Stability of Human Immunodeficiency Virus Serological Markers in Samples Collected as HemaSpot and Whatman 903 Dried Blood Spots

Mark M. Manak,^a Holly R. Hack,^a Ashley L. Shutt,^{a*} Brook A. Danboise,^b Linda L. Jagodzinski,^b Sheila A. Peel^b

^aHenry Jackson Foundation, MHRP-HJF, Silver Spring, Maryland, USA

^bWalter Reed Army Institute of Research, MHRP-WRAIR, Silver Spring, Maryland, USA

ABSTRACT Dried blood spots (DBS) are frequently used in clinical testing for bio-surveillance, infectious disease and confirmatory testing, and clinical trials, particularly for populations in remote areas. The HemaSpot-HF blood collection device (HS) provides an alternative format to the Whatman 903 cards (903) to simplify sample collection and processing. In this study, the performance of the HS was compared to that of the 903 using previously characterized clinical specimens and HIV seroconversion panels known to exhibit markers of early human immunodeficiency virus (HIV) infection. HS and 903 samples were prepared and tested by Bio-Rad GS HIV Combo Ag/Ab enzyme immunoassay (EIA), GS HIV-1/-2 Plus O EIA, GS HIV-1 Western blot, and HIV-1 Geenius assays. Both HS and 903 performed well for up to 6 months at room temperature, but a marked loss of Western blot and low titer antibody signals from early infection samples was observed in samples stored for 180 days at elevated (37 to 45°C) temperatures and high humidity (95%). HemaSpot samples placed in sealed bags with additional desiccant were protected from degradation and showed improved signal recovery relative to that of the 903. HS was easier to use than the 903 and showed higher sensitivity and reproducibility for early infection samples and improved stability.

KEYWORDS dried blood spot, HemaSpot, stability, diagnostics, Whatman 903 cards, human immunodeficiency virus

The ability to easily and efficiently collect, preserve, and transport high-quality biospecimens is critical for the success of clinical screening and biosurveillance programs for infectious pathogen detection, particularly among populations living in remote and/or low-resourced areas. Dried blood spots (DBS) offer a simple and practical alternative to acquire specimens in cases where traditional blood collection methods involving venipuncture by a trained phlebotomist or on-site centrifugation of fresh blood samples may not be feasible (1–4). The collection of DBS by fingerstick is minimally invasive and can be performed by individuals with minimal technical skills. DBS are stable for several weeks at ambient temperature and are considered nonregulated exempt materials that can be easily shipped to laboratories by regular mail without the requirement for cold-chain transportation (5–7).

Dried blood spots have been successfully used for the testing of populations for the prevalence of HIV-1/-2, hepatitis C virus (HCV), hepatitis B virus (HBV), and herpes simplex virus type (HSV), with test results comparable to those from venous blood (8–11). The HIV-1 enzyme immunoassay (EIA) and Western blot (WB) testing of DBS have enabled cost-effective early infant diagnosis (12) and culturally appropriate HIV serosurveillance (1, 13). The surveillance of HIV infection among various high risk groups based on HIV antibody, antigen, and nucleic acid test results of DBS demonstrated a more accurate estimation of HIV prevalence than those based on data from

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Address correspondence to Mark M. Manak, mmanak@hivresearch.org.

* Present address: Ashley L. Shutt, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, Maryland, USA.

prenatal clinics, sexually transmitted infection (STI) clinics, and programs monitoring female sex workers (14, 15). The test results of HIV-1 EIA and HIV-1 WB of matched dried blood spots samples proved them to be a practical and efficient approach for the monitoring of the accuracy of the field performance of HIV rapid tests at peripheral diagnostic centers in Niger and at prenatal clinics in rural Cameroon or the testing of men who have sex with men (MSM) in South Africa (16–18). Dried blood specimens provided a convenient and reliable format for the confirmation of positive HIV rapid tests of Guinean women by Geenius HIV 1/2 in a central lab (19). The testing of DBS with the fourth-generation HIV Ag/Ab Combo assay may enable more sensitive detection of early infections and had been shown to identify more (34/39) HIV early infections than the Determine HIV1/2 rapid test (24/39) (20). With the improvement in technology, DBS are increasingly being used for the clinical monitoring of viral load in HIV-infected individuals on antiviral treatment or in treatment-naïve patients (21, 22) and for drug resistance genotyping analysis, with results similar to those from plasma (23–25).

As DBS devices are more widely employed, the issues attendant to the variability of test results due to the inappropriate handling of specimens, contamination, or inadequate laboratory quality control (26, 27) need to be addressed. A study of infants born to HIV-1-infected women in Equatorial Guinea found discordant results with use of DBS in both serological and molecular assays, with frequent (17/68) false-positive results on viral load testing (28). A DBS HIV surveillance study in India found poor concordance between HIV rapid test and DBS laboratory test results, with many of the initially reactive samples not confirmed on repeat testing (29). DBS and centralized testing revealed higher rates of HIV positivity with the Calypte BED Incidence enzyme immunoassay (EIA) than expected from the local prevalence data (30). Higher unconfirmed positivity rates were also observed when monitoring mother-to-child transmissions by the testing of DBS samples in a central lab than by the use of rapid diagnostic tests (31). In addition to concerns about higher false-positive rates, the issues of analyte sensitivity for the detection of early infection (29) and the stability of analytes such as the HBV surface antigen (HBsAg) and anti-HB core antigen (anti-HBc) upon prolonged storage (32) must be considered when using DBS for clinical applications.

The extensive handling of 903 cards during collection and processing can lead to a variability of results or cross-contamination when specimens are applied too closely together or from accidental splatter or smearing during application. Prior to packaging, 903 DBS samples must be dried for 2 to 18 h in open air (6), which can enable contamination from air and surfaces. The recovery of antibody, antigen, nucleic acid, and other biological analytes from incompletely dried DBS can be quite variable, particularly for markers near the clinical endpoint of detection (6, 33, 34). Additional opportunities for contamination can also occur during the laboratory processing steps, including the excision of DBS by filter punch. In contrast, the HemaSpot-HF blood collection device (SpotOn Sciences, Inc., Austin, TX) is a self-contained unit consisting of a precut segmented 8-wedge HemaForm (HF) membrane positioned in a crush-resistant, moisture-resistant, hard plastic cartridge that can be decontaminated as general biohazard waste and disposed of in the regular trash with no additional safety precautions. The device is simpler to use, less prone to contamination from other samples, and does not require extended drying times. A desiccant within the cartridge ensures efficient drying of the sample, with no refrigeration or cold-chain requirement. Individual wedges can be readily removed by a slight twisting motion with forceps for simplified processing in the laboratory. The HemaSpot is CE marked and is registered as class I with the FDA. In this study, we compared the performance of the HemaSpot-HF blood collection device to that of the Whatman 903 card for the testing of HIV-positive specimens and seroconversion panels containing early HIV infection panel members.

MATERIALS AND METHODS

The specimens used for the testing of DBS were prepared from previously tested HIV-1-positive or -negative serum samples submitted for clinical testing and were supplemented with well-characterized plasma specimens from the PRB204 panel that included weakly reactive HIV EIA

FIG 1 (A) HemaSpot with two wedges torn off. (B) 903 cards with 80 μ l of blood before and after a 13.0-mm (1/2 in.) disc was cut out or 20 μ l before and after a 6.5-mm (1/4 in.) disc was cut out.

members or from PRB910 and PRB947 seroconversion panels containing early HIV-1 infection members (SeraCare, Inc., Milford, MA). Serum or plasma specimens were mixed with equal volumes of whole blood (O negative) from uninfected individuals (Biological Specialty Corporation, Colmar, PA) prior to spotting on the filter membranes.

The samples were applied as 20 μ l, 80 μ l, or one drop to the centers of the spots on Whatman 903 protein saver cards (903) (Whatman 10534612). The cards were placed into a card drying rack, dried overnight in a biological safety cabinet, and placed into Ziploc sandwich bags with 2 to 3 desiccant packs (Fisher 09923360). A duplicate set of samples was prepared on the HemaSpot-HF blood collection device (SpotOn Sciences, Inc., Austin, TX) by applying two drops, or 100 μ l, of blood to the middle of the central disk and allowing the sample to absorb for at least 5 min before the device was snapped shut. The samples were stored at room temperature for 2 to 5 days prior to testing. During preliminary testing, samples from DBS and HemaSpots (HS) were eluted in phosphate-buffered saline-Tween (PBS-T) buffer and were found to work well in the Bio-Rad 1/2/O EIA (1/2/O), but a high background was observed in the Bio-Rad GS HIV Combo Ag/Ab EIA (Combo) test (Bio-Rad, Redmond, WA). The thermo/humidity stability study, in which samples were incubated for up to 180 days at room temperature (20 to 23°C) and ambient humidity (<40%), or in a 37°C or 45°C incubator at high (95%) humidity, was therefore performed using the 1/2/O assay. A duplicate set of spots (in both DBS and HS) were prepared for the Geenius testing and for each time point in the stability study. Once removed from their original bag or cartridge, the samples were tested that same day and not returned to storage. Subsequently, an alternative elution procedure using Western blot buffer was obtained from the CDC (35), which worked well for the Ag/Ab Combo assay, enabling us to complete the performance evaluation of HS/903 DBS for sensitivity, specificity, and reproducibility.

For Bio-Rad HIV-1 Western blot testing, a 6.4-mm (1/4 in.) disc was cut from a 903 card or two HemaSpot wedges were removed by a slight twisting motion with clean forceps, placed directly into the Western blot sample tray, and processed as per the manufacturer's instructions for the assay. For the stability studies, samples from 12.9-mm (1/2 in.) discs cut from the 903 cards or the remainder of the HS (6 wedges plus the center) (Fig. 1) were eluted overnight into 300 μ l of PBS-T at 2 to 8°C, and 75 μ l of eluted material was tested by HIV-1/2/O. For HIV Ag/Ab Combo testing, samples from two HS wedges or a 1/4-in. disc cut from 903 were eluted into 150 μ l Western blot specimen diluent/wash buffer (Bio-Rad; catalog no. 32574) at room temperature for 30 to 60 min, and 75 μ l of eluted material was tested in the Combo, or 40 μ l in the Bio-Rad HIV-1/2 Geenius test. The Ag/Ab Combo cutoff value was set as the mean absorbance for the cutoff calibrators: $(CC\bar{x}) + 0.15$ (35).

Ethical considerations. The results reported represent an analysis of a quality assurance activity on clinical discard samples or commercial panel materials within an existing clinical regulated activity. The WRAIR Human Subjects Protection Branch has determined this study (WRAIR 2188) involves the analysis of anonymized samples with no link to individual patients. As such, the study does not meet the definition of research involving human subjects, and no institutional review board (IRB) review was required.

RESULTS

The recovery of the HIV Ag/Ab Combo EIA signal from HS and 903 DBS was evaluated on serial dilutions of an HIV-reactive clinical sample diluted in normal human blood up to a 1:10,000 dilution (Fig. 2). Values for signal-to-cutoff ratios (S/CO) greater than 1.0 are considered reactive. The original plasma sample yielded the highest S/CO signal and was reactive at up to a 1:1,000 dilution, while the HS and 903 samples were reactive at 1:100 dilutions. Samples which yielded S/CO values in the linear range (3 to 12) in third- or fourth-generation EIAs yielded consistently higher S/CO values (3.05 ± 0.88) when eluted from two HS wedges than from the 1/4-in. discs cut from 903 cards.

The reproducibility of HIV Ag/Ab Combo signals from 903 and HS DBS was evaluated by the replicate testing of spots prepared from the Bio-Rad Virotrol-2 low-copy antibody control. Spots prepared from a 1:1 dilution of Virotrol-2 in negative whole blood were tested in seven replicates per day over three days (21 replicates total). The results

FIG 2 Recovery of HIV-1 Ag/Ab Combo signals from serial dilutions of a reactive plasma sample eluted from a 1/4-in. cutout of a 903 card or two wedges of a HemaSpot. Seventy-five microliters of the 150 μ l eluate was used for testing. An S/CO of ≥ 1.0 designates a reactive sample.

are shown in Fig. 3 and Table 1. The S/CO of HS samples was consistently reactive with a mean of 4.19, while that from 903 was 1.23, with 4 of the 21 replicates below the S/CO value of 1.0. The reproducibility of the signal was also tighter for the HS, with an intra-assay coefficient of variation (CV) at 10.4% and interassay CV at 9.7%, while those for the 903 cards were 20.8% and 21.1%, respectively.

FIG 3 The reproducibility of signal recovery from HS and 903 cards was evaluated for the low-titer Virotest-2 control (Bio-Rad) in 7 replicates per day over 3 days. The HS gave consistently higher signal, with tighter CVs for both intra-assay and interassay reproducibility.

TABLE 1 Precision of recovery of HIV Ag/Ab Combo signal from 903 and HS dried blood spots

Comparison	903			HS		
	Mean	SD ^a	CV (%)	Mean	SD	CV (%)
Intra-assay (<i>n</i> = 3) ^b	1.30	0.27	20.8	4.43	0.46	10.4
Interassay (<i>n</i> = 7)	1.23	0.26	21.1	4.41	0.43	9.7

^aSD, standard deviation.^bThe Bio-Rad Virotrol-2 low-titer HIV control was run in 7 replicates over 3 days (21 total).

The recovery of the HIV Ag/Ab Combo signals from HS or 903 membranes was evaluated in 51 previously tested clinical samples (30 reactive plus 21 nonreactive samples). All 30 reactive samples were reactive in the HS and 903 DBS, while all 21 nonreactive samples were nonreactive. The recovery of HIV Combo was also evaluated in more challenging samples, prepared from each of the 18 members of the PRA204 low-titer HIV panel. The results are plotted in descending order of HS S/CO, with the last two members being the negative panel members (Fig. 4). Again, the original plasma samples gave the highest signals. The S/CO signal of the low-titer samples showed consistently better recovery than those from the 903 cards, except for member number 12. Two HS and three 903 samples prepared from the very-low-plasma-reactive members of PRA204 were nonreactive (Fig. 4). The two negative PRA204 samples were also negative on both HS and 903.

On the basis of the results of the 51 clinical and 18 PRA204 panel members, we calculated the sensitivity of the 903 at 93.5% and of HS at 95.6%, with a specificity and positive predictive value of 100% for both (Table 2). The negative predictive value was 88.5% for 903 and 92.0% HS, with accuracy at 95.7% and 97.1%, respectively.

The recovery of low-titer HIV-1 antibody signal from early infection was also evaluated by the third-generation HIV-1/2/O EIA on both 903 and HS using the SeraCare PRB914 and PRB947 seroconversion panels (Fig. 5). The signal from the HS DBS was consistently higher than from 903, with all positive members being detected except for one member (PRB914-03) that was just below the cutoff value for the 903 sample. The first member of PRB947 was HIV 1/2/O nonreactive on the original plasma and was also nonreactive on both the HS and 903.

The recovery of the HIV-1 Western blot signal from samples collected on HS was evaluated for three plasma samples from individuals with established HIV infection. The signal recovered from one or two HS wedges was indistinguishable from that of the original plasma (Fig. 6). An additional 10 positive samples were also tested by the HIV-1/2 Geenius assay following storage of HS or 903 spots at room

FIG 4 Relative signal intensities for Bio-Rad HIV Ag/Ab Combo obtained by testing the original plasma samples from the PRA204 low-titer panel compared to that recovered from a 1/4-in. cutout of 903 cards or two wedges of HS. Samples are arranged in decreasing order of HS reactivity, with the members at the far right representing the negative panel members.

TABLE 2 Sensitivity and specificity of HS and 903 dried blood^a

Determinant	903 ^b		HS ^c	
	Value (%)	95% CI	Value (%)	95% CI
Sensitivity	93.5	82.0–96.6	95.6	84.9–99.5
Specificity	100	85.2–100	100	85.2–100
Positive predictive value	100		100	
Negative predictive value	88.5	72.0–95.8	92.00	74.8–97.8
Accuracy	95.7	87.8–99.1	97.1	89.8–99.6

^aHS and 903 spots were prepared from 30 reactive and 21 nonreactive clinical samples, plus 16 reactive and 2 nonreactive members from the SeraCare PRA204 low-titer panel.

^bFor 903, 43/46 of the reactive plasma samples were reactive from 903 (3 were nonreactive), and 23/23 nonreactive from plasma were nonreactive from 903.

^cFor HS, 44/46 of the reactive plasma samples were reactive from HS (2 were nonreactive), and 23/23 nonreactive from plasma were nonreactive from 903.

temperature. All samples were reactive for HIV-1, with 10/10 still reactive on HS and 9/10 reactive on 903 spots. The remaining sample was Geenius indeterminate (IND) after 300 days of storage at room temperature.

Since DBS are frequently used for the evaluation of populations in tropical environments, we evaluated the impact of high temperature (37°C and 45°C) and high humidity (95%) on antibody stability as detected by the Bio-Rad HIV-1/2/O assay. Controls were stored at room temperature (20 to 23°C) and ambient humidity (<40%). All 10 of 10 (100%) clinical samples from confirmed HIV infections remained reactive for up to 180 days when stored on 903 cards sealed in Ziploc bags with additional desiccant (903-S) at room temperature (RT) or 37°C in high humidity (Fig. 7). However, 4/10 (40%) 903 samples stored at 45°C in high humidity became nonreactive by 180 days. The corresponding HS samples snapped shut in their cartridges but stored in open bags with no additional desiccant (HS-O) at room temperature remained reactive for 180 days, but 2/5 (40%) HS-O samples stored at 37°C for 180 days, 2/5 (40%) stored at 45°C for 30 days, and 3/5 (60%) stored at 45°C for 180 days became nonreactive. When the HS cartridges were sealed in Ziploc bags without additional desiccant (HS-S), all (100%) remained reactive except for those at 45°C for 180 days. An HS sample stored in a Ziploc bag with additional desiccant remained reactive even at 45°C for 180 days (not shown).

All 903-S samples stored at room temperature for up to 180 days were reactive on HIV-1 WB, but 1/10 (10%) became WB (IND) after 30 days at 45°C or 180 days at 37°C, and 9/10 (90%) became IND or nonreactive by 180 days when stored at 45°C. HS

FIG 5 Recovery of HIV-1/2/O signal in 903 and HS membranes prepared from early seroconversion samples. The HS signal is consistently higher. Member 01 from PRB947 was also negative in the original plasma sample.

FIG 6 Comparison of Western blot signals obtained from three patient plasma samples to those of one or two wedges from the corresponding HemaSpot samples.

samples stored in open bags (HS-O) remained reactive for 180 days at room temperature, but 1/5 (20%) became WB IND after 30 days of storage at 37°C, and 4/5 (80%) were IND or negative when stored at 45°C. By 180 days, 4/5 (80%) samples stored at 37°C and all 5/5 (100%) stored at 45°C became WB IND or negative. All 5/5 (100%) HS samples sealed in Ziploc bags without additional desiccant (HS-S) remained HIV-1 WB positive at 180 days when stored at room temperature or 37°C and high humidity, but all became IND or negative by 180 days at 45°C. HS samples sealed in Ziploc bags with additional desiccant remained 1/2/O and WB reactive even after 180 days at 45°C (not shown).

The stability of the antibody signal (HIV-1/2/O) from early HIV infections was also conducted on initial reactive members from SeraCare HIV-1 seroconversion panels (Fig. 8). Seroconversion member PRB947-04 stored as 903 cards in a sealed Ziploc bag with desiccant (903-S) showed lower reactivity (S/CO) but remained reactive when stored for 30 days at RT, 37°C, or 45°C. Samples stored at 37°C or 45°C for 180 days were nonreactive. The corresponding HS sample (HS-O) was reactive at 180 days if stored at room temperature but nonreactive by 30 days when stored at 37°C or 45°C at high humidity. The stability of the 1/2/O signal was also compared in another seroconversion panel member, PRB910-03, in which the HS sample was stored in a Ziploc bag with additional desiccant (HS-S), while the 903 was left in an open bag with no desiccant (903-O). The HS-S samples were stable up to 180 days at room temperature or 37°C. These samples were also stable for 30 days at 45°C but became borderline reactive by 180 days at 45°C at high humidity. The 903 samples stored in unsealed bags with no additional desiccant (903-O) at room temperature, 37°C, or 45°C at high humidity became nonreactive by 30 days.

FIG 7 Percentages of positive serum samples stored on 903 and HS dried blood spots that remain HIV-1/2/O reactive. Ten replicate aliquots of serum samples from established HIV infections were spiked into whole blood, spotted on HS or 903 DBS, and stored at room temperature/ambient humidity (<40%) or at 37°C or 45°C at 95% humidity for up to 180 days. 903-S samples were stored in sealed Ziploc bags with additional desiccant. HS-O HemaSpot samples snapped shut in their cartridges were stored in open bags, enabling moisture to come in contact with the cartridges. HS-S HemaSpot samples were stored sealed in Ziploc bags without additional desiccant.

DISCUSSION

In this study, we evaluated the suitability of Whatman 903 and HemaSpot DBS for serological testing for HIV infection and their stability under conditions mimicking those in tropical climates. Both the 903 and HS showed good recovery of HIV antibody and antigen signals as detected by the fourth-generation HIV Ag/Ab Combo and the third-generation HIV 1/2/O EIA, as well as by the supplementary HIV-1 Western blot and HIV-1/-2 Geenius assays. The Western blot patterns obtained from the elution of sample from one or two wedges of HS was virtually indistinguishable from that of the original plasma sample. However, the HS and 903 dried blood spots were somewhat less sensitive for the detection of very early infections as shown by decreased sensitivity on commercial panels containing low levels of antibody (low-titer and seroconversion panels). The HS showed a higher

FIG 8 Comparison of 903 DBS to HS for HIV 1/2/O stability from samples acquired at early HIV infection. 903 DBS and HS from early HIV-1-infected seroconversion panel members PRB947-04 and PRB910-03 stored in sealed Ziploc bags with additional desiccant for 903-S or without additional desiccant for HS-S. Control samples were stored in open bags with no added desiccant (903-O or HS-O). Samples stored without additional desiccant at high temperature and high humidity rapidly lost reactivity.

recovery (4.43 versus 1.30 S/CO) and greater consistency (10.4% versus 20.8% CV) for the HS compared to those of 903 when tested on a Bio-Rad Virotest-2 low-titer serological control. Similarly higher HIV Ag/Ab Combo signals were obtained from HS than 903 in low-titer samples of PRA204 panel and HIV 1/2/O signals from early seroconversion members. The lower recovery from 903 cards can translate to decreased sensitivity, particularly for the detection of early infection, as one additional low-titer member (PRA204-22) and at least one seroconversion member (PRB914-03) were detected from the sample stored as HS, but not the corresponding 903 card.

The decreased sensitivity in the Combo and EIAs from both HS and 903 relative to that of the original plasma can be in part accounted for by a smaller sample size. The HIV Combo assay, for example, uses a volume of 75 μ l of plasma for testing. The amount of blood eluted from 2 HS wedges would be approximately one-fifth of the blood applied to HS (approximately 100 μ l), with half of that (75 μ l of the 150 μ l eluted), or an equivalent of 10 μ l, used for testing. In the case of the 903 card, the 1/4-in. cutout represents approximately one-third of the spot created by one drop of blood (50 μ l) and half of that (75 μ l of 150 μ l elution), or approximately 8 μ l, used for testing.

Our results are in agreement with those of Luo et al., which showed good recovery of HIV signal from 903 cards using modified FDA protocols for the GS Combo HIV Ag/Ab EIA and the Geenius HIV-1/2 supplemental assay (35). Although the dried blood spots in their studies performed well on samples from established infections, they were less sensitive at detecting acute HIV infections than the corresponding plasma or serum, consistent with the lower signal observed for seroconversion members in our study. The decreased sensitivity might result, in part, from the use of a smaller volume, although more than one punch did not improve the sensitivity. Their study also showed that Geenius testing of DBS was comparable to that of plasma, with very few HIV-untypable or HIV-indeterminate results (35). An equivalent signal recovery of the HIV-1 p24 antigen was also reported in samples collected as dried plasma spots (DPS) or venous plasma in another study (36). Comparable results were also reported between matched plasma and DBS samples from HIV-1-infected individuals as tested on the BioPlex assay, which measures direct antibody binding and avidity to multiple HIV-1 analytes (37).

Previous studies have shown that 903 DBS are quite stable when stored at room temperature and under low humidity conditions and retain activity for up to several months. HIV-1/2 antibody detection from whole blood spots samples stored 4°C, -20°C, and -70°C showed comparable performance to the gold standard of fresh serum and remained stable for several weeks at room temperature and low humidity as tested by EIA,

immunofluorescence, or HIV-1 Western blot (2, 5, 8, 38). However, antibody stability started to deteriorate in high humidity after 1 month of storage at room temperature or 1 week at 37°C (5, 33). Our studies extend these observations to show that both HS and 903 DBS are stable for up to 6 months at room temperature but may lose reactivity on prolonged storage of 30 days at high humidity at 37 to 45°C, as may be encountered in many tropical climates. Sealing the samples in Ziploc bags with additional desiccant extended the stability, suggesting that humidity rather than temperature alone is the significant factor in the loss of reactivity. The HS sample showed superior recovery and stability to that of 903 cards under these conditions if protected from high humidity and temperature in a Ziploc bag with additional desiccant.

Our studies had several limitations. The use of simulated samples prepared from frozen plasma or serum diluted in whole blood may not perform identically to those collected in the field. Dilutions of sample in whole blood as used in Fig. 2 are not equivalent to low-reactive samples from early infections. The volumes of serum used in the laboratory testing (75 μ l for HIV-1/2/O and HIV Ag/Ab Combo, 5 μ l for Geenius) are higher than the equivalent volumes recovered from HS and 903, which can account at least in part for the lower sensitivity of DBS.

We have shown that samples collected as DBS, including Whatman 903 cards and HemaSpot, provide reliable test results in HIV screening (HIV 1/2/O, HIV-1/2 Ag/Ab Combo) and supplemental/confirmatory (HIV-1/2 Geenius and HIV-1 Western blot) assays, even under harsh environmental conditions. The HemaSpot device (approximately \$6.00 per unit in the United States, or \$2.53 to \$3.00 for global public health clients outside the United States) is easier to handle, requires shorter drying times, and provides more convenient processing in the laboratory, with decreased opportunity for potential for cross-contamination than the standard 903 card (\$2.30 per unit). The HS device demonstrated higher yields and more consistent recovery of analyte for the detection of early infection, with improved stability at high temperature and humidity when stored in Ziploc bags with additional desiccant. Since this study was conducted, SpotOn has reformatted the HemaSpot-HF product packaging to include a Ziploc with additional desiccant, which should further improve the preservation of samples under conditions of high humidity. The use of HemaSpot for molecular testing, including HIV drug resistance studies (39) and other nucleic acids testing applications (SpotOn Sciences; <https://www.spotonsciences.com/knowledge-center/publications/>), is the subject of ongoing studies.

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