay) so volume should be considered “approximate”.

This information has been added to the text

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Sample Buffer comments – We don't disclose the formulation of the Sample Buffer, which is proprietary, beyond the information provided in the product MSDS. We can disclose that the Buffer contains blocking agents and sample conditioning agents.

This information has now been included in the table

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Reader-Asanté™ Rapid Test Strip Reader. Connection to a PC computer is via a USB cable. It should be noted that the RDS-1500 Software has been modified with settings proprietary to Sedia.

This information has been added to the table

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Table 5 (page 19)
The totals in the top half and the bottom half of the table don't seem to match the totals one calculates of the individual DOI categories below those totals. For example, the first column in the top half, I would expect 422 = 283+224+125+72+41. Obviously it doesn't, and it's not clear if the subheadings under "Subject has estimable infection date" are intended to be subsets of that heading.

Table 6 (page 20) and Table 7 (page 21)
Same as above. What am I missing?

These tables were erroneous and out of date and have been corrected.

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Data Analysis Methods (page 23)
2nd Paragraph of this section – references to the Visual Scale. Although we used a scale of 0, 1, 2 ,3, 4, plus half units in between, this is actually an ordinal scale, not a nominal scale, with values assigned subjectively. Thus, no assumption is made about the "distance" between values on this scale.

Additional text has been added to the section

October 1, 2019: Word "subjectively" has been misspelled. Sedia acknowledges the changes as addressing its concerns.

October 4, 2019: Spelling has been corrected in final report.

Conclusions and Recommendations (page 33)

Conclusion 1) As stated previously, the manufacturer does not recommend using the assay on its own in cross-sectional surveys. This conclusion ignores this suggestion. Finally, this conclusion seems to suggest that only assays which meet all ideal criteria of the TPP will be recommended.

This has moved to the 2nd conclusion

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Conclusion 2): Although a tepid "endorsement", we can't help but wonder why this is the secondary Conclusion, which specifically responds to usage of the assay as recommended, comes after Conclusion 1 which specifically responds to usage of the assay not as recommended.

This has now been moved to become the first conclusion

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Conclusion 5) Not a criticism or dispute, we agree. We had asked that we could get some probability tables of what given assay cutoff's corresponded to what MDRI's to be able to provide this information to users, and are still hoping to get that data. The data on pages 27 and 28 make it difficult for a user to determine what cutoff should be used if they desire to target an MDRI of a set time, or conversely, if they have subjects with a specific cutoff, what is the likely to be the duration of infection for that subject. This kind of information would benefit researchers, for example, seeking to identify efficacy of therapeutics relative to duration of infection and develop more sophisticated therapeutic targeting. Is it possible to generate a table or nomograph that would enable users to make this correlation that would also indicate precision of those estimates?

Need for further information added

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Conclusion 6) We have had discussions with several parties since this was first raised by CEPHIA in our meeting in Atlanta in March 2019. It would seem this would require more investigation and is a bit more nuanced than simply assuming that samples with diagnostic tests that cannot be verified by the Asanté™ Verification Line are automatically presumed as recent infections. The recommendation assumes that the Diagnostic test results are correct. This may not necessarily be the case, particularly in populations that may yield more frequent false positives from rapid HIV diagnostic tests when those populations have a low prevalence of infections (https://www.ncbi.nlm.nih.gov/pubmed/28758335) or where 4th generation rapid HIV tests are used that themselves have a higher false positive rate (https://www.ncbi.nlm.nih.gov/pubmed/28372891). In addition, the sensitivity of the Asanté™ test has improved as it has continued to undergo scaleup further tightening of the process with experience gained in multiple lots of products that have enabled variabilities in the manufacturing process to be reduced. This increased sensitivity has been observed both internally and by CDC during their qualification of our assay at lot release. We are open to

suggesting that the discordant specimens should be considered possibly recent infections, with an appropriate discordant analysis that may include, for example, PCR testing of such specimens, however in many settings, such analysis is not readily available.

Further clarification added. Our recommendation is based on the test lots we have received but we welcome the described improvement in performance.

October 1, 2019: We believe that the final wording in the report more effectively reports our concern about whether or not the possibility to miss samples that are recently infected if the Verification Line is below its detection threshold and does not confirm other common diagnostic assays with different sensitivity. This information must also be balanced with the specificity of those assays and the positive predictive value of such assays, for example, in low prevalence populations, when considering how to treat specimens that are discordant between the diagnostic assays and the Verification Line of the Asanté™ assay. In such cases, additional investigation and characterization of the specimens is appropriate to resolve the discordance. We therefore propose to add wording to address this concern raised in this report in the product insert at its next revision.

Technical Conclusions (page 34).

Although the second paragraph states that “it is unlikely that specialist training would be required for all individuals performing the assay", it is well known that there is wide variability in the sensitivity of the visual acuity of human operators running lateral flow assay tests in general, especially when reaction lines are read close to the threshold. For this reason, we do recommend that training be provided, at a minimum, by an experienced user, especially in terms of bringing the new operator’s visual acuity to a level close to that of an experienced user. This may be more important for this test than most diagnostic lateral flow assays, whose results in the clinical setting, predominantly result in clear results, while the LT/R line on the Asanté™ will frequently have borderline results in the clinical setting, especially in a population more prone to recent infections.

Additional comment added.

October 1, 2019: As we've gained experience with the assay, including doing multiple trainings of new users, it has continued to be apparent that training is very important for those users relying on visual results, which we believe is less consistent than results obtained with the Asanté™ Reader. For example, users experienced in running and reading other lateral flow assays, when first performing the Asanté™ HIV-1 Rapid Recency® Assay typically will see Verification and LT/R Lines down to around 3.2 to 3.5 as measured by the Asanté™ Reader. After more experience with the assay, (or additional training reading borderline specimens which CDC or we provide to new users) operators become skilled enough to typically have a visual threshold of 2.8 or 3.0 on the Verification Line and LT/R Line, respectively. (We've observed this shift in increased visual acuity with the assays numerous times with experienced lateral flow assay/inexperienced Asanté™ users, including when we first trained CDC's Atlanta Staff who have run thousands of other lateral flow assays.) Individuals that continue to read the assay from time to time on a fairly frequent basis, and would generally be considered "skilled" in the assay typically have visual acuity at

this level. However, in laboratories where hundreds of the assays are run every day over weeks or month, often gain even greater visual acuity as low as 2.5 or 2.6, due to hypersensitive detection of the lines. This happens in Sedia's own QC staff who can see lines remarkably well and run tests more frequently than other employees, and have to be "trained" not to over-read the assay. Although use of the Asanté™ Reader is not practical in many settings, we do recommend that it be used as a training tool to prevent visual acuity "creep" over time and experience, a widely known phenomenon in "high volume QC Circles" of lateral flow assay operators. We recommend the use of a training panel composed of a 1:2 serial dilution of a positive specimen containing perhaps 8-12 dilutions, where the titer midway in the series yields a result of approximately 2.8 on the Verification Line. (This doesn't work as well on the LT/R Line since it is limiting and doesn't dilute in direct relationship to the dilution of antibody concentration). Coding the series, scrambling them, and presenting to trainees, to determine the visual acuity of the trained, is very useful when the series is uncoded, and trainees are asked to look at those specimens they read the Verification Line as negative but which were above 2.8 (or similarly, read results as positive that were below 2.8). Repeating this process several times to align all visual readers to a common visual acuity periodically is a useful and effective tool to prevent visual acuity creep (and gradually increasing sensitivity) over time among laboratory workers. This phenomenon is quite reproducible and this training approach quite effective in quickly training new users to a level of visual acuity equal to their experienced peers.

Technical Conclusions, Page 36. Paragraph 2

October 1, 2019: We agree that lighting conditions can play an important role in the ability to correctly read visual test results, and will incorporate instructions into the next product insert revision that the assay should be visually read in a well-lit area to help ensure accurate reading.

Contact Details (page 41)
The email address for Sedia is incorrect. It should be: customerservice@sediabio.com. (sediabio.com spelled wrong)

Email address updated

October 1, 2019: Sedia acknowledges the changes as addressing its concerns.

Enquiries

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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.