

ing the observed differences between the two methods. Our study is even more intriguing if we consider that, very recently, a new molecular form of PTH, with structural integrity of the PTH(1–4) region and a modified PTH(15–20) region, has been identified by HPLC in primary and secondary hyperparathyroidism (21). In fact, this newly discovered form of PTH appears to be immunoreactive and detectable by the whole-PTH assay but not by the intact-PTH assay.

In conclusion, the unexpected constant differences in PTH values among immunoassays, observed in both uremic and PHPT patients and in healthy individuals, could be attributable to the different calibration procedures in addition to the presence of PTH fragments. It would be useful for manufacturers to reduce the systemic variability among methods by use of a more standardized method of calibration and use of antibodies that recognize the only biologically active PTH molecule.

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Protein Bead Array for the Detection of HIV-1 Antibodies from Fresh Plasma and Dried-Blood-Spot Specimens, Sylvie Faucher,^{1*} Alexandre Martel,¹ Alice Sherring,¹ Tao Ding,¹ Laurie Malloch,² John E. Kim,² Michèle Bergeron,¹ Paul Sandstrom,³ and Francis F. Mandy¹ (¹ National HIV Immunology Laboratory, National HIV and Retrovirology Laboratories, Centre for Infectious Disease Prevention and Control, Health Canada, Ottawa, ON, Canada; ² National Laboratory for HIV Reference Services, National HIV and Retrovirology Laboratories, Centre for Infectious Disease Prevention and Control, Health Canada, Ottawa, ON, Canada; ³ National HIV and Retrovirology Laboratories, Centre for Infectious Disease Prevention and Control, Health Canada, Ottawa, ON, Canada; * address correspondence to this author at: National HIV Immunology Laboratory, National HIV and Retrovirology Laboratories, Centre for Infectious Disease Prevention and Control, Bldg. 6, P.L. 0603B1, Tunney's Pasture, Ottawa, Ontario, Canada K1A 0L2; fax 613-946-3237, e-mail sylvie_faucher@hc-sc.gc.ca)

Simultaneous multianalyte immunoassays offer advantages, including reduction of technical operations, execution time, and overall costs. The suspension array technology (SAT), which uses microfluorospheres with flow cytometry, is a multianalyte immunoassay that requires small specimen volumes, such as those obtained by less-invasive blood sampling approaches such as heel or finger sticks, as well as those obtained in pediatric specimens. The dried-blood-spot (DBS) technology has become an important screening tool for clinical and epidemiologic testing (1–3). It is particularly convenient in rural, resource-limited settings where trained personnel and adequate facilities for blood collection, processing, transport, and storage may not be available. This approach, however, provides limited specimen volume (~5–6 μ L of serum/6-mm punch) (2). Fortunately, SAT is well suited to support such a format (4, 5).

The range of applications for SAT includes antibody, oligonucleotide, peptide, and protein bead arrays (PBAs) (4, 6–17). PBAs have been used for the simultaneous detection of serum antibodies to infectious agents for systemic candidiasis (18), herpes viruses (19), measles, mumps (20), and HIV (21, 22). The successful detection of

HIV-1 serum antibodies by a four-protein bead array was first reported by Scillian et al. (21), who used beads of various sizes and proteins noncovalently coupled to beads. Reagents are now available that permit covalent coupling of proteins to beads and simultaneous analysis of up to 100 color-coded bead sets in a high-throughput, 96-well plate format. In addition, a new generation of flow cytometers compatible with SAT has recently been introduced, e.g., the Luminex-100 System (Luminex Corp.) and the FACSArray (BD Biosciences). We have developed and evaluated a HIV-1 PBA for the rapid detection of antibodies to seven viral proteins from fresh plasma and DBS specimens.

Seven recombinant HIV-1 proteins from the gag, pol, and envelope regions and a nonviral antigen control [bovine serum albumin (BSA)], all from commercial sources (see the Appendix in the Data Supplement that accompanies the online version of this Technical Brief at <http://www.clinchem.org/content/vol50/issue7/>), were covalently coupled to individual sets of color-coded microfluorospheres (Luminex Corp.). Diluted plasma and DBS specimens (in 50 μ L) were incubated for 1 h with the mixture of microfluorospheres, to which seven or eight proteins had been coupled, in a filter-bottomed 96-well plate (Nunc). Bound antibodies were detected with biotinylated anti-human IgG conjugate (BD Biosciences; 1 h) and streptavidin-phycoerythrin conjugate (Sigma; 15 min). The beads were resuspended in 20 mL/L paraformaldehyde in phosphate-buffered saline and analyzed directly from the filter-bottomed, 96-well plate with a Luminex-100 System equipped with the Luminex Data Collector, Ver. 1.7, software. The median fluorescence intensity was used as the read-out. The HIV-1 PBA test was completed within 3 h. The interassay variation (CV) for antibody measurements for all seven proteins was 3–10% as evaluated by measuring a HIV-1 antibody plasma pool in duplicate in 13 consecutive assays.

We tested the HIV-1 PBA against two commercial enzyme immunoassays (EIAs), the GSTM rLAV HIV-1 EIA (Bio-Rad) and the DETECT HIV EIA (Adaltis), and a Western blot (WB) assay (Calypse Biomedical Corp.), using fresh plasma from six HIV-1-infected individuals, obtained from the National Laboratory for HIV Reference Services (Health Canada). The results obtained with the PBA and the two commercial EIAs were similar (Table 1). Samples analyzed with the PBA were interpreted as reactive only when antibodies to proteins derived from at least two of the three gene regions were detected. In fresh plasma samples, the PBA detected antibodies to individual HIV-1 proteins more frequently than the WB (see Table 1 in the online Data Supplement). Antibodies to every viral protein were detected (endpoint titer ≥ 20), with the exception of p17 in sample 2. In contrast, the WB failed to detect antibodies to one or more proteins in one-half of the samples. Five of the six samples met the WB criteria for positive interpretation, i.e., they showed reactivity to at least two of the following three proteins: p24, gp41, and gp120/160. One sample (sample 3) showed antibody reactivity to p24 only (indeterminate reactivity)

Table 1. Comparison of HIV-1 antibody detection by the HIV-1 PBA, EIAs, and WB.

Specimens	EIA		WB	PBA
	GSrLAV (Bio-Rad)	Detect-HIV (Adaltis)		
Fresh HIV+ plasma ^a (n = 6)	6	6	5 (1 IND) ^b	6
DBS ^c				
HIV- (n = 5)	0	NT	NT	0
HIV+ (n = 10)	10	NT	10	10

^a Fresh plasma samples were tested with the HIV-1 PBA as serial 10-fold dilutions (1:20–1:200 000; see the Appendix in the online Data Supplement). Samples were considered positive when antibody reactivity to proteins derived from at least two of the three different gene regions, gag, pol, or envelope, were detected (see Table 1 in the online Data Supplement).

^b IND, indeterminate; NT, not tested.

^c Paper disks (6 mm) from DBS specimens were incubated overnight in elution buffer (see Table 1 in the online Data Supplement) before testing with the EIA and the PBA. For WB testing, the paper disk was incubated with the WB strip overnight in blotting buffer. Results of the HIV-1 PBA are expressed as the ratio of the median fluorescence intensity for the test samples to the cutoff, with values ≥ 1 considered reactive (see Table 1 in the online Data Supplement).

when tested with WB, whereas it was reactive for all seven HIV-1 proteins in the PBA (Table 1 in the online Data Supplement). Consistent with early seroconversion, blood samples taken from this patient at a later time were reactive when tested by WB (data not shown). The apparent increased sensitivity of the HIV-1 PBA for the detection of envelope antibodies would constitute a definite advantage over the WB for weakly reactive plasma samples. Antibody reactivity to BSA was detected in one of six fresh plasma samples (endpoint titer, 189; see Table 1 in the online Data Supplement). However, in any given individual, the antibody profile to BSA appeared to be independent of HIV antibody profile, as illustrated in the longitudinal testing of seroconverters with the HIV-1 PBA (Fig. 1A).

We evaluated the PBA for the detection of HIV-1 antibodies eluted from 15 DBS specimens (National Laboratory for HIV Reference Services, Health Canada). The HIV-1 PBA performed as well as the EIA and the WB for the determination of HIV-1-reactive DBS specimens obtained from HIV-1-infected individuals (Table 1). Five samples determined to be nonreactive with the EIA were also interpreted as nonreactive with the PBA. Antibodies to HIV-1 p24 were detected in two of the samples nonreactive with the PBA, but these samples were not tested with the WB (see Table 1 in the online Data Supplement). Antibodies to p17 and p55 proteins were detected more frequently with the PBA (p55, 10 of 10; p17, 8 of 10) than with the WB (p55, 7 of 10; p17, 7 of 10), but gp41 and gp120 antibodies were not detected more often (Table 1 in the online Data Supplement). In four DBS specimens, gp41 and/or gp120 antibodies were not detected with the PBA, whereas they were detected as weak bands on the WB. Overnight incubation of the paper disk with the WB strip might partly explain the discrepancy observed for the detection of envelope protein antibodies with the

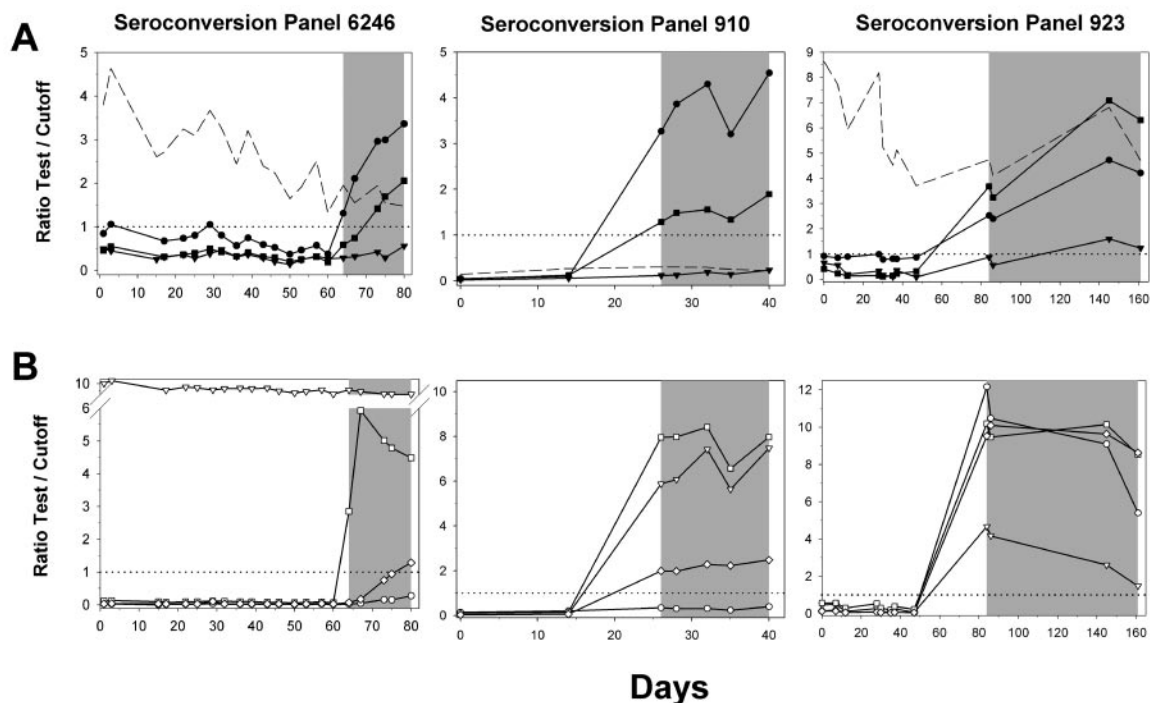


Fig. 1. Antibody reactivity to HIV-1 proteins and BSA from three HIV-1 seroconversion panels.

Plasma specimens were tested with the HIV-1 PBA (1:50 dilution; see the Appendix in the online Data Supplement). Results are shown as the ratio of the median fluorescence intensity of the test samples to the cutoff with values ≥ 1 considered positive. The cutoff for positivity is indicated by the dotted line in each panel. The shaded areas denote specimens reactive with two commercial EIAs (GS rLAV HIV-1 EIA and the DETECT HIV EIA). (A), profiles of antibody reactivity to HIV-1 envelope proteins and BSA from three HIV-1 seroconversion panels. ●, gp41 antibodies; ■, gp120 antibodies; ▼, gp160 antibodies. The dashed line corresponds to antibodies to BSA. (B), profiles of antibody reactivity to HIV-1 gag/pol proteins from three HIV-1 seroconversion panels. ○, p17 antibodies; ▽, p24 antibodies; □, p55 antibodies; ◇, p66 antibodies. The three seroconversion panels tested were BCP6246 (a 21-specimen panel collected serially from a single plasma donor over a period of 82 days), PRB910 (a 7-specimen panel collected serially from a single plasma donor over a period of 40 days), and PRB923 (a 13-specimen panel collected serially from a single plasma donor over a period of 161 days).

HIV-1 PBA. A shorter incubation period (1 h) was chosen for the PBA to evaluate its potential as a rapid test. Incubation of the microfluorospheres with the paper disk as the elution takes place or prolonging the incubation period with the eluate would likely improve the detection of the envelope antibodies.

The HIV-1 PBA performed well for the early detection of HIV-1 antibodies in seroconverters (submitted for publication). In the present study, the PBA was compared with two EIAs for the early detection of HIV-1 antibodies in three seroconverters. Fig. 1 shows the HIV-1 antibody profiles (1:50 dilution) of the ratio of the median fluorescence intensity for the test samples to the cutoff for all seven viral proteins. The first reactive samples detected with the PBA in all three seroconverters were identical to those detected with the two EIAs (Fig. 1, shaded areas). Antibodies to gp41 (Fig. 1A) and p55 (Fig. 1B) were often the first to be detected. As reported by others (23, 24), nonspecific antibody reactivity to the capsid protein p24 was observed in one donor (BCP6246).

The PBA described here used only seven HIV-1 proteins. The inclusion of other HIV proteins from HIV-1/2 and various HIV-1 subtypes would be essential for widespread serology screening. HIV-specific peptides, as opposed to full-length recombinant proteins, could also potentially be used to resolve antibody cross-reactivity to

the products of the GAG gene, which is encountered occasionally in uninfected individuals.

Effort has been invested to improve the algorithm for confirming HIV-1/2 infection (25–28). The currently used WB can produce false-negative results in cases of recent infection and false-positive results as a result of contaminating cellular antigens or cross-reactive antibodies (24, 29–31). Alternative immunoblot assays use recombinant HIV-1/2 proteins and peptides deposited as bands on a nitrocellulose membrane (25, 27, 28). Reactivity is estimated qualitatively from the intensities of the individual bands. This type of assay typically requires 20 μ L of specimen per strip and 6–7 h for analysis. The use of recombinant proteins and peptides, free of cellular contaminants, has been shown to reduce the number of indeterminate results seen with WB. The HIV-1 PBA parallels the principle of these immunoblots in that it uses recombinant proteins or peptides. It potentially offers the same advantage of the recombinant immunoblot regarding the reduction of indeterminate results. However, the HIV PBA offers additional advantages compared with WB and recombinant immunoblots, such as consistency in the composition and concentration of viral proteins, numeric read-outs, smaller specimen volume requirements ($\leq 5 \mu$ L), and less hands-on time (completion time ~ 3 h).

The HIV PBA offers the high throughput of EIA com-

bined with the individual protein testing capacity of the WB. The assay can be tailored to the requirements of screening or confirmatory testing as well as to the needs of specific vaccine studies. Vaccine trials could benefit greatly from a PBA that differentiates protected from infected individuals. Because large-scale vaccine studies are likely to be conducted in settings where conditions may not permit easy handling of fresh blood specimens, the DBS technology constitutes an attractive alternative for specimen collection and the PBA appears to be well suited for this purpose. The HIV PBA offers substantial advantages over conventional immunoassays, but standardization, validation, and performance testing remain to be done before the assay can be used for clinical testing.

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Serum Copper Is Decreased in Premature Newborns and Increased in Newborns with Hemolytic Jaundice, Kleopatra H. Schulpis,¹ Theodoros Karakontantakis,² Stavroula Gavrili,³ Christos Costalos,³ Eleftheria Roma,⁴ and Ioannis Papassotiriou^{2*} (¹Institute of Child Health and ²Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, Athens, Greece; ³Neonatology Department, "Alexandra" Maternity Hospital, Athens, Greece; ⁴First Department of Pediatrics, Athens University, Athens, Greece; *address correspondence to this author at: Department of Clinical Biochemistry, "Aghia Sophia" Children's Hospital, 115 27 Athens, Greece; fax 30-210-7467171, e-mail biochem@paidon-agiasofia.gr or jpapasotiriou@ath.forthnet.gr)

Copper is an active component of several enzyme systems, including cytochrome *c* oxidase and superoxide dismutase (1), and is essential for the prevention of anemia and leucopenia. It is important for the maturation of collagen and the maintenance of skeletal and vascular integrity because of its involvement in the lysyl oxidase enzyme system (2). Deficiency of copper is associated with an increased incidence of infection, as seen in patients with Menke kinky-hair disease, an inherited disease with low copper concentrations (3). The mechanism by which high copper concentrations interact with genetic

APPENDIX 1

HIV-1 Protein Bead Array

Full length HIV-1 p24 capsid protein, p66 reverse transcriptase protein, p55 gag protein, gp160_{LAV} envelope protein (Protein Sciences Corp., CT, USA) and gp120_{IIIB} envelope protein (donation from ImmunoDiagnostics, EU Programme EVA/MRC Centralised Facility for AIDS Reagents, UK, contract BMH4 97/2515) were expressed in the insect cell/baculovirus expression system. P17_{IIIB} matrix protein (donation from Dr S Adams, EU Programme EVA/MRC Centralised Facility for AIDS Reagents, UK, contract BMH4 97/2515) was expressed as a glutathione s-transferase fusion protein and cleaved with thrombin before purification. P17_{IIIB} and HIV-1 gp41 (a.a. 1-241 minus a.a. 6-30 and 169-193) (Trinity Biotech, CA, USA) were expressed in the E. coli expression system. Recombinant proteins were covalently coupled to individual set of carboxylated microfluorospheres (Luminex Corp., TX, USA) through carbodiimide coupling using EDC. The microfluorospheres ($2-4 \times 10^6$) were resuspended in MES 0.1M pH 6.1 (2-[N-Morpholino]ethanesulfonic acid, Sigma-Aldrich Canada, Ltd., Canada) and activated for 20 min. at room temperature with 0.5 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, Pierce Biotechnologies, Inc., IL, USA) and 0.5 mg of sulfo-NHS (N-Hydroxysulfosuccinimide, Pierce Biotechnologies) in a total volume of 100 μ L. After washing with MES 0.1 M pH 7.2, the microfluorospheres were coupled with 0.0625-0.375 nmoles protein/ 10^6 microfluorospheres for 2 hours at room temperature in a final volume of 100-200 μ L MES 0.1 M pH 7.2. The microfluorospheres were washed twice with PBS (Canadian Life Technologies Inc., ON, Canada) containing 0.05% Tween-20 (Sigma-Aldrich) and 0.05% NaN₃ and resuspended at 10^7 /mL in PBS containing 1% BSA (Sigma-Aldrich) and 0.05% NaN₃. Protein coupled microfluorospheres were kept at 4° C in the dark. On the day of the assay, the 7 protein coupled- microfluorospheres mix was prepared and washed in PBS containing 0.05% Tween-20 and 0.05% NaN₃. For fresh

plasma samples, 50 μ L of serial tenfold dilutions (1: 20 - 1: 200000) of each sample were prepared in PBS containing 1% BSA and 0.05% NaN_3 . For DBS specimens, the paper disks (6 mm punch) were incubated overnight at 2-8° C in 150 μ L Dilsim II buffer (BioMérieux, USA) and the eluate was further diluted 1/6 in 50 μ L of PBS containing 1% BSA and 0.05% NaN_3 (final dilution of $\sim$ 1/150). The diluted samples were incubated for 1 hour at room temperature with the protein coupled microfluorospheres mix (2500 microfluorospheres for each protein) in a filter-bottomed 96-well plate (Nunc, VWR International, Inc., ON, Canada). The microfluorospheres were washed in PBS containing 0.05% Tween-20 and 0.05% NaN_3 and biotinylated anti-human IgG conjugate (BD Biosciences, CA, USA) was added to the well and incubated for 1 hour at room temperature. The microfluorospheres were washed in PBS containing 0.05% Tween-20 and 0.05% NaN_3 and bound antibodies were revealed with streptavidin-phycoerythrin conjugate (Sigma-Aldrich) for 15 minutes. The microfluorospheres were washed in PBS containing 0.05% Tween-20 and 0.05% NaN_3 and resuspended in 100 μ L of 2% paraformaldehyde (Polysciences, Inc., PA, USA) in PBS. The microfluorospheres were analysed on a Luminex-100 System (Luminex) equipped with the Luminex Data Collector version 1.7 software using the median fluorescence intensity (MFI) as the read-out.

Supplemental Data Table 1 . Antibody reactivity to individual HIV proteins using the HIV-1 PBA and WB.

SPECIMENS	ENVELOPE						GAG						POL		BSA	
	gp41		gp120		gp160		p17		p24		p55		p66		WB	PBA
	WB	PBA	WB	PBA	WB	PBA	WB	PBA	WB	PBA	WB	PBA	WB	PBA		
Fresh plasma *																
1	wk	11,757	wk	6,898	st	100,054	wk	261	st	>200,000	wk	>200,000	st	>200,000	NT	<20
2	tr	66	tr	77	wk	191	tr	<20	wk	514	-	728	st	1,357	NT	<20
3	-	264	-	120	tr	86	-	28	st	3,028	-	3,317	-	98	NT	189
4	wk	5,749	wk	1,457	st	12,224	wk	2,737	st	20,178	wk	29,307	st	>200,000	NT	<20
5	wk	3,864	wk	1,650	st	14,175	wk	1,192	st	>200,000	wk	>200,000	st	16,695	NT	<20
6	wk	1,844	wk	366	st	6,323	-	22	wk	1,274	tr	1,525	wk	715	NT	<20
DBS **																
1	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	NT
2	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	NT
3	NT	-	NT	-	NT	-	NT	-	NT	2.8	NT	-	NT	-	NT	NT
4	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	-	NT	NT
5	NT	-	NT	-	NT	-	NT	-	NT	5.9	NT	-	NT	-	NT	NT
6	wk	-	wk	1.0	wk	2.5	wk	1.0	wk	12.8	tr	19.6	wk	3.1	NT	NT
7	wk	2.0	wk	9.5	st	11.9	wk	5.9	st	25.5	wk	26.3	st	22.4	NT	NT
8	wk	3.8	wk	13.1	st	14.8	wk	4.0	st	21.5	wk	22.7	st	20.1	NT	NT
9	wk	3.6	st	9.1	st	14.9	tr	3.5	wk	13.9	wk	24.0	st	22.8	NT	NT
10	st	3.5	st	14.3	st	17.9	st	16.7	st	22.9	st	25.2	st	20.6	NT	NT
11	wk	1.5	wk	4.3	st	8.4	wk	6.2	st	23.1	st	24.2	st	11.6	NT	NT
12	wk	1.4	wk	2.3	st	8.1	-	-	wk	8.7	tr	15.1	st	12.4	NT	NT
13	wk	-	wk	-	st	2.3	wk	4.3	wk	18.9	wk	22.4	st	8.5	NT	NT
14	tr	-	wk	-	wk	1.6	tr	-	wk	17.3	wk	21.1	wk	7.2	NT	NT
15	wk	-	wk	1.1	st	3.0	wk	1.1	wk	11.2	tr	16.9	wk	2.9	NT	NT

* Fresh plasma were tested with the HIV-1 PBA using serial tenfold dilutions (1/20 – 1/200,000) (see Appendix 1). Results shown represent antibody end point titers. End point titers were determined from the linear portion of log-log plots of MFI v reciprocal dilution of plasma using as the endpoint, cutoff values defined for each protein coupled bead set. Cutoff values were defined as the mean MFI of a plasma pool from 12 HIV-1 negative individuals plus 1/15 the value of a positive control. The following cutoff values were used: BSA, 181; p17, 332; p24, 430; p55, 440; p66, 593; gp41, 200; gp120, 224; gp160, 487. (The mean of the MFI for the pooled HIV-1 negative plasma range from 32 to 111 with a CV% of less than 5). Samples were considered positive when antibody reactivity to proteins derived from at least two of the three different gene regions, gag, pol, or envelope was detected.

** Paper disks (6 mm) from DBS specimens were incubated overnight in 200 µL of rLAV diluent before testing with the EIA at a dilution of 1/2.5 (final dilution of ~1/83). For WB testing, the paper disk was incubated with the WB strip overnight in 2 mL of blotting buffer (final dilution of ~1/333). For the HIV-1 PBA, the paper disks were incubated overnight in 150 µL Dilsim II buffer (BioMérieux, USA) and tested at 1/6 dilution (final dilution of ~1/150). Results of the HIV-1 PBA are expressed as test/cutoff with values ≥ 1 considered reactive (see above for cutoff values).

NT, not tested; IND, indeterminate; -, non reactive; +, reactive; tr, trace; wk, weak; st, strong.