Published in final edited form as:

J Acquir Immune Defic Syndr. 2015 October 1; 70(2): 109–114. doi:10.1097/QAI.0000000000000726.

Increased sensitivity of HIV-1 p24 ELISA using a photochemical signal amplification system

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Abstract

In this study we describe a photochemical signal amplification method (PSAM) for increasing of the sensitivity of enzyme-linked immunosorbent assay (ELISA) for determination of HIV-1 p24 antigen. This method can be used for both commercially available and in-house ELISA tests, and has the advantage of being considerably simpler and less costly than alternative signal amplification methods. The photochemical signal amplification method is based on an autocatalytic photochemical reaction of a horseradish peroxidase (HRP) substrate, orthophenylenediamine (OPD). To compare the performance of PSAM-boosted ELISA with a conventional colorimetric ELISA for determination of HIV-1 p24 antigen we employed a PerkinElmer HIV-1 p24 ELISA kit, using conventional ELISA alongside ELISA + PSAM. In the present study, we show that PSAM technology allows one to increase the analytical sensitivity and dynamic range of a commercial HIV-1 p24 ELISA kit, with and without immune-complex disruption (ICD and Non-ICD ELISA), by a factor of approximately 40-fold. ELISA + PSAM is compatible with commercially available microtiter plate readers, requires only an inexpensive illumination device, and the PSAM amplification step takes no longer than 15 min.

Keywords

HIV-1 p24 antigen; ELISA; sensitivity; immunoassay; PSAM signal amplification; illumination

INTRODUCTION

HIV-1 RNA, anti-HIV antibodies, and HIV-1 capsid protein (p24 antigen) are the main viral markers used to detect HIV infection, and to monitor disease progression. HIV-1 p24 tests are used in combination with anti-HIV antibody testing for early detection of HIV-1 infection [1, 2]. Anti-HIV antibody/p24 antigen combination assays can reduce the seroconversion “window period” to an average of 17 days post-infection and aid in identification of acute infection [2]. Assays for HIV-1 p24 are also useful for diagnosing HIV infection in infants since HIV antibody tests can yield false positives due to maternal

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Parts of the data were presented at Oak Ridge Conference 2013, Baltimore, MD.

Conflicts of Interest and Source of Funding: S.B. and R.S. were supported by the National Institutes of Health, grant No R44AI083169.

HIV antibodies. Although nucleic acid testing (NAT) is the gold standard for pediatric samples, NAT assays require complex instrumentation and are sensitive to contamination, which ultimately limits their use in resource-limited settings.

Most commercial HIV-1 p24 assays employ a standard ELISA format for the capture and detection of p24 antigen. A crucial disadvantage shared by these commercially available ELISAs is that they are capable of detecting only 5–25 pg/mL of HIV-1 p24 antigen in the absence of signal amplification [3]. At this level of sensitivity, p24 antigen cannot be detected consistently in HIV-positive subjects, particularly in those with asymptomatic infections. In fact, only about 50–60% of AIDS patients, 30–40% of AIDS-related complex (ARC) patients, and 10% of asymptomatic patients will have p24 antigenemia that is detectable via standard ELISA [4]. One of the reasons for poor sensitivity of p24 antigen tests in HIV infected persons is that free p24 antigen in serum is complexed with p24 antibody. A conventional ELISA test cannot detect complexed antigens, unless an immune-complex dissociation (ICD) step is used. Thus, free p24 is measured using a Non-ICD ELISA procedure, whereas the detection of bound p24 antigen requires pretreatment with acid or heat to dissociate the complex. When an immune-complex disruption (ICD) procedure is used, the analytical sensitivity of the assay may actually decrease because of the dilution of sample with the ICD reagent. For example, in Perkin Elmer's HIV-1 p24 assay, manufacturer claims that the sensitivity of detection is 3.5 pg/mL without the acid-mediated ICD treatment, and 26 pg/mL with acid-mediated ICD treatment [5]. Thus limited analytical sensitivity of ICD ELISA tests is also an impediment to clinical implementation of p24 antigen tests.

In recent decades several signal amplification systems have been developed in order to increase the analytical sensitivity of ELISA [6–10]. Each of these approaches has its advantages and limitations. In this study, we present a photochemical signal amplification method, PSAM, which is based on an autocatalytic, photochemical reaction of a commonly-used colorimetric substrate for ELISA assays [11]. In brief, ELISA + PSAM consists of two steps. The first step is a conventional ELISA based on the use of one of the most sensitive chromogenic detection systems: the combination of horseradish peroxidase (HRP) enzyme and its substrate, ortho-phenylenediamine (OPD). Within this step, HRP catalyzes oxidation of OPD, yielding a yellow solution containing the product of this reaction, the dye 2, 3-diaminophenazine (DAP) (Fig. 1, reaction (1.1)). In the second step, the PSAM system takes advantage of the fact that DAP is a good photosensitizer, and the small amount of DAP produced during the enzymatic reaction acts as a catalyst for a subsequent autocatalytic photosensitized amplification reaction (Fig. 1, reaction (1.2)). The possible mechanism of the photosensitized reaction (1.2) is discussed in [11, 12].

Here we show that ELISA + PSAM allows one to increase the analytical sensitivity and dynamic range of ELISA-based p24 antigen tests by a factor of 40. Since ELISA + PSAM is compatible with most widely used colorimetric ELISA format and requires only an inexpensive illumination device, PSAM can become a valuable tool in the field of medical diagnostics.

MATERIALS AND METHODS

Alliance® HIV-1 p24 ELISA kit for detection and quantification of the major structural core component of HIV-1 virus, p24 antigen, was purchased from PerkinElmer Life Sciences (Boston, MA). Pathogen-free human serum and OPD substrate tablets were obtained from Sigma (St Louis, MO). Standard 96-well clear microplates were obtained from R&D Systems (Minneapolis, MN). A Labsystems Multiscan® MCC/340 microplate reader was used for optical density measurements.

Perkin Elmer HIV-1 p24 ELISA Procedure

Conventional Non-ICD ELISA was performed in accordance with the manufacturer's instructions [5]. HIV-positive control provided by the manufacturer was diluted in pathogen-free human serum. The conventional ELISA calibration curves for quantification of HIV-1 p24 antigen were both provided by the manufacturer and prepared by us.

Heat-mediated ICD ELISA was performed using the modified version of the manufacturer's Ultrasensitive HIV-1 p24 assay protocol (PerkinElmer), as described in [13] and employing a virus disruption buffer (VDB) (30 mM Tris-HCl (pH 7.2), 450 mM NaCl, 1.5% Triton X-100, 1.5% deoxycholic acid sodium salt, 0.3% sodium dodecyl sulfate, 10 mM EDTA) as described in [14]. Briefly, 50 µl of serum was mixed with 25 µL of VDB and incubated at room temperature for 10 min. These samples were then treated with 225 µL pre-diluted kit complex disruption buffer and heat denatured as described in the manufacturer's instructions. We also excluded the tyramide signal amplification step [15] that requires the use of PerkinElmer's ELAST® p24 ELISA Amplification System.

All core ELISA steps were performed as described in manufacturer's instructions. Analytical sensitivity of the conventional ELISA tests was determined via the least squares fit to the standard curve at an absorbance equal to the cutoff defined by the manufacturer (i.e., mean negative control O.D. + 0.050).

Perkin Elmer HIV-1 p24 ELISA + PSAM Procedure

Minor adjustments to the p24 ELISA reaction conditions were made so as to minimize background in the p24 ELISA + PSAM system. This was necessitated because the PSAM reactions performed in conjunction with the conventional p24 ELISA were rather "noisy"; that is, the conventional p24 ELISA was characterized by a high level of Non-specific binding of reagents, which caused amplification of both signal and background. In the Non-ICD p24 ELISA + PSAM assays, the steps were the same as for the conventional assay procedure described above, except that we used Sigma OPD tablets instead of the tablets provided by Perkin Elmer and decreased incubation time with OPD substrate solution (10 min instead of 30 min in conventional assay). The OPD substrate solution contained OPD and H₂O₂ in concentrations of 6.0×10^{-3} M and 4×10^{-3} M, respectively in 0.05 M phosphate-citrate buffer, pH 5.0 (PCB). After 10 min incubation with the OPD substrate solution, samples were transferred to the clean microtiter plate (R&D Systems, Minneapolis, MN). Thirty µl of the PSAM reagent solution (250 µM of ascorbic acid (AA) in PCB) [11] was added to 100 µl of OPD substrate solution, and samples were irradiated in 2 min

increments for the first 4 min, and in 1 min increments after that for a total of 12 min using an in-house made illumination device configured for even illumination of 96-well microtiter plates (see Text, Supplemental Digital Content 1, that provides the description of the illumination device). The samples were illuminated at an average power density of 0.044 W/cm². Calibration curves for determination of p24 antigen were prepared by double dilution of positive control in negative control (normal human serum).

In the heat-mediated ICD p24 ELISA + PSAM assays, the steps were the same as in the modified version of Ultrasensitive HIV-1 p24 assay protocol (PerkinElmer) described in [13, 15] except that two changes were made: 1) the tyramide signal amplification step was omitted; and, 2) Sigma OPD tablets were used instead of the tablets provided by PerkinElmer. After a 30 min enzymatic step of OPD oxidation, samples were transferred to R&D Systems microtiter plate, and 30 µl of the PSAM reagent solution was added. The samples were irradiated in 2 min increments for the first 4 min and in 1 min increments after that for a total of 9 min as described above.

Differences in ELISA procedures used for conventional PerkinElmer ELISA and Table 1 ELISA+ PSAM assays are gathered in Table 1.

RESULTS

Comparison of the Non-ICD ELISA + PSAM and conventional Non-ICD ELISA

The calibration curves for Non-ICD detection of HIV-1 p24 antigen prepared using Fig. 2 ELISA+ PSAM are shown in Figs. 2A and 2B as compared to conventional Non-ICD ELISA (Fig. 2B, closed squares). All data is plotted on log 2 scales to show the best fitted lines for the signal-amplified ELISA. The regression line for the conventional ELISA was obtained after adding stop solution (signal readout at 492 nm) and has the best fit curve if data is plotted on the linear x scale ($y = 0.0135x + 0.1023$, the best fit curve is not shown). For ELISA+ PSAM, the calibration curve obtained after 12 min of illumination (Fig. 2A, closed circles) represents the curve yielding the best sensitivity of the assay. Indeed, the lower detection limit was calculated as the analyte concentration corresponding to twice the value of the background signal. Using a regression analysis for the p24 antigen ELISA+ PSAM (12 min illumination procedure), we calculated the lower limit of detection as 0.08 pg/mL. The detection limits of the conventional Non-ICD HIV-1 p24 ELISA (Perkin Elmer, Boston, MA) provided by the manufacturer and according to our calculations are 3.5 pg/mL [5] and 3.3 pg/mL, respectively. Thus, the analytical sensitivity of the ELISA+ PSAM is more than 40-fold higher than that for the conventional assay.

It should be noted that HIV-1 p24 ELISA+ PSAM results can be read in both kinetic (0 min and 4 min of illumination) and endpoint mode (12 min of illumination). Curves represented by open and closed triangles (Fig. 2B) correspond to signal-amplified ELISA at 0 and 4 min of illumination and are suitable for determination of high HIV-1 p24 antigen concentrations. Indeed, the first OD reading is done before illumination (Fig. 2B, open triangles). Note that in the ELISA + PSAM procedure, stop solution is not added before the photo-amplification step, since the addition of the stop solution inhibits the photochemical amplification reaction. Also, samples are incubated with OPD for only 10 min prior to amplification and

measurement (Table 1). The shortened period of incubation with OPD causes the slope of the ‘before illumination’ calibration curve (i.e. the PSAM assay at 0 min illumination) to be lower than that for the corresponding curve prepared according to manufacturer’s instructions (Fig. 2B, closed squares). While this causes the PSAM assay to be less sensitive prior to amplification, the net effect is to extend the dynamic range of the assay. In this configuration, the estimated dynamic range of the ELISA + PSAM assay at 0 min illumination is between 80–2,560 pg/mL (upper detection limit not shown). Here we did not attempt to get the best fitting curve for data points acquired before illumination. Data points obtained during the second OD reading at 4 min of illumination (Fig. 2B, closed triangles) were used to construct a calibration curve for measuring p24 antigen concentrations in the range between 5.0–500 pg/mL ($y = 0.4111 \log(x) + 0.0433$, where x is the antigen concentration and y is OD). The final calibration curve (endpoint data) obtained after 12 min of illumination (Fig. 2A, closed circles) is used for calculation of p24 antigen concentration in the range between 0.08–5.00 pg/mL ($y = 0.5663 \log(x) + 0.8685$). If all three calibration curves (lines obtained at 0, 4, and 12 min of illumination) are combined, the estimated total dynamic range of the assay boosted with PSAM is more than four logs, between 0.08–2,506 pg/mL. Data acquired during kinetic and endpoint readings can easily be pooled using QuantiKin software [17]. Thus, the dynamic range of this configuration of the Non-ICD ELISA+ PSAM is two orders of magnitude larger than that of the conventional Perkin Elmer Non-ICD ELISA (3.5–100 pg/mL) [5]. It should be emphasized, however, that the dynamic range of the conventional ELISA could be increased in two ways: 1) samples with high p24 concentrations can be diluted in such a way that analyte concentrations fall within the dynamic range of the conventional Perkin Elmer Non-ICD ELISA; and 2) using a kinetic mode in which the signal is measured at different times of the substrate solution incubation. Thus, the same upper limit of detection could be achieved by using both the conventional and the ELISA + PSAM assay. Since the lower limit of detection of the ELISA+ PSAM is increased approximately 40-fold as compared to that for the conventional assay, the ELISA+ PSAM’s dynamic range is increased at least 40-fold.

Comparison of the heat-mediated ICD ELISA without signal amplification, ICD ELISA + PSAM and conventional Ultrasensitive p24 ELISA

Fig. 3 shows calibration curves for heat-mediated ICD ELISA+ PSAM (Figs. 3A and 3B) Fig. 3 as compared to the conventional heat-mediated ICD ELISA (Fig. 3B, closed squares). The regression line for the conventional ICD ELISA has the best fit curve if data is plotted on the linear x scale ($y = 0.0064x + 0.0817$, the best fit curve is not shown). Heat-mediated ICD ELISA + PSAM assay was most sensitive after 9 min of illumination (Fig. 3A, closed circles) with a lower limit of detection of approximately 0.1 pg/ml estimated as twice the value of the background signal as described above. In contrast, the lower detection limit for ICD ELISA without signal amplification is equal to 4.0 pg/mL. Thus, the ELISA+ PSAM has 40-fold higher analytical sensitivity as compared with the conventional ELISA. It is worth noting that the documented lower detection limit of the PerkinElmer Ultrasensitive HIV-1 p24 ELISA, which uses a rather complex tyramide amplification system, is from 0.5 to 1 pg/mL [15, 18–25]. Therefore, the analytical sensitivity of the ELISA + PSAM is 5–10-fold higher than that of the conventional Ultrasensitive assay, which uses the labor intensive and expensive ELAST amplification system.

Heat-mediated HIV-1 p24 ELISA + PSAM results can be read in both kinetic and endpoint mode the same way as described for Non-ICD ELISA. The first OD reading is done before illumination (Fig. 3B, open triangles). The estimated dynamic range of this variant of the ELISA+ PSAM assay at 0 min illumination is between 128 and 8,196 pg/mL (upper detection limit not shown) whereas the linear range of conventional heat-mediated ICD ELISA is between 4.0–256 pg/mL. Data points obtained during the second OD reading at 4 min of illumination (Fig. 3B, closed triangles) were used to construct a calibration curve for measuring p24 antigen concentrations in the range between 8.0–512 pg/mL ($y = 0.4102 \log(x) - 0.093$, where x is the antigen concentration and y is OD). The final calibration curve obtained after 9 min of illumination (Fig. 3A, closed circles) is used for calculation of p24 antigen concentration in the range between 0.1–8.0 pg/mL ($y = 0.4215 \log(x) + 0.5425$). All three calibration curves (lines obtained at 0, 4, and 9 min of illumination) can be combined to get an estimated total dynamic range of the assay between 0.1–8,196 pg/mL, which is more than four logs in magnitude.

It is worth noting that the conventional heat-mediated ICD ELISA has a 2 log dynamic range. Thus, ELISA+ PSAM has almost 2 orders of magnitude larger dynamic range than the conventional assay. The Ultrasensitive ICD ELISA has a 4 log dynamic range, if sample optical density is measured in both kinetic and endpoint mode. This range is comparable to the estimated dynamic range of the ICD ELISA+ PSAM.

DISCUSSION

Our results obtained using the p24 ELISA system confirm that ELISA + PSAM could dramatically increase the detection sensitivity and extend the dynamic range of a commercially-available assay. Given the clinical challenges involved in detection and treatment of AIDS, PSAM technology holds the potential to significantly improve the diagnostic utility of p24 immunoassays. Indeed, our experiments show that the analytical sensitivity of the p24 antigen assays and, consequently, their dynamic range, can potentially be increased approximately 40-fold as compared to the conventional tests. PSAM technology increases analytical sensitivity of conventional ELISA and requires only one additional short step of illumination (9–12 min) of the microtiter plate using an inexpensive illumination device. Amplification occurs as a result of the photochemical amplification of 2,3-diaminophenazine (DAP) produced within enzymatic reaction of the regular colorimetric ELISA [11].

It is worth noting that in all instances the error (coefficient of variation, CV) of the measurements using the PSAM system did not exceed 15%, which is acceptable for immunoassays (Figs. 2–3).

It should also be emphasized that the aforementioned calibration curves and calculations of the analytical sensitivity for ELISA and ELISA + PSAM assays are based on the use of “ideal” negative (normal) sera. In order to determine a clinical sensitivity (real cut-off) of the assay, at least several dozen clinical negative samples must be run. An increase in cut-off value of the assay in real clinical samples as compared to its analytical sensitivity is a common phenomenon. For example, the cut-offs for p24 positivity and for p24 negativity for

the Ultrasensitive PE HIV-1 p24 assay using VDB and Tanzanian plasma samples were 3.5 pg/ml and 2.3 pg/mL, respectively [16]. These cut-offs are 2.5–7-fold higher than those for the analytical sensitivity (0.5 – 1 pg/ml) of the same assay. With respect to ELISA + PSAM, pre-clinical studies including examination of hundreds clinical negative and positive samples are underway, and the results on the ELISA + PSAM clinical sensitivity and specificity will be presented in the near future elsewhere [26]. We should mention here, however, that our preliminary data obtained for 25 negative sera shows that real cut-offs for Non-ICD and Heat-mediated ELISA + PSAM are 0.15 pg/ml and 0.3 pg/ml, respectively [26], and these cut-off values are 2–3 fold higher than the corresponding analytical sensitivities. We should also note that the real cut-off for Non-ICD ELISA + PSAM is equal to its analytical sensitivity when the method is used for determination of recombinant p24 antigen in cell culture supernatants.

Several signal amplification strategies have been applied to p24 ELISA assays, in an effort to address the need for more sensitive detection of p24. One approach is to boost the signal intensity by using amplification systems. For example, the aforementioned Tyramide Signal Amplification (TSA) is the method that has been applied to the p24 antigen test in an attempt to increase its analytical sensitivity [9]. It should be noted, however, that the TSA is labor intensive, and includes a time-consuming TSA step (see Table 1), which introduces a high coefficient of variation. Moreover, the cost of TSA is much higher than that for PSAM: TSA and PSAM reagents add approximately \$15 and \$1 per sample, respectively, whereas the projected PSAM illumination device's cost, \$2,000, will approach zero in a clinical setting performing tens of thousands tests.

Another approach, immuno-PCR (IPCR) amplification method, uses an oligonucleotide as a surrogate marker and polymerase chain reaction (PCR) for its amplification. This method has been applied to the p24 antigen assay and, at present, is claimed to be the most sensitive p24 antigen test with the detection limit of 10–100 HIV-1 p24 molecules per reaction (in ag/mL concentration range) and 4-log dynamic range [28]. This test has a potential to detect HIV-1 infection earlier than nucleic acid tests [28]. However, this assay is very intricate, extremely sensitive to contamination, has reproducibility issues and requires complex equipment. Moreover, it is not commercially available, perhaps due to the aforementioned shortcomings.

Several new p24 tests that use different platforms have the potential to offer enhancement in the detection limit over the traditional colorimetric ELISA and have been described in the literature. Gold nanoparticle-based bio-barcode amplification (BCA) assay claims sensitivity of 0.1 pg/mL [29–31]. A novel HIV-1 p24 antigen single-molecule immunosorbent assay (SMISA) has the detection limit of 0.1 pg/mL of p24 antigen and a linear dynamic range of the assay is over three orders of magnitude between 0.1 and 100 pg/mL [32]. An Ultrasensitive Microsphere immunoassay with tyramide signal amplification (TSA) and the heat-mediated immune complex disruption procedure using commercially available anti-p24 has a sensitivity of 1 pg/ml [33]. And, an immunofluorescent cytometric bead assay is another flow cytometry-based assay that offers a wide dynamic measurement range and allows for the detection of p24 concentrations over 4 orders of magnitude from less than 0.4 pg/mL to up to 20,000 pg/mL [27]. These techniques uniformly require complex optical or

flow cytometric equipment that complicates their use in the resource-limited settings. It should also be emphasized that all aforementioned techniques are complex, involve numerous incubation and wash steps, and are labor-intensive. Consequently, none have yet been implemented in commercially available formats.

While a number of methods that allow for ultrasensitive detection of protein analytes have been developed, these methods are generally characterized by complex protocols, expensive reagents, or a combination of both. The advantages of the ELISA + PSAM assay over other signal amplification methods include ultrasensitive detection capabilities and extended dynamic range of the assay without the use of the complex and expensive equipment and without significant changes in assay procedure. Unlike other ultrasensitive techniques, the PSAM is simple and straightforward, requiring minimal effort to achieve markedly improved results from commercially available ELISA reagent systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to acknowledge the financial support of National Institutes of Health (Grant No R44AI083169). In addition, we would like to thank Dr. Addison D. Ault for assistance in the preparation of this manuscript.

References

- Speers D, Phillips P, Dyer J. Combination assay detecting both human immunodeficiency virus (HIV) p24 antigen and anti-HIV antibodies opens a second diagnostic window. *J Clin Microbiol.* 2005; 43:5397–5399. [PubMed: 16208030]
- Fiebig EW, Wright DJ, Rawal BD, Garrett PE, Schumacher RT, Peddada L, et al. Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS.* 2003; 17:1871–1879. [PubMed: 12960819]
- Miedouge M, Greze M, Bailly A, Izopet J. Analytical sensitivity of four HIV combined antigen/antibody assays using the p24 WHO standard. *J Clin Virol.* 2011; 50:57–60. [PubMed: 20926337]
- Constantine, NT.; Saville, R.; Dax, E. *Retroviral Testing and Quality Assurance: Essentials for Laboratory Diagnosis.* Malloy Printers; 2005.
- HIV-1 P24 ELISA [package insert]. PerkinElmer Life and Analytical Sciences B.V; Groningen, The Netherlands: 2009. OPD0024-06/09 - REV.B
- Finan JE, Zhao RY. From molecular diagnostics to personalized testing. *Pharmacogenomics.* 2007; 8:85–99. [PubMed: 17187512]
- Funke SA, Birkmann E, Willbold D. Detection of Amyloid-beta aggregates in body fluids: a suitable method for early diagnosis of Alzheimer's disease? *Curr Alzheimer Res.* 2009; 6:285–289. [PubMed: 19519310]
- Schweitzer B, Kingsmore SF. Measuring proteins on microarrays. *Curr Opin Biotechnol.* 2002; 13:14–19. [PubMed: 11849952]
- Bobrow MN, Harris TD, Shaughnessy KJ, Litt GJ. Catalyzed reporter deposition, a novel method of signal amplification. Application to immunoassays. *J Immunol Methods.* 1989; 125:279–285. [PubMed: 2558138]
- Vera-Cabrera L, Handzel V, Laszlo A. Development of an enzyme-linked immunosorbent assay (ELISA) combined with a streptavidin-biotin and enzyme amplification method to detect anti-2,3-di-o-acetylrehalose (DAT) antibodies in patients with tuberculosis. *J Immunol Methods.* 1994; 177:69–77. [PubMed: 7822839]

11. Bystryak, S.; Santockyte, R. Methods for Improving Analyte Detection Using Photochemical Reactions. US Patents No. 8,916,341 and 8,951,722.
12. Mekler VM, Bystryak SM. *Khim Phys.* 1991; 10:1469–1474.
13. Jennings C, Fiscus SA, Crowe SM, Danilovic AD, Morack RJ, Scianna S, et al. Comparison of two human immunodeficiency virus (HIV) RNA surrogate assays to the standard HIV RNA assay. *J Clin Microbiol.* 2005; 43:5950–5956. [PubMed: 16333081]
14. Schupbach J, Boni J, Bisset LR, Tomasik Z, Fischer M, Gunthard HF, et al. HIV-1 p24 antigen is a significant inverse correlate of CD4 T-cell change in patients with suppressed viremia under long-term antiretroviral therapy. *J Acquir Immune Defic Syndr.* 2003; 33:292–299. [PubMed: 12843739]
15. P24 Ultrasensitive Assay Protocol [package insert]. PerkinElmer Life, Inc; Boston, MA: PC5032–0603
16. Knuchel MC, Jullu B, Shah C, Tomasik Z, Stoeckle MP, Speck RF, et al. Adaptation of the ultrasensitive HIV-1 p24 antigen assay to dried blood spot testing. *J Acquir Immune Defic Syndr.* 2007; 44:247–253. [PubMed: 17146373]
17. Giacomini M, McDermott JL, Giri AA, Martini I, Lillo FB, Varnier OE. A novel and innovative quantitative kinetic software for virological colorimetric assays. *J Virol Methods.* 1998; 73:201–209. [PubMed: 9766891]
18. Schupbach J, Boni J, Tomasik Z, Jendis J, Seger R, Kind C. Sensitive detection and early prognostic significance of p24 antigen in heat-denatured plasma of human immunodeficiency virus type 1-infected infants. Swiss Neonatal HIV Study Group. *J Infect Dis.* 1994; 170:318–324. [PubMed: 8035017]
19. Schupbach J, Flepp M, Pontelli D, Tomasik Z, Luthy R, Boni J. Heat-mediated immune complex dissociation and enzyme-linked immunosorbent assay signal amplification render p24 antigen detection in plasma as sensitive as HIV-1 RNA detection by polymerase chain reaction. *AIDS.* 1996; 10:1085–1090. [PubMed: 8874624]
20. Boni J, Opravil M, Tomasik Z, Rothen M, Bisset L, Grob PJ, et al. Simple monitoring of antiretroviral therapy with a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma. *AIDS.* 1997; 11:F47–52. [PubMed: 9143600]
21. Nadal D, Boni J, Kind C, Varnier OE, Steiner F, Tomasik Z, et al. Prospective evaluation of amplification-boosted ELISA for heat-denatured p24 antigen for diagnosis and monitoring of pediatric human immunodeficiency virus type 1 infection. *J Infect Dis.* 1999; 180:1089–1095. [PubMed: 10479135]
22. Pascual A, Cachafeiro A, Funk ML, Fiscus SA. Comparison of an assay using signal amplification of the heat-dissociated p24 antigen with the Roche Monitor human immunodeficiency virus RNA assay. *J Clin Microbiol.* 2002; 40:2472–2475. [PubMed: 12089264]
23. Sutthent R, Gaudart N, Chokpaibulkit K, Tanliang N, Kanoksinsombath C, Chaisilwatana P. p24 Antigen detection assay modified with a booster step for diagnosis and monitoring of human immunodeficiency virus type 1 infection. *J Clin Microbiol.* 2003; 41:1016–1022. [PubMed: 12624024]
24. Schupbach J, Tomasik Z, Knuchel M, Opravil M, Gunthard HF, Nadal D, et al. Optimized virus disruption improves detection of HIV-1 p24 in particles and uncovers a p24 reactivity in patients with undetectable HIV-1 RNA under long-term HAART. *J Med Virol.* 2006; 78:1003–1010. [PubMed: 16789014]
25. Schupbach J. Viral RNA and p24 antigen as markers of HIV disease and antiretroviral treatment success. *Int Arch Allergy Immunol.* 2003; 132:196–209. [PubMed: 14646380]
26. Bystryak S, Acharya C. Detection of HIV-1 p24 antigen in patients with varying degrees of viremia using an improved and highly sensitive ELISA with a photochemical signal amplification system. Manuscript in preparation.
27. Biancotto A, Brichacek B, Chen SS, Fitzgerald W, Lisco A, Vanpouille C, et al. A highly sensitive and dynamic immunofluorescent cytometric bead assay for the detection of HIV-1 p24. *J Virol Methods.* 2009; 157:98–101. [PubMed: 19100289]

28. Barletta J, Bartolome A, Constantine NT. Immunomagnetic quantitative immuno-PCR for detection of less than one HIV-1 virion. *J Virol Methods*. 2009; 157:122–132. [PubMed: 19138706]
29. Kim EY, Stanton J, Korber BT, Krebs K, Bogdan D, Kunstman K, et al. Detection of HIV-1 p24 Gag in plasma by a nanoparticle-based bio-barcode-amplification method. *Nanomedicine (Lond)*. 2008; 3:293–303. [PubMed: 18510425]
30. Tang S, Hewlett I. Nanoparticle-based immunoassays for sensitive and early detection of HIV-1 capsid (p24) antigen. *J Infect Dis*. 2010; 201(Suppl 1):S59–64. [PubMed: 20225948]
31. Tang S, Zhao J, Storhoff JJ, Norris PJ, Little RF, Yarchoan R, et al. Nanoparticle-Based biobarcode amplification assay (BCA) for sensitive and early detection of human immunodeficiency type 1 capsid (p24) antigen. *J Acquir Immune Defic Syndr*. 2007; 46:231–237. [PubMed: 17693896]
32. Li J, Xie W, Fang N, Yeung ES. Single-molecule immunosorbent assay as a tool for human immunodeficiency virus-1 antigen detection. *Anal Bioanal Chem*. 2009; 394:489–497. [PubMed: 19267241]
33. Ondo P, Vereecken C, Asahchop EL, Litzroth A, Diallo A, Fransen K, et al. Proof of principle: an HIV p24 microsphere immunoassay with potential application to HIV clinical diagnosis. *Cytometry B Clin Cytom*. 2009; 76:231–236. [PubMed: 19012321]

Fig. 1.

A scheme of oxidation of OPD catalyzed by HRP (1.1) and autosensitized by DAP (1.2).

Fig. 2.

Calibration curves for determination of HIV-1 p24 antigen using Non-ICD ELISA + PSAM at different times of illumination (A and B) as compared to conventional Non-ICD ELISA (B, closed squares). The calibration curve obtained after 12 min of illumination (A, closed circles) provides the highest sensitivity of the assay, with a lower limit of detection of 0.08pg/ml. Curves represented by open and closed triangles (B) correspond to signal-amplified ELISA at 0 and 4 min of illumination and can be used to calculate high HIV-1 p24 antigen concentrations.

Fig. 3.

Calibration curves for determination of HIV-1 p24 antigen using heat-mediated ICD ELISA + PSAM at different times of illumination (A and B) as compared to conventional heat-mediated ICD ELISA (B, closed squares). The calibration curve obtained after 9 min of illumination (A, closed circles) provides the highest sensitivity, with a lower limit of detection of approximately 0.1 pg/ml). Curves represented by open and closed triangles (B) correspond to signal-amplified ELISA at 0 and 4 min of illumination and can be used to calculate high HIV-1 p24 antigen concentrations.

Table 1

Differences in ELISA conditions between conventional and ELISA + PSAM assays.

	Non-ICD ELISA	Non-ICD ELISA + PSAM	Heat-mediated ICD ELISA ^a	Heat-mediated ICD ELISA ^a + PSAM	PE Ultrasensitive ELISA ^b
Strep-HRP incubation	30 min	30 min	30 min	15 min	15 min
ELAST Steps:	N/A	N/A	N/A	✓	15 min
Biotin-Tyramide					10 washes
Washing					15 min
FP105 Strep-HRP					10 washes
Washing					
OPD substrate	PerkinElmer	Sigma	PerkinElmer	Sigma	PerkinElmer
OPD substrate dark incubation	30 min	10 min	30 min	30 min	30 min
PSAM signal amplification	N/A	12 min	N/A	9 min	N/A

^aUltrasensitive ELISA procedure without ELAST signal amplification. The virus disruption buffer was used to optimize the assay performance, see *Materials and Methods*.

^bLiterature data [13–15]

N/A - Not Applicable.