Antibody detection by agglutination–PCR (ADAP) enables early diagnosis of HIV infection by oral fluid analysis

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Oral fluid (OF) is a highly effective substrate for population-based HIV screening efforts, as it is noninfectious and significantly easier to collect than blood. However, anti-HIV antibodies are found at far lower concentrations in OF compared with blood, leading to poor sensitivity and a longer period of time from infection to detection threshold. Thus, despite its inherent advantages in sample collection, OF is not widely used for population screening. Here we report the development of an HIV OF assay based on Antibody Detection by Agglutination–PCR (ADAP) technology. This assay is 1,000–10,000 times more analytically sensitive than clinical enzyme-linked immunoassays (EIAs), displaying both 100% clinical sensitivity and 100% specificity for detecting HIV antibodies within OF samples. We show that the enhanced analytical sensitivity enables this assay to correctly identify HIV-infected individuals otherwise missed by current OF assays. We envision that the attributes of this improved HIV OF assay can increase testing rates of at-risk individuals while enabling diagnosis and treatment at an earlier time point.

protein–DNA conjugation | PCR | immunoassay | oral fluid | infection diseases

Eliminating HIV from the human population will require innovative diagnostic and therapeutic strategies (1). Currently, large-scale population screening efforts remain the most effective public health mechanism to identify and funnel HIV-infected people to treatment (1). Early identification of newly infected individuals permits the timely initiation of antiretroviral therapy (ART) to reduce transmission rates and improve health outcomes (1). During this "acute" period immediately following infection, patients are up to 26 times more infectious and over 50% of new transmissions are thought to occur in this window (2, 3).

While blood-based assays efficiently diagnose HIV infection during the acute phase, these tests suffer from poor compliance rates due to their invasive nature (4, 5). In contrast, noninvasive assays such as oral fluid (OF) antibody tests have higher levels of compliance but lack the analytical sensitivity to detect very low levels of antibodies in the OF of acutely infected individuals (6–10). Currently, no existing test meets the pressing medical need to noninvasively detect HIV during acute infection, which is essential to maximize the number of people screened and to intervene at the earliest time.

HIV tests that analyze easily collected OF increase the numbers of individuals tested in situations where needle-mediated blood drawing is inefficient or unsafe (4, 5). The use of oral specimens has facilitated testing in many populations including (*i*) populations for whom it is inconvenient or unsafe to test using needles (e.g., prisons), (*ii*) patients whose veins are difficult to draw from (e.g., drug users, infants), and (*iii*) people who are averse to having blood drawn using needles (e.g., children, adolescents) (10–12). Furthermore, antibodies in OF are stable for several weeks at ambient temperature, thus decreasing the likelihood of false results when cold chain shipping is not available (13, 14). Finally, OF is much safer to handle on a large scale, as HIV cannot be transmitted by OF thanks to significantly lower viral loads and the presence of naturally occurring enzymes and other inhibitors that deactivate the virus (15). OF therefore represents an ideal sample type for large-scale screening of HIV incidence in many groups, including those hard-to-reach populations.

Antibodies are the most reliable markers of HIV infection in OF (16). While HIV-derived RNA and proteins (i.e., p24) are considered powerful blood-borne markers for detecting early infections, these HIV components do not consistently appear in OF (16). Thus, assays that measure HIV RNA by quantitative PCR (qPCR) or HIV protein (p24) by enzyme-linked immunoassays (EIAs) are unsuitable for OF screening. By contrast, HIV-associated immunoglobulins are reliable markers of infection in OF (17, 18). Indeed, detection of OF IgG in EIA-type formats forms the basis of the FDA-approved OraQuick test. Unfortunately, however, this test cannot detect disease until at least 40 d after infection (18). This unacceptably long window period is attributed in part to the ∼1,000-fold lower antibody concentration in OF relative to serum/plasma (19). As a point of comparison, blood-based tests can detect infection as soon as 14–25 d after exposure (20). The diminished antibody titers along

Significance

There is a substantial public health interest in identifying HIV-infected individuals through population-based screening. Oral fluid (OF) is easier to collect than blood and therefore ideal for such screening efforts. Unfortunately, OF has a very low concentration of anti-HIV antibodies (markers of HIV infection), which current assays cannot detect during the early stage of this disease. Here we report an assay for anti-HIV antibodies in OF that is up to 10,000 times more sensitive than current alternatives. This assay, called Antibody Detection by Agglutination–PCR (ADAP), could be broadly deployed to screen at-risk populations using OF in many settings, including those where cold chain shipping is not available (low-resource settings) and where needles are inconvenient (pediatrics) or unsafe (prisons).

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with much lower antibody production in the early phase of the disease pose significant analytical challenges for current HIV OF antibody tests (19).

Here we report an ultrasensitive OF HIV antibody detection method based on Antibody Detection by Agglutination–PCR (ADAP) technology (Fig. 1) (21). The ADAP platform, similar in nature to proximity ligation assay (PLA) (22), leverages multivalent binding of antibodies to drive the agglutination of antigen–DNA conjugates. The induced proximity enables ligation of DNA fragments to form a full-length DNA amplicon, which can then be quantified by qPCR. As reported previously, this amplification permits detection of antigen-specific antibodies at high zeptomole levels in 1-μL samples (21). Since ligation is only triggered following a productive antibody–antigen interaction, ADAP does not require washing steps to remove unbound antigen–DNA conjugates and is thus well-equipped to detect low-affinity antibodies. Furthermore, ADAP can detect antibodies of any isotype, including IgM, the earliest antibody marker of acute infection (20). Importantly, DNA barcoding allows multiplexing by linking the identity of each antibody to a unique DNA sequence. Thus, antibodies specific for multiple antigens can be detected in a single sample.

Accordingly, we developed an HIV OF test based on ADAP using DNA conjugates of the HIV proteins p24, gp41, and gp120 (Fig. 1*A*), the standard antigens for clinical HIV antibody testing (23). The ADAP test was found to be 1,000–10,000-fold more sensitive than the EIAs used in clinical settings. We analyzed OF samples from the Alameda County (California) Public Health Laboratory's HIV screening program. We confirmed previously assigned HIV diagnoses with 100% accuracy. To further evaluate the assay, we tested a panel of eight OF samples that were classified as "indeterminate" by current assays. ADAP analysis reclassified six of these samples as HIV positive due to the presence of two or more anti-HIV antibodies. Critically, one such patient was confirmed to be HIV-infected by a follow-up blood test. Thus, ADAP-based HIV testing may enable population-based screening for early HIV infection using OF, especially for those who will not normally get tested.

Results

Synthesis of Viral Antigen–DNA Conjugates. We used commercially available full-length recombinant p24, gp120, and gp160 (precursor of gp41 and gp120), all from HIV-1 clade C, as substrates for DNA conjugation (Fig. 1*A*). For gp41, we instead used a recombinant gp41-derived peptide fragment. We then synthesized the antigen–DNA conjugates by lysine modification with sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC), followed by reacting the newly installed maleimide groups with thiolated oligonucleotides that had been prereduced by treatment with DTT (Fig. 1*B*). Unreacted reagents were removed by extensive purification with size-exclusion filter columns. Viral antigen–DNA conjugation ratios were determined by UV-Vis spectroscopy and gel analysis (Fig. S1). The conjugates were then diluted in buffer and stored at 4 °C until use. Importantly, in a singleplex experiment, all viral antigen–DNA conjugates contained the same DNA sequence (Table S1), whereas in the multiplexed experiment, each viral antigen–DNA conjugate had a unique DNA sequence (Table S1).

ADAP Workflow for HIV Antibody Detection. In an ADAP experiment, pairs of viral antigen–DNA conjugates are first diluted in buffer. One antigen–DNA conjugate bears the 5′ half of a PCR amplicon, while the other conjugate bears the 3′ half that is 5′ phosphorylated to enable ligation. The pooled conjugates are added to 1 μL of OF sample and incubated for 30 min to allow antibody binding. Next, DNA ligase and a bridge oligonucleotide are added and incubated for 15 min. The ligation mixture is then preamplified by PCR, and the resulting products are quantified by qPCR (Fig. 1*C*). As high Ct values of qPCR are associated

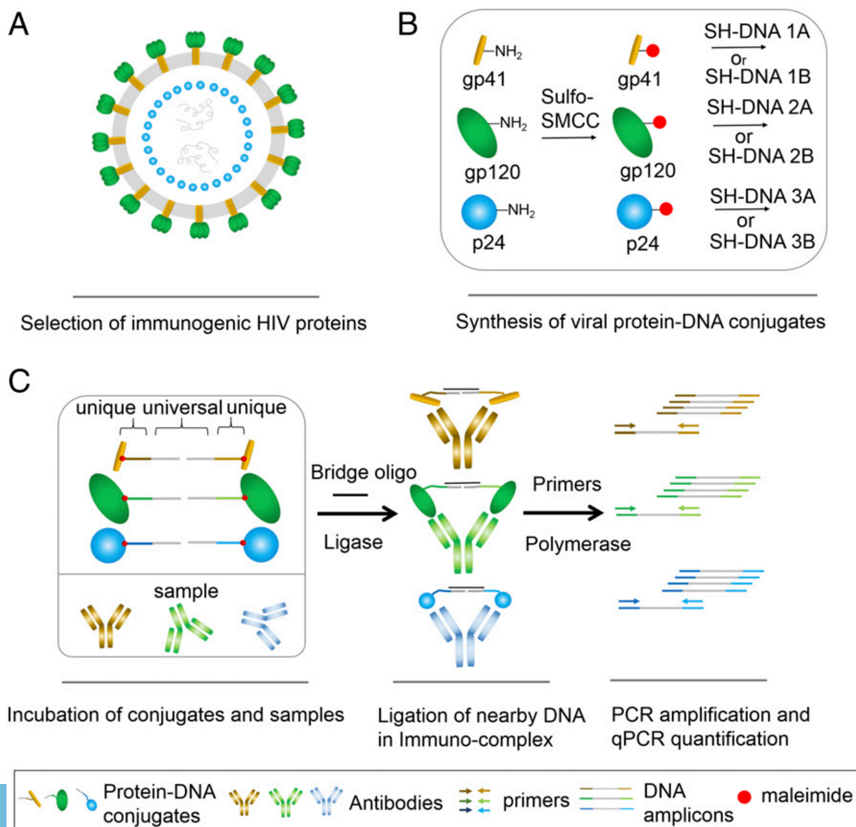

Fig. 1. Principle scheme of antibody detection by ADAP for HIV diagnosis. (*A*) HIV virus contains many immunogenic proteins, including viral capsid protein p24 (blue) and envelope glycoprotein gp160, which can be cleaved into gp41 (brown) and gp120 (green). (*B*) Recombinant viral proteins are activated by installation of maleimides onto lysine residues via the small-molecule cross-linking agent sulfo-SMCC. Thiol-functionalized DNA covalently ligates to these maleimides by Michael addition to form protein–DNA conjugates. (*C*) Upon incubation with antibody-containing samples, antibodies and conjugates form immune complexes, allowing nearby DNA to be ligated into a full-length amplicon upon addition of a universal bridge oligonucleotide and DNA ligase. Each amplicon bears unique primer binding sites for independent amplification and quantification by real-time qPCR. Critically, DNA conjugates alone without ligation bear only one primer binding site and are therefore PCR-incompetent. Only successful ligation into a full-length amplicon enables exponential amplification by PCR. This "turn-on" mechanism allows ADAP to leverage PCR's analytical sensitivity while preserving assay specificity.

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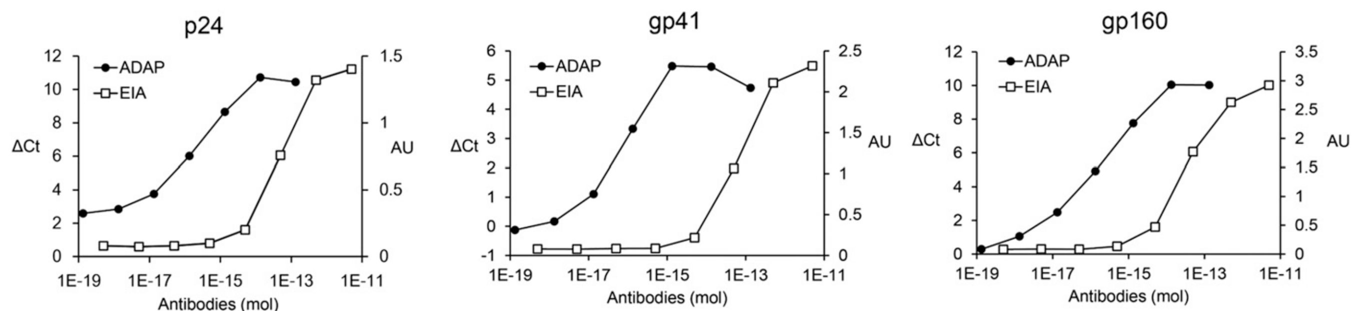

Fig. 2. ADAP's analytical sensitivity outperformed commercial EIA by several orders of magnitude. Purified human anti-HIV antibodies were serially diluted in buffer. The dilution series was assayed by ADAP and EIA (Avioq microelisa). The *x* axis displays the amount of antibody, while the left *y* axis shows the signal from an ADAP qPCR experiment and the right *y* axis shows the signal from commercial EIA. For most data points, the error bars were too small to be visualized.

with low assay reproducibility, we include a preamplification step in the ADAP protocol to ensure high assay reproducibility as reported previously (21, 22).

ADAP Showed Enhanced Analytical Sensitivity Compared with a Standard OF EIA Used in Public Health Laboratories. To demonstrate that viral antigen–DNA conjugates were capable of detecting their cognate antibodies, we obtained a panel of highly purified human antibodies against p24, gp41, and gp160 derived from HIV patients (Immunodx). We then serially diluted each HIV antibody into buffer and quantitated them using ADAP or a clinical EIA (Avioq microelisa, FDA approved). We observed concentration-dependent signals for all three antibodies using ADAP with a dynamic range up to 10^5 (Fig. 2). The detection limits were 110, 880, and 550 zeptomoles (10^{-21} moles) of anti-p24, anti-gp41, and anti-gp160, respectively. In contrast, with the EIA assay, the detection limits were 8.5, 9.2, and 11 femtomoles for anti-p24, anti-gp41, and anti-gp160, respectively. Collectively, ADAP showed 1,000–10,000-fold enhanced analytical sensitivity compared with a clinical EIA assay.

Singleplex Clinical Sensitivity and Specificity Using Archived OF Samples from a Public Health Screening Program. Next, we evaluated the clinical sensitivity and specificity of the ADAP method using OF samples obtained as part of an HIV screening effort by the Alameda County (California) Public Health Laboratory. The OF samples were remnant test samples that were not individually identifiable and complied with FDA guidance on their use (24). We selected 22 EIA-reactive ("positive") OF samples from HIV-infected patients and 22 EIA-nonreactive ("negative") OF samples from non-HIV donors. The positive OF samples were selected to represent a range of EIA assay signal intensities, with signal-to-cutoff (S/C) ratios from 1.7 to 6.7 measured by EIAs (Avioq microelisa). A confirmatory Western blot assay (Orasure) was also performed on the OF samples to ensure the presence of two or more reactive antibodies, as recommended by Centers for Disease Control and Prevention (CDC) guidelines (25).

We incubated the OF samples with each viral antigen–DNA conjugate (p24, gp41, gp120, and gp160) and followed the ADAP protocol as outlined above. For all four viral antigens, a clear difference ($P < 0.05$) between positive and negative OF was observed (Fig. 3*A*). We then used two SDs of negative OF to establish a positivity threshold for each antibody marker. We defined HIV positivity as two or more antibodies with signal intensities above threshold values. Under these stringent criteria, all 22 negative OF samples were correctly classified as HIV-negative and all 22 positive OF samples were classified as HIV-positive, achieving 100% sensitivity and specificity (Fig. 3*A*).

Additionally, we sought to compare the signal intensities measured using the ADAP and clinical EIA assays (Avioq microelisa). The EIA assay measured total antibody-derived signal. To approximate this composite measurement, we summed the ADAP signal intensities derived from three viral antigens (p24, gp41, and gp120, Fig. 3*B*). As shown in Fig. 3*C*, the correlation between the two assays was high ($R = 0.80$, $P < 0.05$). Signal correlations of individual HIV components to EIA were shown in Fig. S4. These results further validated ADAP as a means to detect anti-HIV antibodies.

A final concern we sought to address was whether the difference in signal between HIV-positive and HIV-negative individuals was simply the result of intrinsic differences in their OF compositions. For example, OF from immunosuppressed HIV-positive patients might contain unknown factors that elevated ADAP signal intensity in an antibody-independent manner. To preclude this possibility, we synthesized negative-control GFP (green fluorescent protein)–DNA conjugates. As there were no naturally occurring anti-GFP antibodies within human OF, we expected that no signal should be observed from ADAP analysis using GFP–DNA conjugates. Indeed, HIV-positive and HIV-negative OFs were indistinguishable following ADAP analysis with GFP–DNA conjugates ($P = 0.5$) (Fig. S2). This experiment supports the assignment of signals such as those shown in Fig. 3 *A* and *B* to the presence of anti-HIV antibodies in OF.

Multiplexed Clinical Sensitivity and Specificity Using Archived OF Samples from a Public Health Screening Program. Our previous experiments showed that ADAP faithfully detected individual HIV-specific antibodies from OF. Next, we sought to create a multiplexed ADAP assay to simultaneously analyze three of these antibodies in a single test. We synthesized each viral antigen–DNA conjugate with unique DNA barcodes (Fig. 1*B* and Table S1). Only the correct antibody markers would agglutinate the related antigen–DNA conjugates, leading to amplification of the associated barcodes. Unique primer pairs within different wells of a qPCR plate could then be used to quantify the amount of each DNA barcode (Fig. 1*C*).

We reanalyzed the 22 positive OF and 22 negative OF samples using this multiplexed ADAP strategy for antibodies against p24, gp41, and gp120 in a single assay. Gp160 was not included due to the fact that gp41 and gp120 are the cleavage products of gp160. These two antigens together cover the entire amino acid sequence of gp160. Thus, the use of p24, gp41, and gp120 should yield a near-complete landscape of the antibody response to HIV infection. As before, we defined HIV-positivity by detection of two or more antibodies above a cutoff threshold. By this metric, a clinical profile identical to our previous singleplex analysis was obtained, with clinical sensitivity and specificity of 100% (Fig. 4). We observed the same patterns for individual antigens, with 91% positivity for anti-p24 and 100% for anti-gp41 and anti-gp120 (Fig. 4). Correlations of signals between singleplex and multiplexed

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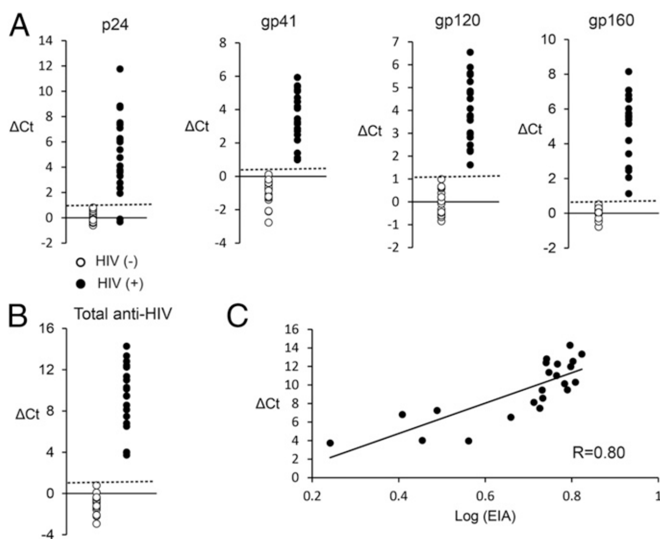

Fig. 3. Singleplex ADAP analysis of OF samples. (*A*) OF samples from HIV-negative ($n = 22$) and HIV-positive ($n = 22$) patients were analyzed by ADAP. Using cutoff values established from HIV-negative samples, HIV-positive samples showed 91% positivity for p24 and 100% for gp41, gp120, and gp160. (*B*) Cumulative signal from all anti-HIV antibodies. All 22 HIV-positive samples showed a higher signal than 22 HIV-negative samples. By defining positivity as the presence of two or more HIV antibodies, singleplex ADAP analysis yields 100% clinical sensitivity and specificity in comparison with the clinical gold-standard EIA. (*C*) The cumulative signal intensities of ADAP correlated well with the signal intensities of EIA ($R = 0.80$, $P < 0.05$).

experiments were also very high ($R = 0.99$ for p24, 0.97 for gp41, and 0.95 for gp120) (Fig. 5).

These data demonstrated that ADAP detection of HIV antibodies in OF was at least as sensitive and specific as a current clinical standard assay employed in public health laboratories. Importantly, the ADAP OF assay allowed multiplexed profiling of the HIV immune response in a single assay. It is also of considerable note that the only FDA-approved confirmatory test for positive OF EIA test results (OraSure HIV-1 Western Blot) is poised to leave the market. An assay such as ADAP, which can detect antibodies to multiple HIV antigens simultaneously, might be an effective replacement. The absence of an OF confirmatory test will force laboratories to adapt existing blood-based tests for this purpose, which could be a less than ideal proposition for the diagnostic community.

ADAP May Detect HIV Earlier than Current OF Assays. Finally, we performed a pilot study to determine whether ADAP analysis of OF could detect HIV infection earlier than the clinical OF assay (Avioq Microelisa). We obtained a panel of eight OF samples that showed indeterminate results using the current OF test. The indeterminate status was defined by S/C values measured by EIA (Avioq microelisa) between 0.6–0.9 and showed a single weak band in Western blot (Orasure). OF samples that met these criteria displayed "HIV-positive–like" qualities but did not meet the full criteria for HIV positivity. These samples might be derived from patients in the early seroconversion phase of HIV infection and thus might harbor very low levels of antibodies that traditional assays could not detect.

ADAP analysis revealed that six of eight indeterminate samples were positive for two or more HIV-associated antibodies and thus reclassified these patients as HIV-positive (Fig. 4 and Table 1). Critically, we obtained a blood sample from one ADAP-positive/EIA-indeterminate individual (follow-up blood samples were not available for other indeterminate individuals). As blood contains 1,000-fold higher levels of antibodies compared with OF, current tests can analyze such samples with improved confidence. Analysis of this blood sample revealed the patient to be HIV-positive, in agreement with ADAP's classification. These results suggested that ADAP's enhanced analytical sensitivity might enable early detection of HIV infection from OF.

Discussion

Since the first FDA-approved tests for detection of HIV antibodies became available in 1985 (26), assays for blood-based HIV diagnostics have evolved enormously. The first-generation HIV serum assays used whole HIV lysates as antigens, with anti–human-IgG secondary antibodies as reporters. This assay design suffered from low specificity due to contaminants present in the lysates (25). To address this problem, a second-generation assay instead employed recombinant peptides and proteins to improve the purity of antigen probes. However, second-generation assays still required a long period of up to 40 d after exposure before antibody detection was possible (25). A third-generation assay sought to improve the window period by detecting both IgM and IgG anti-HIV antibodies in a sandwich EIA format (25). Since IgM antibodies appear much earlier than IgG in the seroconversion process, this technology shortened the window period to ∼20–25 d. Finally, fourth-generation assays not only detected IgM and IgG anti-HIV antibodies but also detected viral protein p24, which allowed HIV diagnosis ∼14–16 d after infection (25). In addition to these protein detection assays, HIV RNA assays have been developed to accurately quantify viral loads at the earliest stage of infection (25). Moreover, highly sensitive immunoassays including digital ELISA have also been reported to enable early diagnosis of HIV (27). These innovations together have greatly improved the sensitivity and specificity of HIV diagnosis by blood samples.

In contrast, the performance of OF-based HIV assays has remained relatively stagnant, with window periods persisting at 40–50 d postinfection. Counterintuitively, the diagnostic technology used in first- and second-generation blood-based assays outperforms more recent-generation assays when analyzing OF (19). The much lower antibody concentration and inconsistent presence of viral proteins or RNA in OF samples surely contribute to this problem (19). As OF-based assays can greatly improve HIV screening rates and compliance, there remains an unmet need for improved OF assays to advance HIV management.

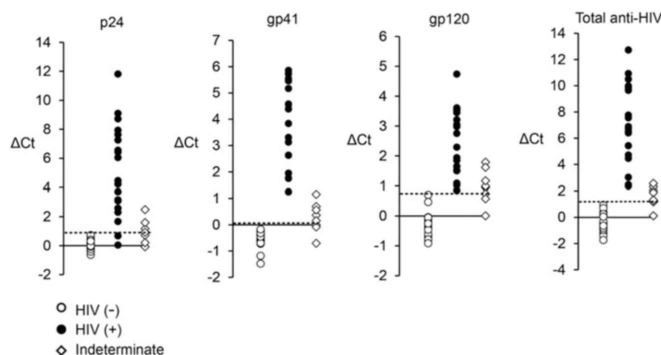

Fig. 4. Multiplexed ADAP analysis of clinical OF samples. A multiplexed version of ADAP simultaneously detected antibodies against p24, gp41, and gp120. We reanalyzed the OF samples from before and observed identical performance to the singleplex analysis. HIV-positive samples again showed 91% positivity in p24 and 100% in gp41 and gp120. Applying the same criteria for positivity (two or more antibodies), multiplexed ADAP analysis demonstrated 100% clinical sensitivity and specificity as seen with singleplex. In addition, we analyzed several indeterminate OF samples ($n = 8$) with multiplexed ADAP. Detailed ADAP analysis results for eight indeterminate samples are summarized in Table 1.

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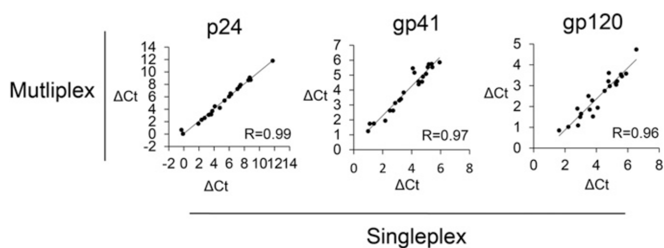

Fig. 5. Singleplex and multiplexed ADAP signal intensities showed strong correlation. The signal intensities from singleplex and multiplexed ADAP analysis of the same samples were plotted and analyzed for correlation. Correlation coefficients of 0.99, 0.97, and 0.96 were observed for p24, gp41, and gp120, respectively.

Our ADAP OF assay leverages the analytical sensitivity of PCR to achieve highly sensitive, specific, and multiplexed detection of antibodies against several HIV antigens with low sample consumption. Notably, the ADAP test likely reports on the presence of both IgGs and IgMs, the earliest antibody infection marker, as both species agglutinate DNA-conjugated antigens (Table S2). We demonstrated that our ADAP assay was 1,000–10,000× more sensitive than a standard clinical OF EIA test. The potential clinical utility of this sensitivity was demonstrated by identifying an HIV infection that was otherwise missed by current OF tests.

These results established the basis for further development and validation of ADAP HIV OF assays. First, most viral antigens employed in this report were derived from HIV-1 clade C. These antigens were well-suited to develop assays for centers at the front lines of the HIV pandemic, including South Africa (28). We envision our ADAP HIV OF assays best applied as an incidence screening test in public health settings. As such, future experiments should focus on whether ADAP HIV OF assays retain clinical sensitivity and specificity for HIV-1 of other clades. Second, specific antigens for HIV-1 group N, O, and P and HIV-2 (e.g., gp36) were not incorporated into current multiplexed panels (29). Including these antigens would strengthen an ADAP assay for HIV diagnosis. Third, we demonstrated a promising but preliminary example of ADAP's ability to detect HIV during the acute infection phase, where other tests might fail. Analysis of paired OF and blood samples throughout the seroconversion processes would be required to show that ADAP assays have a shorter window period in comparison with other assays.

In summary, ADAP presents the unique potential to detect early HIV infection using easily acquired OF samples on a standard qPCR machine. ADAP meets the pressing public health need to identify HIV and treat it as early as possible to both improve interruption of disease transmission and enhance patient outcomes.

Materials and Methods

Materials. Chemicals were purchased from Sigma Aldrich unless otherwise specified. DTT and sulfo-SMCC were purchased from Life Technologies. DNA ligase was purchased from EpiCentre (A8101). Platinum Taq polymerase (10966026) and SYBR qPCR 2X master mix (4385610) were purchased from Thermo Fischer. Other reagents were detailed in the *Materials and Methods* section as appropriate.

Synthesis of Protein–DNA Conjugates. The p24 (Immunodx), gp120 (Immunodx), and gp160 (Avioq) antigens in this study were full-length recombinant proteins. The gp41 antigen was a recombinant peptide fragment (Fitzgerald Industry International). The above proteins were suspended in reaction buffer (55 mM sodium phosphate, 150 mM sodium chloride, 20 mM EDTA, pH 7.2) to make 1 mg/mL solutions. Sulfo-SMCC (Thermo Scientific) (1 μL of 8 mM solution in anhydrous DMSO) was added to 10 μL of the protein solution. The reaction mixture was incubated at room temperature (RT) for 2 h. Thiolated-DNA (IDT) was suspended in reaction buffer to 100 μM. A 3-μL aliquot of thiolated-DNA solution and 4 μL of a 100-mM solution of DTT were mixed to reduce oxidized thiolated-DNA. The solution was then incubated at 37 °C for 1 h. The excess sulfo-SMCC in the protein solution and DTT in the thiolated-DNA solution were removed by 7K MWCO Zeba spin column (Thermo Fischer). The thiolated-DNA and viral protein solutions were then pooled and incubated overnight at 4 °C. The DNA-to-protein incubation ratio was 3-to-1 for all proteins and peptides used in this study. Finally, protein–DNA conjugates were purified by 30K MWCO filter (Millipore). Conjugate concentrations were determined by BCA assay (Life Technologies). Conjugation efficiencies were analyzed by SDS/PAGE and silver staining as described previously (21). A representative silver stain was shown in Fig. S1. DNA-to-protein ratios of the conjugates were estimated by UV-VIS absorption and typically fell in the range of 2-to-1. Protein–DNA conjugates were stored at 4 °C for short-term usage or aliquoted for long-term storage at −80 °C.

DNA Sequences. All DNA sequences used in this study are provided in Table S1.

Clinical Samples. The study protocol (ID 36631) was approved by Stanford University Institutional Review Board. OF samples used in this study were deidentified remnant test samples (from the HIV screening program at Alameda County) that would otherwise be discarded. These individually non-identifiable leftover samples met the full criteria of FDA guidance on their use, and informed consent requirements were waived (24). OF samples were collected using Orasure oral specimen collection devices according to the manufacturer's instructions. An OF collection pad was inserted between the cheek and gums for 5–7 min. Thereafter, the collection pad was stored in the collection tube containing storage buffer. The OF specimen was then transferred to Alameda County Public Health laboratory at RT. Once received, OF specimens were eluted by centrifugation at 800 *g* for 15 min. Each OF specimen contained 0.7–1.5 mL of liquid. HIV status was determined by a two-tier algorithm. Avioq HIV-1 Microelisa was used as a first-tier assay, and positive samples were confirmed by Orasure Western blots. All OF samples had been stored at −20 °C.

Singleplex ADAP. Paired protein–DNA conjugates (1 femtomole) were suspended in 2 μL of buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA, 100 μM of competition DNA, 1 mg/mL goat IgG in PBS). A 1-μL aliquot of analyte was added to the conjugates and then incubated at 37 °C for 30 min. A 117-μL aliquot of ligation mix (20 mM Tris, 50 mM KCl, 20 mM MgCl2, 20 mM DTT, 25 μM NAD, 0.025 U/μL ligase, 100 nM bridge oligonucleotide, 0.01% BSA, pH 7.5) was added and incubated at 30 °C for 15 min. A 25-μL aliquot of the solution was added to 25 μL of 2× PCR Mix with 10 nM primers and then amplified by PCR (95 °C for 10 min, 95 °C for 15 s, 56 °C for 30 s, 12 cycles). The PCR was then diluted 1:20 in H_2O. A 8.5-μL aliquot of the diluted PCR samples was added to 10 μL of 2× qPCR Master Mix with 1.5 μL of a primer solution (final primer concentration 690 nM). SYBR Green-based qPCR was performed on a Bio-Rad CFX96 real-time PCR detection system (95 °C for 10 min, 95 °C for 30 s, 56 °C for 1 min, 40 cycles).

A 1-μL sample volume was used, as larger volumes lead to increased background signals in some clinical samples. In addition, data generated from sample volumes below 1 μL suffered from reduced reproducibility.

Importantly, to correct potential drift in qPCR signal across different experiments, a blank sample containing buffer C was always run concurrent to the actual samples of interest. The rest of the procedure then followed the protocol outlined above.

Table 1. Multiplexed ADAP assay identified HIV infection missed by commercial EIA

Individual ID	p24	gp41	gp120	Status
13AC6953	**0.9254**	**0.3633**	**1.785**	Positive
13AC9465	**1.1654**	**1.1533**	**1.615**	Positive
14AC10527	−0.0746	**0.6933**	**0.945**	Positive
14AC7304	0.7154	−0.7067	0.005	Negative
15AC4639	**1.5954**	−0.0067	**0.985**	Positive
15AC8637	0.2054	−0.0867	1.165	Negative
16AC1148	**0.9254**	**0.5633**	**0.735**	Positive
16AC6294	**2.4854**	**0.1533**	**0.575**	Positive
Cutoff	0.75622	0.12831	0.70333	

Six out of eight indeterminate OF samples were reclassified as HIV-positive (highlighted yellow) by multiplexed ADAP analysis. Strikingly, a follow-up blood draw confirmed one such sample (16AC6294, highlighted red) to be HIV infected, which was otherwise missed by commercial EIA (bold font for positive signals).

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The ADAP assay readout ΔCt is defined as the Ct value of blank minus Ct value of actual samples (Fig. S3). The value of ΔCt is proportional to the initial amplicon concentrations in the PCR plate well. This amplicon concentration is also proportional to the amount of target antibodies present in the samples. (For each curve, the PCR cycle number with fluorescence value corresponding to the chosen threshold value is defined as the cycle threshold, Ct.)

Multiplexed ADAP. The protocol was similar to singleplex ADAP analysis with minor modifications. Briefly, 1 femtomole of all protein–DNA conjugates was suspended in 2 μL of buffer C. Then, analyte and ligation mix were added and incubated sequentially as described above. Then, 25 μL of ligated solution was aliquoted into different wells of PCR tubes that each contained PCR master mix and one primer pair. The preamplified products were then quantified by different primer pairs in a 96-well qPCR plate. Finally, ΔCt for each DNA amplicon/primer was calculated and therefore allowed multiplexed quantification of multiple antibody targets from a single sample.

Analysis of Purified HIV-Patient Antibodies. Purified patient-derived human HIV antibodies against p24, gp41, and gp160 (ImmunoDx) were serially diluted 10-fold in buffer C and subjected to either ADAP analysis in our laboratory or clinical EIA testing (Avioq microelisa) at the Alameda County Public Health Laboratory. Each sample was run in triplicate. The dilution curve was modeled by 4 parameter logistic fit (30). The limit of detection was then defined as the average ΔCt value of the buffer C-only blank plus 3 SDs of the blank (31). In this work, we measured buffer C-only blank in triplicate to derive the SDs. The limit of detection was calculated relative to the blank (31).

Analysis of Clinical HIV OF Samples. HIV OF samples were obtained from Alameda County Public Health Laboratory. The 22 HIV-positive OFs showed EIA signals above the cutoff and two or more reactive bands on Western blot. These positive samples were carefully selected to display a wide range of EIA signal intensities to challenge ADAP's assay performance (S/C ratio ranged from 1.7 to 6.7). The 22 HIV-negative OF samples showed signal below the EIA cutoff (S/C ratio ranged from 0.1 to 0.3). The eight indeterminate HIV samples showed EIA signal close to the cutoff (S/C ratio ranged from 0.6 to 0.9). Furthermore, these samples showed one weakly reactive band in Western blot analysis (Orasure), therefore rendering them HIV-indeterminate. The Alameda County Public Health Laboratory obtained a follow-up finger prick for one indeterminate sample donor and confirmed the patient HIV-positive.

In singleplex ADAP analysis, p24–, gp41–, gp120–, and gp160–DNA conjugates were used separately to detect antibody reactivity in OF. In a multiplexed experiment, p24–, gp41–, and gp120–DNA conjugates that contained unique DNA barcodes were used simultaneously to profile anti-HIV antibody response in OF samples. Gp160 was not employed in a multiplexed experiment because gp41 and gp120 displayed the entire amino acid sequence of gp160. We thus did not foresee the additional benefit of including gp160.

Data Analysis. In the ADAP to EIA correlation analysis, the ADAP signal was the geometric sum of the ΔCt value of the p24, gp41, and gp120 assays. The summed signals were then correlated to the logarithm of EIA signals. The use of logarithm was necessary as ΔCt is a logarithmic parameter (32). [For instance, consider a sample of ΔCt value of 2 and another sample of ΔCt of 4; their amplicon quantities differ by fourfold ($2^4/2^2$) rather than twofold.]

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