

Ultrasensitive Human Immunodeficiency Virus Type 1 p24 Antigen Assay Modified for Use on Dried Whole-Blood Spots as a Reliable, Affordable Test for Infant Diagnosis

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The ultrasensitive human immunodeficiency virus (HIV) p24 antigen assay was modified for use on pediatric dried whole-blood spots on Whatman no. 1 filter paper. The modified assay was found to be reliable and accurate, making it an affordable tool for pediatric HIV diagnosis in developing countries.

In countries with limited resources there is an urgent need for an affordable, reliable diagnostic test for human immunodeficiency virus type 1 (HIV-1) infection in infants in the first months of life. Nucleic acid tests (NAT), the “gold standards” against which other diagnostic tests are measured, are expensive and technically challenging (3, 7, 11), making their use in developing countries problematic.

The HIV-1 p24 antigen (Ag) enzyme-linked immunosorbent assay (ELISA) is a commercially available kit performed with equipment found in most laboratories. The improved ultrasensitive p24 Ag assay performed on plasma, used for pediatric HIV diagnosis, has sensitivities and specificities ranging from 97 to 100%, comparable to HIV DNA and RNA PCR (8, 12, 14, 18). There is, additionally, evidence that the assay is more sensitive to diversity in viral genome than HIV PCR (3, 7, 8, 18).

The advantages of blood collection as dried whole-blood spots (DBS) on filter paper are well documented (9, 10). Importantly, DBS could provide increased access to HIV testing for children in resource-poor countries.

The U.S. Food and Drug Administration-approved Schleicher and Schuell (S&S) Grade 903 paper (10) is currently not widely available in Southern Africa. Whatman no. 1 filter paper, however, can be found in most laboratories on the continent.

The present study examines the performance in terms of precision, reliability, and accuracy of the p24 Ag ELISA on DBS relative to nucleic acid testing for HIV infection in infants. The reliability of Whatman no. 1 filter paper for the collection of whole-blood samples is also investigated.

DBS were from two sources. Ninety-three were from infants born to HIV-infected women attending clinics for routine check-ups at the Coronation Women and Children’s Hospital and the Chris Hani Baragwanath Hospital, Johannesburg, South Africa. The HIV status of these infants was determined

by qualitative HIV DNA PCR (Amplicor HIV-1 DNA Version 1.5 Assay; Roche Molecular Systems, Inc., Branchburg, NJ). To adequately test the sensitivity of the assay, an additional 48 DBS were made from pediatric anticoagulated blood specimens with a known HIV DNA or RNA PCR (Roche Amplicor HIV-1 Monitor Assay, version 1.5) or NASBA (nucleic acid sequence-based amplification) HIV-1 RNA (Nuclisens; bioMérieux, Boxtel, The Netherlands) result. Of the 48, 34 had an HIV DNA PCR result, and 14 had a known viral load. The age range of the sample population was 34 days to 12 years. (University of the Witwatersrand Human Ethics clearance number M00-01-07).

Four separate drops of blood of unknown volume were collected on Whatman no. 1 filter paper, air dried at ambient temperature for a minimum of 3 h. To simulate local collection conditions, filter papers were stored in individual plastic zip-lock bags at room temperature without a desiccant sachet. Blood spots that contained insufficient blood, or appeared “layered,” crusty, clotted, or wetted were discarded (13).

In addition to the reagents supplied with the Ultrasensitive p24 Ag ELISA kit (Perkin-Elmer Life Sciences, Boston, Mass.), a virus-lysing (VL) buffer (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) was used (17).

The stock standard was serially diluted with sterile phosphate-buffered saline (PBS; Roche Diagnostics, Mannheim, Germany) plus 1% bovine serum albumin (BSA), in a range from 12,500 to 3.052 pg/ml.

Washed erythrocytes were prepared from EDTA-anticoagulated whole blood from a consenting HIV-negative individual. Whole blood was centrifuged (1,500 × g, 10 min), plasma and buffy coats were removed, and red blood cells (RBC) were washed three times with sterile PBS. Equal volumes of serially diluted standards and washed erythrocytes were mixed to give standards ranging from 6,250 to 1.526 pg/ml. Aliquots were dropped onto Whatman no. 1 filter paper and allowed to air dry completely (3 h, room temperature). The blank DBS consisted of RBC mixed with an equal volume of sterile PBS. A negative control, prepared from equal volumes of washed RBC and the negative control supplied with the kit was used to

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calculate the thresholds for positivity and negativity. A high and a low in-house control (2,000 and 50 pg/ml, respectively) were prepared in a similar fashion from the stock standard.

Preliminary studies to determine the optimal eluting efficiency of different buffers on DBS showed the best recovery (91.3% of the concentration of p24 in the high DBS control) was with the Triton X-100 supplied with the kit in conjunction with the VL buffer (data not shown). This combination was used for all subsequent experiments.

With some modifications, the assay was performed according to the manufacturer's instructions and as described elsewhere (2). Using an ordinary paper punch duplicate 6-mm disks were punched from blanks, standards, controls, and samples DBS, and these substituted for diluted liquid blanks, standards, controls, and plasma samples in the assay. The punch was swabbed with 70% ethanol after each use to prevent cross-contamination.

Briefly, disks were incubated with 25 μ l of the VL buffer (10 min, room temperature) (16) and then with 275 μ l of the supplied Triton X-100 (0.5%, overnight, 4°C). Tubes were heated to 100°C for 5 min and allowed to cool naturally to room temperature. Aliquots (250 μ l) of the boiled eluates were transferred to a 96-well plate coated with HIV-1 p24-specific monoclonal antibody and incubated for 60 min at 37°C. Following the protocol supplied by the manufacturer, bound p24 Ag was detected, the signal was amplified and detected, and the reaction was stopped with 4 N sulfuric acid. The plate was washed (10 cycles, 0.05% Tween 20) after each step. Absorbances were read at 490 nm against a reference wavelength of 630 nm using an EL_x 808 Bio-Tek microplate reader with onboard software. Quantification of p24 Ag was by endpoint evaluation from a best-fit four-parameter curve constructed from DBS standards. Four negative control DBS were included on each plate to determine the cutoff baseline for positivity (greater than the average absorption plus five standard deviations), and negativity (less than the average absorption plus three standard deviations) (15).

Assay performance was tested by evaluating assay precision and reliability (intra- and interassay coefficients of variance [CV]), and accuracy in terms of sensitivity and specificity. The effect of time on p24 concentration and the relationship between log₁₀-transformed DBS p24 Ag values (pg/ml) and log₁₀ plasma RNA (copies/ml) were also examined on the small subsample of DBS that had a quantitative plasma viral load value.

The standard curve constructed from a range of DBS standards from 1.526 to 6,250 pg/ml consistently showed a good fit with values for R^2 of never less than 0.997. This compares favorably with the fit for the curve constructed from liquid standards (Fig. 1). The presence of any agents likely to elevate DBS absorbances (e.g., heme), found by others attempting to measure DBS p24 Ag using liquid blanks and standards (4), was negated by using DBS blank and standards with incorporated RBC.

Intra-assay variation for 10 replicates of the low- and high-DBS control was 4.8% (range, 45.4 to 53.3 pg/ml for the 50-pg/ml DBS control) and 8.0% (range, 2,064 to 2,636 pg/ml for the 2,000-pg/ml DBS control), respectively. These values rival those reported for the assay performed on plasma (5.3 and 28.4%, respectively, for a low and a high control), showing a

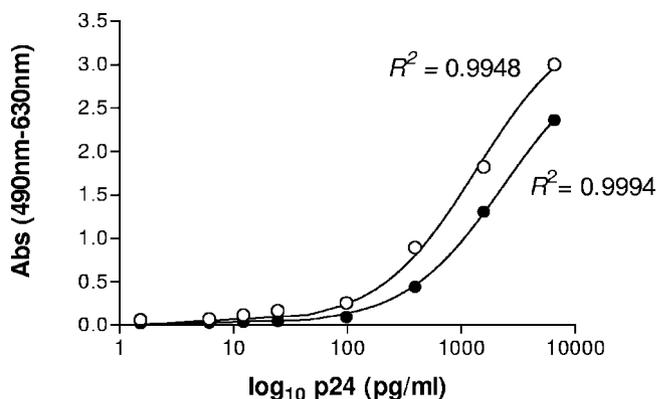


FIG. 1. Comparison between the p24 Ag standard curve constructed from a range of standards diluted in a plasma-like matrix (○) and that constructed from standards eluted from dried blood spots (●). The standard range was 1.526 to 6,250 pg/ml for both conditions. Absorbance values ranged from 0.056 to 3.0 nm for the liquid standards and from 0.018 to 2.362 nm for the DBS standards.

high a degree of precision for the assay when performed on DBS. These results additionally support the argument that the volume of plasma in each 6-mm disk excised from the Whatman DBS is relatively constant as has been shown for DBS on S&S paper (10), thus abolishing the need to apply a known volume of blood to the filter paper.

Interassay CV for the low and high DBS controls ($n = 9$) were 9.0% (range, 43.5 to 60.9 pg/ml for the 50-pg/ml DBS control) and 20.4% (range, 1,707 to 2,571 pg/ml for the 2,000-pg/ml DBS control), respectively. The reported CVs for a low- and a high-plasma sample were 6.4 and 9.9%. As discussed later, the higher DBS values may be due to deterioration of the p24 Ag protein in DBS over time (the assays were performed over 3 months) or the filter paper type or storage conditions used.

Of the 141 DBS assayed for p24 Ag, 83 were from children diagnosed as HIV infected and 58 HIV uninfected on the basis of HIV-1 DNA or RNA PCR or NASBA HIV-1 RNA tests. The results for the p24 Ag ELISA on DBS were 81 positive, 59 negative, and one result regarded as "indeterminate" by the criteria suggested by Schupbach et al. (15). With no false-positive values, the diagnostic specificity for the p24 Ag assay was 100%. Since the vast majority of HIV infections in South Africa are by subtype C virus (18, 20), this result supports the finding of others that the assay is sensitive to viral subtype C (3, 4, 5, 8, 17).

A single value that tested positive by PCR was not detected by the p24 Ag ELISA giving the assay a diagnostic sensitivity of 98.8%, which equals that for HIV DNA PCR performed on plasma and DBS (8, 12, 14, 18). The indeterminate result fell in the "gray" area between diagnostically positive and diagnostically negative.

The relationship between log₁₀ DBS p24 Ag values (pg/ml) and log₁₀ plasma RNA (copies/ml) was tested on the small subsample of DBS that had a known HIV viral load. The Pearson correlation coefficient and analysis of variance indicate a significant positive correlation between log₁₀ DBS p24 Ag and log₁₀ plasma RNA (Fig. 2, $r = 0.79$, $P < 0.001$). Although only on a small sample, this compares well with the

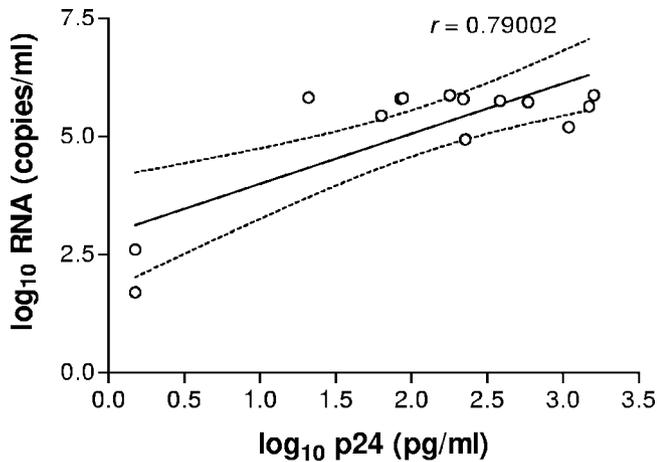


FIG. 2. Correlation between \log_{10} plasma HIV RNA copy number and \log_{10} DBS p24 Ag concentration, respectively, in the small subsample with a known viral load ($n = 14$). The Pearson correlation coefficient of 0.79 indicates a significant positive correlation ($P < 0.001$) between the two measures of HIV infection. The 95% confidence intervals are indicated by the broken lines.

correlation found between \log_{10} plasma p24 Ag and \log_{10} plasma RNA ($r = 0.74$, $P < 0.0001$) (G. Stevens, N. Rekhvashvili, L. E. Scott, and W. Stevens, Abstr. 2nd IAS Conf. HIV Pathog. Treatment, abstr. 475, 2003).

Using the same 14 DBS the effect of time on p24 Ag concentration in DBS in our storage conditions was examined (Table 1). Within 6 weeks of collection, the DBS showed 100% specificity and sensitivity in complete agreement with the HIV RNA results. When re-assayed 6 weeks later, 2 of the 14 DBS remained negative and 2 had slightly higher p24 Ag values. Of the remaining 10 DBS, 4 that had previously tested positive were negative 6 weeks later, and 6 DBS had p24 Ag concentrations substantially lower than when initially measured (mean decrease \pm the standard deviation, 69.85% \pm 33.15%). This observation agrees with the work of Behets et al. (1), who found in Zaire that HIV-1 antibody concentrations in S&S 903 DBS were stable for 6 weeks but declined steadily thereafter.

TABLE 1. Effect of time on the stability of p24 Ag protein in DBS

HIV RNA PCR (copies/ml)	Amt of p24 Ag (pg/ml)		Direction of % change
	First assay within 6 wk of collection	Second assay 6 wk later	
<400	Negative	Negative	No change
567,000	382.8	Negative	-100
<50	Negative	Negative	No change
158,000	1,088.4	368.7	-66.1
750,000	178.5	120.6	-22.8
86,000	225.7	252.9	+12.05
613,000	219.3	Negative	-100
276,000	63.0	54.7	-13.2
660,000	20.9	21.6	+3.3
532,000	585.6	125.9	-78.5
627,000	84.4	Negative	-100
429,000	1,482.1	842.2	-43.1
750,000	1,588.3	400.7	-74.8
648,000	87.4	Negative	-100

Possible causes for the protein decrease are currently being investigated.

In a recent study undertaken in the Democratic Republic of the Congo, de Baets and coworkers found the ultrasensitive p24 Ag assay to be 100% sensitive and specific using a known volume of plasma dried on S&S 903 paper (5). Our results, using 6-mm disks punched from an unknown volume of whole blood dried on Whatman no. 1 filter paper show that blood collection and processing can be further simplified with little loss of sensitivity or specificity, with the proviso that the assay is performed within a 6-week window.

For reasons of expense and technical complexity most vertically exposed babies in developing countries do not have access to an HIV diagnosis until at least 12 months of age, when as few as 15% present for testing (6, 19). We report here a simple, inexpensive method for accurate testing for HIV-1 infection in infants using ordinary, easily obtained equipment.

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REFERENCES

- Behets, F., M. Kashamuka, M. Pappaioanou, T. A. Green, R. W. Ryder, V. Batter, J. R. George, W. H. Hannon, and T. C. Quinn. 1992. Stability of human immunodeficiency virus type 1 antibodies in whole blood dried on filter paper and stored under various tropical conditions in Kinshasa, Zaire. *J. Clin. Microbiol.* **30**:1179-1182.
- Boni, J., O. Opravil, Z. Tomasik, M. Rothen, L. Bisset, P. J. Grob, R. Luthy, and J. Schupbach. 1997. Simple monitoring of antiretroviral therapy with a signal-amplification-boosted HIV-1 p24 antigen assay with heat-denatured plasma. *AIDS* **11**:F47-F52.
- Burgisser, P., P. Vernazza, M. Flepp, J. Boni, Z. Tomasik, U. Hummel, G. Pantaleo, and J. Schupbach. 2000. Performance of five different assays for the quantification of viral load in subjects infected with various subtypes of HIV-1. *J. Acquir. Immun. Defic. Syndr.* **23**:138-144.
- Chung-Chen, L., K. L. Seidel, R. W. Coombs, and L. M. Frenkel. 2005. Detection and quantification of human immunodeficiency virus type 1 p24 antigen in dried whole blood and plasma on filter paper stored under various conditions. *J. Clin. Microbiol.* **43**:3901-3905.
- De Baets, A. J., B. S. Edidi, M. J. Kasali, G. Beelaert, W. Schrooten, A. Lizroth, P. Kolsteren, and K. Franssen. 2005. Pediatric human immunodeficiency virus screening in an African district hospital. *Clin. Diagn. Lab. Immunol.* **12**:86-92.
- Doherty, T., M. Besser, S. Donohue, N. Kamoga, N. Stoops, L. Williamson, and R. Visser. 2003. An evaluation of the prevention of mother-to-child transmission (PMTCT) of HIV initiative in South Africa: lessons and key recommendations. [Online] www.hst.org.za/uploads/files/pmtct_national.pdf.
- Goldschmidt, P. L., A. Devillechabrolle, Z. Ai-Arkoub, and J. T. Aubin. 1998. Comparison of an amplified enzyme-linked immunosorbent assay with procedures based on molecular biology for assessing human immunodeficiency virus type 1 viral load. *Clin. Diagn. Lab. Immunol.* **5**:513-518.
- Lyamura, E., U. Bredberg-Raden, A. Massawe, E. Urassa, G. Kawo, G. Msemo, T. Kazimoto, A. Osborn, K. Karlsson, F. Mhalu, and G. Biberfeld. 1996. Performance of a modified HIV-1 p24 antigen assay for early diagnosis of HIV-1 infection in infants and prediction of mother-to-infant transmission of HIV-1 in Dar es Salaam, Tanzania. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **12**:421-426.
- McDade, T. W., and B. Shell-Duncan. 2002. Whole blood collected on filter paper provides a minimally invasive method for assessing human transferrin receptor level. *J. Nutr.* **132**:3760-3763.
- Mei, J. V., J. R. Alexander, B. W. Adam, and W. H. Hannon. 2001. Use of filter paper for the collection and analysis of human whole blood specimens. *J. Nutr.* **131**:1631S-1636S.
- Michael, N. L., S. A. Herman, S. Kwok, K. Dreyer, J. Wang, C. Christopher, J. P. Spadoro, K. K. Y. Young, V. Polonis, F. E. McCutchan, J. Carr, J. R. Mascola, L. L. Jagodzinski, and M. L. Robb. 1999. Development of calibrated viral load standards for group M subtypes of human immunode-

- iciency virus type 1 and performance of an improved Amplicor HIV-1 Monitor test with isolates of diverse subtypes. *J. Clin. Microbiol.* **37**:2557–2563.
12. **Nadal, D., J. Boni, C. Kind, O. E. Varnier, F. Steiner, Z. Tomasik, and J. Schupbach.** 1999. Prospective evaluation of amplification-boostered ELISA for heat-denatured p24 antigen for diagnosis and monitoring of pediatric human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **180**:1089–1095.
 13. **National Committee of Clinical Laboratory Standards.** 2003. Blood collection on filter paper for newborn screening programs, 4th ed. Approved Standard. NCCLS publication LA4-A4. NCCLS, Villanova, Pa.
 14. **Respass, R. A., A. Cachafeiro, D. Withum, S. A. Fiscus, D. Newman, B. Branson, O. E. Varnier, K. Lewis, and T. J. Dondero.** 2005. Evaluation of an ultrasensitive p24 antigen assay as a potential alternative to human immunodeficiency virus type 1 RNA viral load assay in resource-limited settings. *J. Clin. Microbiol.* **43**:506–508.
 15. **Schupbach, J., J. Boni, Z. Tomasik, J. Jendis, R. Seger, C. Kind, et al.** 1994. Sensitive detection and early prognostic significance of p24 antigen in heat-denatured plasma of human immunodeficiency virus type 1-infected infants. *J. Infect. Dis.* **170**:318–324.
 16. **Schupbach, J., Z. Tomasik, D. Nadal, B. Ledergerber, M. Flepp, M. Opravil, and J. Boni.** 2000. Use of HIV-1 p24 as a sensitive, precise and inexpensive marker for infection, disease progression and treatment failure. *Int. J. Antimicrob. Agents* **16**:441–445.
 17. **Schupbach, J., J. Boni, L. R. Bisset, Z. Tomasik, M. Fischer, H. F. Gunthard, B. Ledergerber, M. Opravil, et al.** 2003. 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.* **33**:292–299.
 18. **Sherman, G. G., G. Stevens, and W. Stevens.** 2004. Affordable diagnosis of human immunodeficiency virus infection in infants by p24 antigen detection. *Pediatr. Infect. Dis. J.* **23**:173–175.
 19. **Sherman, G. G., S. A. Jones, A. H. Coovadia, M. F. Urban, and K. D. Bolton.** 2004. PMTCT from research to reality: results from a routine service. *S. Afr. Med. J.* **94**:289–292.
 20. **Van Harmelen, J. H., E. van der Ryst, A. S. Loubser, D. York, S. Madurai, S. Lyons, D. R. Wood, and C. Williamson.** 1999. A predominantly HIV type 1 subtype C-restricted epidemic in South African urban populations. *AIDS Res. Hum. Retrovir.* **15**:395–398.