Rapid, Fully Automated Digital Immunoassay for p24 Protein with the Sensitivity of Nucleic Acid Amplification for Detecting Acute HIV Infection

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BACKGROUND: Nucleic acid testing (NAT) has become the standard for high sensitivity in detecting low levels of virus. However, adoption of NAT can be cost prohibitive in low-resource settings where access to extreme sensitivity could be clinically advantageous for early detection of infection. We report development and preliminary validation of a simple, low-cost, fully automated digital p24 antigen immunoassay with the sensitivity of quantitative NAT viral load (NAT-VL) methods for detection of acute HIV infection.

METHODS: We developed an investigational 69-min immunoassay for p24 capsid protein for use on a novel digital analyzer on the basis of single-molecule-array technology. We evaluated the assay for sensitivity by dilution of standardized preparations of p24, cultured HIV, and preseroconversion samples. We characterized analytical performance and concordance with 2 NAT-VL methods and 2 contemporary p24 Ag/Ab combination immunoassays with dilutions of viral isolates and samples from the earliest stages of HIV infection.

RESULTS: Analytical sensitivity was 0.0025 ng/L p24, equivalent to 60 HIV RNA copies/mL. The limit of quantification was 0.0076 ng/L, and imprecision across 10 runs was <10% for samples as low as 0.09 ng/L. Clinical specificity was 95.1%. Sensitivity concordance vs NAT-VL on dilutions of preseroconversion samples and Group M viral isolates was 100%.

CONCLUSIONS: The digital immunoassay exhibited >4000-fold greater sensitivity than contemporary immunoassays for p24 and sensitivity equivalent to that of NAT methods for early detection of HIV. The data indicate that NAT-level sensitivity for acute HIV infection is possible with a simple, low-cost digital immunoassay.

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Nucleic acid amplification techniques have become established as indispensable tools in medical research and diagnostics, including the detection and diagnosis of infectious disease. With implementation for blood screening in the US in 1999 (1), nucleic acid testing (NAT) for detection of viral pathogens has helped safeguard blood for transfusion by providing the most sensitive, economically feasible detection of infected blood donations possible. Qualitative NAT methods for screening pooled blood donor units for HIV are capable of analytical sensitivities of approximately 60 HIV RNA copies/mL (30 viruses/mL) with individual donors (2). NAT has significantly shortened the window between initial infection and detection by amplifying viral nucleic acid rather than detecting the presence of antibody after seroconversion (3). Although NAT has become the mainstay for high-sensitivity virus detection for blood donor screening, the technology is relatively complex and expensive (4), making it difficult to implement in settings where access to high sensitivity could be clinically advantageous. These scenarios include blood donor screening in low-resource settings and clinical screening in high-incidence areas, where a substantial number of cases of acute infection can be missed by less sensitive immunoassay tests. Rapid detection and reporting of acute HIV infection represents a key opportunity to control the spread of the disease, because viral transmission is 10 times more likely during the acute phase than the chronic phase (5).

Immunoassays are simpler and lower in cost than NAT and have the potential to be more easily deployed in diverse settings. Immunoassays to HIV antibodies are straightforward, but they require an immune response and are unable to detect acute infection when antibodies are not yet present. Third-generation antibody assays (6) detect the earliest stage of the immune response (immunoglobulin M), reducing the serological window to approximately 3 weeks (7). Viral proteins are present at the

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Nonstandard abbreviations: NAT, nucleic acid testing; S/(2)G, streptavidin-S/(2)galactosidase; RGP, β-D-galactopyranosidase; 4PL, 4-parameter logistic; GS, goat serum; LoD, limit of detection; VL, viral load; CLSI, Clinical and Laboratory Standards Institute; LoQ, limit of quantification; AEB, average enzymes per bead.
start of infection, and each HIV particle is composed of approximately 2000 copies of p24 capsid protein and 2 viral RNA copies (8). A titer of 30 viruses per mL equates to 60 000 p24 molecules, or a concentration of 3 femtograms per microliter, which is well below the detection limit of 11 000–70 000 fg/mL with contemporary p24 and fourth-generation HIV combination immunoassays (9, 10). Although contemporary p24 immunoassays can reduce the detection window by another 4 or 5 days relative to antibody tests, they remain thousands-fold less sensitive for virus than RNA detection, which reduces the window an additional week or more relative to antigen detection (7). Thus, contemporary immunoassays are blind to most of the acute-phase window that is detectable by NAT. A simple immunoassay capable of the sensitivity of NAT for HIV could represent a step forward in broader, more cost-effective detection of acute HIV infection, which would help further control the spread of the disease.

Previously, we reported a prototype digital immunoassay for p24 that used an early version of single-molecule-array (Simoa) technology (11, 12). The assay was a bead-based ELISA requiring 7–8 h to complete a 96-well plate. Here we describe a more advanced investigational version of the assay enabled by the automation of Simoa technology (13). The object of our studies was to demonstrate the potential for a rapid and simple immunoassay alternative to NAT for detection of acute HIV infection.

Materials and Methods

APPARATUS

The fully automated Simoa HD-1 Analyzer uses Simoa technology for isolation and counting of single molecules. The instrument pipettes sample directly from sample tubes and processes immunoassays and data reduction with a throughput of 66 tests/h. Details of the instrument are given elsewhere (13).

ASSAY PRINCIPLE AND PROTOCOL

The high sensitivity enabled by Simoa technology has been discussed (12). In brief, Simoa is a digitized bead-based ELISA whereby diffusion of fluorescent reporter molecules at the signal step is constrained to 40-femtoliter wells in a microarray. By restricting diffusion to such a small volume, fluorophores generated by a single enzyme label can be detected in the array in 30 s. This extreme sensitivity permits use of low quantities of labeling reagent, which lowers nonspecific interactions and dramatically increases the signal-to-background ratio relative to conventional ELISAs.

The Simoa p24 assay is a fully automated 2-step sandwich immunoassay for p24 measurement in preseroconverted (acutely infected) serum or plasma with the Simoa HD-1 Analyzer. The assay does not perform dissociation of immune complexes that form after seroconversion, so it is unsuitable for viral load monitoring without dissociation pretreatment. In the first step, 144 μL sample is drawn from the sample tube by the instrument pipette, and the sample, anti-p24–coated paramagnetic capture microbeads (2.7 μm), and biotinylated detector antibodies are combined in a reaction cuvette and incubated for 35 min. p24 molecules present in the sample are captured by the beads and labeled with detector. Capture beads are collected with a magnet and washed. A conjugate of streptavidin-β-galactosidase (SβG) is then mixed with the capture beads for the labeling step. After a second wash, the beads are resuspended in a resorufin β-o-galactopyranoside (RGP) substrate solution. Digital processing occurs when beads are transferred to the Simoa array disc (14). Individual beads are then sealed in microwells in the array. If p24 has been captured and labeled, the βG hydrolyzes the RGP substrate into a fluorescent product that provides the signal for measurement. At low p24 concentrations, the percentage of bead-containing wells in the array with a positive signal is proportional to the amount of p24 present in the sample. At higher p24 concentrations, the total fluorescence signal is proportional to the amount of p24 present in the sample (15). The concentration of p24 in unknown samples is interpolated from a standard curve obtained by 4-parameter logistic (4PL) regression fitting. Total time to first result is 69 min.

REAGENTS

We developed 4 reagents: paramagnetic p24 capture beads, biotinylated detector, SβG conjugate, and assay diluent. The capture beads comprised a monoclonal antip24 antibody (Capricorn HIV-018-48304) covalently attached by standard coupling chemistry to 2.7-μm carboxy paramagnetic microbeads (Agilent Technologies). The antibody-coated beads were diluted to a concentration of 3 × 10⁶ beads/mL in Tris with a surfactant and BSA. Biotinylated detector reagent comprised a monoclonal anti-p24 antibody (Zeptometrix 801136) that was biotinylated with standard methods and diluted to a concentration of 0.2 μg/mL in a PBS diluent containing surfactant and BSA. We prepared SβG by covalent conjugation of purified streptavidin (Thermo Scientific) and βG (Sigma) with standard coupling chemistry and dilution to 300 pmol/L in phosphate buffer with surfactant and BSA. Assay diluent was formulated in Tris with heterophilic blockers, goat serum (GS), and surfactants.

CALIBRATION

We calibrated the assay with purified p24 antigen (Advanced Biotechnologies) diluted in pooled human serum (SeraCare HS210). The antigen is soluble native p24 gag
protein of HIV type 1 (IIIB strain) isolated from culture medium of HIV-1–infected HuT 78 cells. Calibrator levels were 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, and 30.0 ng/L.

CULTURED VIRUS
Cultured HIV-1 Group M, Subtype B virus for limit of detection (LoD) estimates was purchased from SeraCare (PN242B, lot 2701LN-10, 2.3 \times 10^6 HIV-1 RNA copies/mL by Roche Amplicor® version 1.5) and diluted in delipidated, defibrinated human serum (Seracare HS-210). Limits of detection (LoDs) (RNA copies/mL by Amplicor) were interpolated from 4PL fits of the titration data at 2.5 SDs above background.

PRESEROCONVERSION SAMPLES
Serum samples from HIV-infected individuals were purchased from SeraCare. Four samples from 3 infected individuals (PRB973-03, PRB974-02, PRB974-03, RB975-05) reactive for HIV by NAT but nonreactive for HIV antibody were diluted to 300, 150, and 100 RNA copies/mL in negative serum. RNA value assignments reported by SeraCare for the samples differed by >2-fold between 3 NAT viral load (NAT-VL) methods (Siemens Versant HIV-1 RNA 3.0, Abbott RT HIV-1 RNA m2000, and Roche COBAS Ampiprep TaqMan HIV-1 version 2.0). The NAT method giving the middle RNA values among the 3 methods (Abbott m2000) was chosen for RNA assignments. LoDs (RNA copies/mL by m2000) were interpolated from 4PL fits of the titration data at 2.5 SDs above background.

NAT YIELD SAMPLES
Comparisons between the Simoa p24 assay, 2 quantitative NAT-VL methods, and 2 contemporary p24 immunoassays were performed in 2 studies with NAT yield samples (blood donor samples that are RNA positive/antibody negative). For the first study, 26 surplus blood donor plasma samples were obtained from Blood System Research Institute. Measured viral loads ranged from <40 to 10 000 000 RNA copies/mL by Abbott m2000 (product version with LoD of 40 RNA copies/mL). The samples were also tested for reactivity with 2 fourth-generation HIV combination immunoassays: GS HIV Combo Ag/Ab EIA (Bio-Rad) (LoD for p24 15 pg/mL) and Architect HIV Ag/Ab Combo (Abbott) (LoD for p24 50 pg/mL). For the second study, 22 surplus blood donor samples were obtained from Abbott Diagnostics. Measured viral loads ranged from 339 to 660 000 RNA copies/mL by Abbott m2000 (product version with LoD of 100 RNA copies/mL). Samples selected for this set were all nonreactive by HIV Ag/Ab combination assay. The 2 highest NAT yield samples in this set were also diluted 100-fold and 1000-fold with negative serum. To amplify the sensitivity challenge, 10-fold dilutions of 13 virus culture isolates obtained from Abbott were tested with the Abbott m2000 and Simoa p24 assay. The isolates were composed of 12 HIV-1 Group M of mixed subtypes and 1 Group O. RNA yields for the isolates ranged from 158 489 to 78 copies/mL (below the LoD of the m2000), as calculated on the basis of dilution factors. All samples were stored at −80 °C in single-use aliquots before testing to avoid potential freeze–thaw effects.

Results
DOSE RESPONSE AND LINEARITY
Fig. 1 shows a representative dose response across a 4.1-log range. The low background typical for Simoa immunoassays is highlighted in Fig. 1A. Linearity, conducted with guidance from the Clinical and Laboratory Standards Institute (CLSI) protocol EP6-A (16), was evaluated with admixtures of serum supplemented with p24 and negative serum (Fig. 1B). Deviation from linearity was <10%.

SENSITIVITY
We evaluated sensitivity by dilution of p24-spiked serum, cultured virus, and seroconversion samples from HIV-infected individuals. The limit of quantification (LoQ) was estimated from CVs of dose values obtained from assaying diluted p24-spiked serum in replicates of 3 across multiple days and instruments (11 runs, 58 determinations). An LoQ of 0.0076 ng/L was estimated at a 20% dose CV (Fig. 2A). Analytical LoD was estimated as 2.5 SDs above antigen-free serum background. The LoD was determined for each of 10 calibration runs from triplicate measurements of the zero calibrator and the lowest calibrator (0.01 ng/L). The mean LoD was 0.0025 ng/L (SD 0.0017 ng/L), corresponding to 63 RNA copies/mL (Fig. 2B). Representative detectability of p24 from cultured and native HIV is depicted in Fig. 3. LoDs on the basis of NAT value assignment ranged from 23 to 45 RNA copies/mL, corresponding to 0.0009–0.0017 ng/L p24. Differences between LoDs estimated in RNA copies/mL may be a result of differences in RNA quantification between the different NAT-VL methods used for RNA value assignment.

PRECISION
We assessed repeatability with guidance from CLSI Protocol EP5-A (17). Two QC controls (same composition as calibrators) and 2 serum samples (serum supplemented with p24 antigen) were assayed in replicates of 3 twice a day for 5 days with a single stored calibration curve and a single lot of reagents. The data and nested ANOVA (Minitab) are depicted in Fig. 4. CVs were <10% for all samples. The lowest sample tested was >100-fold below the LoD of contemporary p24 immunoassays.
SPECIFICITY

We conducted a preliminary specificity study with 55 serum and 99 EDTA plasma samples from HIV-free donors (Bioreclamation IVT). The distribution of p24 results is depicted in Fig. 5. The median signal was 0.0041 (SD 0.0013) units of average enzymes per bead (AEB). With a preliminary cutoff of 3 SD above mean negative calibrator background across 13 calibration runs, 137 of
144 samples gave signals below the cutoff, for a specificity of 95.1%. Samples above the cutoffs were not retested to confirm reactivity. There was no detectable bias between serum and EDTA plasma (Fig. 5 inset).

**EARLY DETECTION OF HIV**

A comparison with NAT-VL and conventional immunoassay methods (qualitative fourth-generation HIV combination) for concordance in early detection of HIV is depicted in Fig. 6. In these studies, the sensitivities of the methods were challenged with low VL NAT yield samples and dilutions of samples and viral isolates. In the first study of 26 NAT yield samples (Fig. 6A), 24 of 26 samples were reactive with the NAT-VL method (LoD 40 RNA copies/mL), with the lowest measured sample at 126 RNA copies/mL. All 24 samples were also reactive in the Simoa p24 assay, whereas 2 of the samples were below the LoD of both methods. Eight of 26 samples were reactive in the GS HIV Combo Ag/Ab kit (Bio-Rad), and 9 of 26 samples were reactive in the Architect HIV Ag/Ab Combo kit (Abbott). Compared with the NAT-VL method, the sensitivity concordance of the Simoa test was 100%, whereas the contemporary immunoassays exhibited sensitivity concordances of 33%–38%. In the second study (Fig. 6B), 22 of 22 NAT yield samples were reactive with NAT-VL (Abbott m2000, LoD 100 RNA copies/mL), with the lowest measured sample at 126 RNA copies/mL. All 22 samples were also reactive in the Simoa assay, with the lowest measured sample 0.0088 ng/L p24. Fourteen negative controls were included in the sample set, and 13 of 14 were <LoD of the p24 method (all were negative by NAT-VL). One of the negative controls read an apparent 0.0037 ng/L p24, very close to the LoD of 0.0025 ng/L. The sample was not retested to confirm reactivity. Two of the NAT yield samples were diluted 1:100 and 1:1000. All 4 dilutions were positive in both the NAT-VL and p24 assays. All NAT yield samples and dilutions (n = 26) were nonreactive in the HIV Ag/Ab Combo kit. The set of 13 viral isolates ranged from 18 197 to 158 489 RNA copies/mL before 10-fold dilutions (to 1:1000). p24 was detected in 37 of 37 Group M samples (12 isolates and dilutions, n = 37 samples), whereas RNA was detectable by the NAT-VL method in 35 of 37 samples. Two of the Group M isolate dilutions (calculated 78 and 81 copies/mL) were below the LoD of the NAT-VL method, but both of these samples were reactive in the Simoa method (0.0031 and 0.0079 ng/L p24, respectively). One of the 13 viral isolates (#13) was Group O, which caused difficulty for the Simoa method. One of 3 dilutions of #13 was detectable in the Simoa method but significantly below the Group M method comparison trend (Fig. 6B), whereas the 2 lowest dilutions were undetectable.

**Discussion**

We report development of a rapid, fully automated investigational immunoassay with the sensitivity of NAT-VL methods for detection of early HIV infection. The assay requires no sample pretreatment or enrichment steps. The LoD was estimated to be 0.0025 ng/L, approximating 60 RNA copies/mL (30 virus/mL). With an assay sample volume of 144 μL, this equates to detection of only 4 virus particles. Multiple approaches to test sensitivity were consistent in demonstrating the assay to be capable of matching or even exceeding the sensitivity of NAT-VL, and approaching the sensitivity of qualitative NAT screening methods for detection of Group M HIV-1. In the sensitivity comparison, the Simoa assay matched a NAT-VL method with an LoD of 40 RNA copies/mL sample-for-sample, and exceeded the sensitivity of a NAT-VL method with an LoD of 100 RNA copies/mL by detecting 2 additional samples among Group M isolate dilutions. On the other hand, contemporary immunoassays exhibited sensitivity concordance vs NAT-VL of 33%–38% with a random set of NAT yield samples. A strength of this study was the use of multiple approaches to estimate sensitivity with well-characterized materials and samples. A study limitation is the preliminary nature of the data (particularly specificity), as well as relatively small sampling size. More robust
validation of the method requires much larger numbers of samples in a clinical setting, with NAT as the reference method.

The Simoa p24 immunoassay uses a novel, nondedicated digital immunoassay analyzer. The HD-1 Analyzer is a completely automated system designed to minimize operator intervention. Samples require no pretreatment, no special handling, and no molecular laboratory measures against carryover contamination associated with amplification methods. The assay uses standard low-cost bead-based ELISA reagents, calibration can be stored and reused to save calibration costs, and the system is price-competitive with conventional immunoassay analyzers.

A low-cost, easily deployable alternative to NAT could have important implications for HIV screening economics and efficacy. NAT economics in different screening settings is a key issue (18–25). In blood bank screening, despite donor pooling to reduce cost, NAT is cost ineffective for serology-negative detection of acute HIV infection (19, 20), with normal medical cost-benefit standards greatly exceeded (23). In high-income countries, public expectation of maximum blood safety has led to the establishment of NAT for blood bank
screening despite its cost, but this is not the case for many European and developing countries (24, 25). The use of NAT-VL for routine clinical screening has also been explored and concluded to be impractical except in high-risk settings, such as sexually transmitted disease clinics (22, 23).

Timely detection and reporting represent another issue limiting the clinical impact of NAT detection of acute HIV infection. The acute phase represents a period of high viremia and high infectious risk, contributing disproportionately to the spread of the disease (5, 26). Turnaround time for executing a pooling strategy, testing, and deconvolution of a positive pool can delay reporting of an acute infection for days, making difficult the timely intercession of a patient’s potential to transmit virus. If the method by which early-stage infection is caught were capable of a short, patient-specific turnaround of results, the reduction of the detection window could have greater practical significance, as there would be greater likelihood of conveying acute status to the patient and preventing transmission during the hyperinfectious stage. Results from a rapid automated immunoassay can be turned around while the patient waits.

Whereas studies on the practicality of NAT in the clinical setting have focused on immunoassay-negative samples, recent HIV screening recommendations use NAT in a confirmatory capacity (27, 28). Expanded screening is aimed at the 20% of HIV-infected individuals who are unaware of their HIV status (29), and an algorithm proposed in the US by the CDC starts with a fourth-generation HIV combination immunoassay. A premise of the algorithm is to use combination reactivity to sufficiently increased p24 before seroconversion (30) and to anti-HIV antibodies after seroconversion. Ideally, a screening test should offer 3 things: high sensitivity for both acute and chronic infection, low cost, and short turnaround time to facilitate patient intervention. NAT provides the highest sensitivity for acute infection but can miss chronic infection when viral load is undetectable. Antibody assays detect chronic infection and offer cost and turnaround-time advantages, but can miss acute infection. With reactivity to sufficiently increased p24, combination assays provide the best tradeoff, but a sensitivity gap remains vs NAT, and failure to diagnose acute HIV infection represents a key public health problem (31, 32).

HIV screening in developing countries and low-resource settings with high HIV incidence faces the issues described above, but on a larger, more urgent scale. NAT is a sophisticated technology with adoption barriers on both supplier and user sides. Although the screening of donated blood with NAT has been in practice since the late 1990s, 40% of worldwide blood donations remain unscreened or are screened by less-sensitive immunoassays (33, 34). In South Africa, the prevalence of HIV infection in the general population is almost 11% (35), but diagnosis of acute HIV infection is rare (36). Feasibility of NAT implementation in these very high-incidence settings (36, 37) has highlighted both the epidemiological benefits and the
challenges around adoption of these methods. A low-
cost immunoassay with the sensitivity of NAT could
represent an opportunity to further affect the spread of
HIV in these settings.

Although the data presented here demonstrate the
potential of Simoa to provide an alternative to NAT for
high-sensitivity HIV-1 detection, 2 areas for further
development are apparent. First, Group O is an uncommon
HIV strain seen mainly in west central Africa, and its
sensitive detection can be added by inclusion of Group
O-specific antibodies in the reagents. Second, the cur-
rent specificity of the assay is insufficient for donor
screening. Improving specificity requires additional re-
agent blocker optimization to minimize nonspecific in-
teractions leading to false positives.

Because the assay does not perform immune com-
plex dissociation, its clinical use requires an adjunctive
HIV antibody test. However, the assay format readily
lends to addition of reagents enabling detection of HIV
antibodies. In such a configuration, both acute and
chronic infection would be detected with 1 test. Further,
by using the HD-1’s multiplexing capability, a “fifth-
generation” HIV test that combines NAT sensitivity for
virus and identification of reactive antibodies to the virus
is possible. As a multiplex, the reactive entities can be
identified separately, in contrast to combination tests
that do not discriminate between p24, HIV-1, or HIV-2
antibody reactivity. With fast turnaround time and low
cost, such an assay could present an ideal consolidated
alternative to current approaches for detection of both
acute and chronic HIV infection with the highest sen-
sitivity possible.

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