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Detection of Human Immunodeficiency Virus Type 1 DNA in Dried Blood Spots by a Duplex Real-Time PCR Assay

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A dried blood spot (DBS) is a well-accepted means for the collection, transport, and storage of blood samples for various epidemiologic, serologic, and molecular assays for human immunodeficiency virus (HIV) studies. It is particularly important for mother-to-infant-transmission studies of affected individuals living in remote areas. We have developed a real-time PCR method to detect HIV type 1 (HIV-1) DNA in dried blood spots. A cellular gene, RNase P, was coamplified with the HIV-1 DNA in the same tube to monitor the DNA extraction efficiency and the overall assay performance. Our assay is a one-tube, single-step closed-system assay and uses a dUTP/uracil DNA glycosidase anti-PCR contamination control. The HIV-1 primers and probe were derived from a conserved region of the long terminal repeat. The detection of RNase P is attenuated by lowering the forward and reverse primer concentrations so that its amplification will not overwhelm the HIV-1 amplification and yet will provide a semiquantitative measurement of the quality of the isolated DBS DNA. We examined 103 HIV-1-seropositive and 56 seronegative U.S. adults and found that our assay has a sensitivity of 98.1% (95% confidence interval [CI], 95.5% to 100%) and specificity of 100% (95% CI, 99% to 100%). The positive and negative predictive values are 100% and 96.6%, respectively. This duplex PCR assay may be useful in identifying HIV-1-infected persons, particularly infants born to seropositive mothers in remote areas of the world.

Filter papers were initially used for blood collection, transportation, storage, and screening for newborn metabolic disorders (9, 10). With the availability of PCR-based nucleic acid amplification technology, their use has been extended to the detection and identification of a variety of genetic mutations and infectious pathogens, including human immunodeficiency virus (HIV) (2, 3–6, 7, 11, 12, 16, 17, 19). Filter papers are especially useful for blood collection in resource-poor settings with limited access to diagnostic facilities. A small quantity of blood, typically 50 to 100 μl, is required to make a dried blood spot (DBS). The DBS DNA samples are stable for long periods of time and can be transported to a reference laboratory at minimal cost. Already, DBS has been widely used in serologic quality assurance programs for HIV. Moreover, it has been used for the detection of both RNA and DNA (2, 3–6, 7, 11, 12, 14, 16, 17, 19) for projects involving participants living in geographically remote areas and for persons whose infection status cannot be revealed by serologic means. For instance, the passively acquired maternal antibodies in infants born to seropositive mothers may persist up to 18 months; these infants have antibodies to HIV regardless of their true infection status. Also, newly infected persons may be seronegative for a short period of time. Molecular assays used to detect HIV proviral DNA in DBS must be very sensitive because the level of HIV proviral DNA in circulating white blood cells is extremely low. Furthermore, in the case of pediatric patients, the difficulty in measuring HIV proviral DNA is compounded by the small volumes of blood that can be obtained. To increase the sensitivity of HIV detection, many laboratories use nested amplification (7, 17). This may enhance the chance of the false-positive error because it does not include procedures or reagents that effectively prevent PCR-related amplicon contamination. In this report, we describe the design and performance of a single-tube, real-time PCR duplex assay to detect HIV proviral DNA in DBS specimens derived from adults residing in the United States. This method is highly sensitive and specific, and it incorporates provisions for monitoring the entire DNA isolation and HIV DNA detection processes, as well as safeguards for DNA contamination.

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MATERIALS AND METHODS

Human specimens. EDTA-containing whole-blood specimens from 103 HIV type 1 (HIV-1)-seropositive intravenous drug users and 56 HIV-1-seronegative persons, all residing in the United States, were used in this study. Informed consents were obtained for HIV serologic and nucleic acid-based testings. The seropositive status of these persons was determined by standard enzyme immunoassay and Western blotting. These specimens were tested anonymously, and CD4 cell counts and viral load data were not available.

DBS standard panel. Cultured 8E5 cells (8), which contain a single copy of the HIV genome per cell, were used to make positive controls. 8E5 cells were counted using a hemocytometer and diluted in whole blood from an HIV-seronegative donor to construct a standard DBS panel with various numbers of HIV-1 DNA copies in a constant human blood background. The respective 8E5 cell numbers in this panel were 0, 494, 1,482, 4,444, 13, 333, and 400,000 per ml.

DBS and DNA extraction. Fifty μl of EDTA venous blood specimens from human subjects and from the DBS standard panel were spotted onto the preprinted circle of filter paper (no. 903; Schleicher & Schuell) in a biosafety hood. The filter was air-dried at room temperature for 4 h and then placed in a liquid-tight bag (Saranex Series; VWR International, Atlanta, GA) with the desiccant and stored at −20°C. HIV-1 proviral DNA in DBS specimens stored at −20°C can be stable for at least up to 18 months (4). In this report, all the DBS samples were extracted within the first 6 months of their storage at −20°C. The entire DBS circle was punched with a paper punch (6 mm in diameter) several times to dislodge the blood-containing disks into a clean disposable weighing boat. Several paper punches were employed to speed up the punching process and were washed with water and dried between uses. To ensure that HIV-1 proviral DNA in DBS is detected, we devised an efficient DBS DNA extraction procedure followed by a DNA affinity isolation procedure (QIAGEN, Valencia, CA). The blood filter disks were transferred into a 2-ml tube containing 280 μl

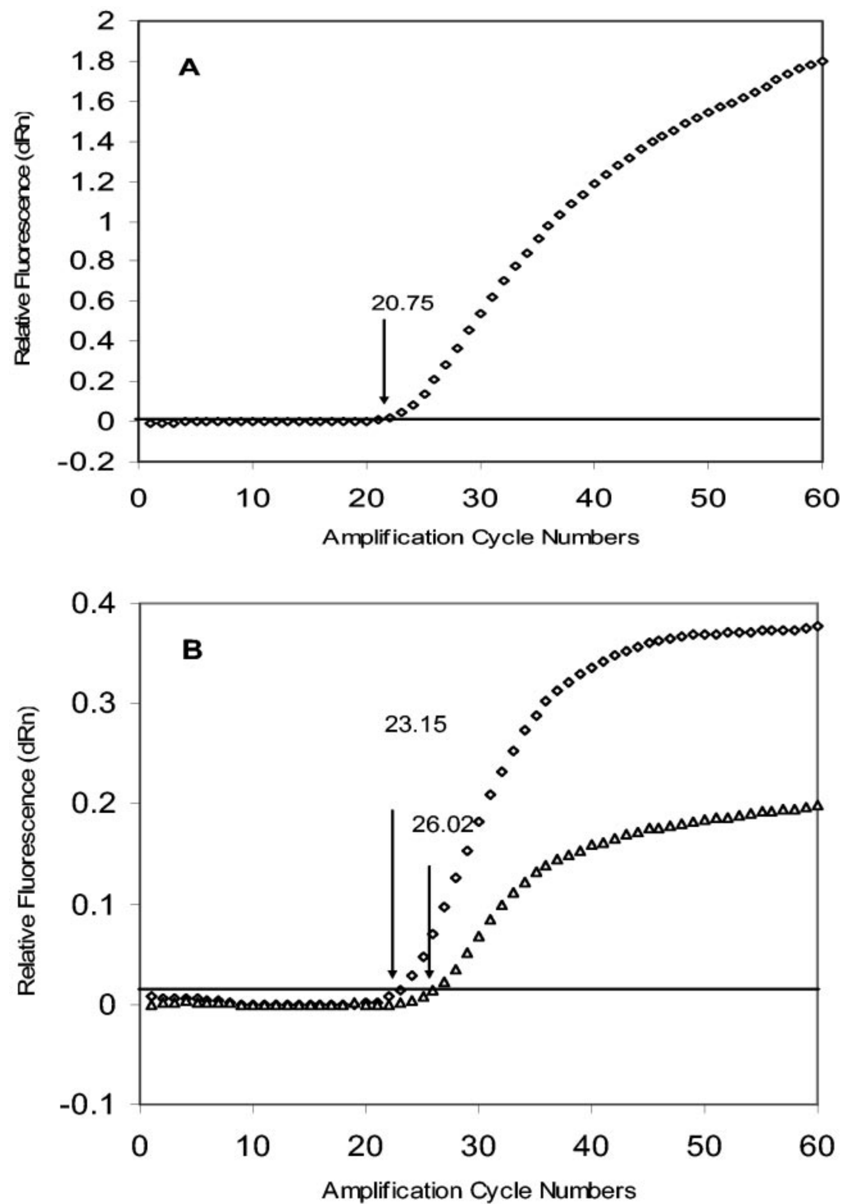

FIG. 1. Amplifications of HIV-1 LTR and RNase P DNA with 8E5 cellular DNA. (A) Amplification profiles of HIV-1 LTR. Chromosomal DNA was extracted from cultured 8E5 cells and amplified using an HIV LTR primer and probe set. The HIV forward primer, reverse primer, and probe are 200 nM, 400 nM, and 400 nM, respectively. The horizontal cutoff (threshold) lines are determined by MX4000 software and are 0.054. C_T (20.75; one standard deviation, ± 0.25) is the cycle number where the fluorescence output exceeds the threshold. The relative fluorescence (dRn) was the fluorescence output normalized by Rox, which serves as the passive dye in the assay system. (B) Amplification profiles of HIV-1 LTR. The same isolated 8E5 DNA as shown in (A) was amplified with the RNase P primer and probe set. The typical RNase P forward primer, reverse primer, and probe are 200 nM, 200 nM, and 200 nM (diamonds), and the attenuated concentrations are 40 nM, 100 nM, and 200 nM (triangles). The cutoff for RNase P is 0.02. The RNase P C_T at the typical concentrations is 23.15 (± 0.03), and that at the attenuated conditions is 26.02 (± 0.29). (C) The effect of the RNase P forward and reverse primer concentrations on RNase P amplification. The experiment was carried out with forward primer (solid circles) or reverse primer (open circles) concentrations varying from 20 to 200 nM, while the concentrations of their pairing primers were kept at 200 nM. The C_T value obtained from each set of amplification conditions was plotted against the concentrations of the forward or reverse primer. Solid downward arrow, when the forward RNase P primer concentration was lowered from 200 nM to 40 nM, the RNase P C_T increased slightly by 0.6 cycles; left open arrow, when the reverse primer concentration was lowered from 200 nM to 40 nM, the RNase P C_T increased markedly by 4.8 cycles; the C_T only increased by 0.9 cycles if the reverse primer was adjusted to 100 nM (right open arrow).

of extraction buffer (10 mM Tris HCl, pH 8.0, 50 mM KCl, 0.45% Tween 20, and 0.45% NP-40) and 20 μl of proteinase K (20 mg/ml; QIAGEN). The DBS disks were completely submerged in the extraction buffer and incubated first at 56°C for 30 min and then at 90°C for 20 min with constant agitations at 1,000 rpm in an Eppendorf thermomixer (Model R; Brinkmann Instruments, Inc., Westbury, N.Y.). The entire contents were transferred into a QiaShredder column (QIAGEN) and centrifuged in a tabletop Eppendorf microfuge at 14,000 rpm for 1 min to remove the filter disks and large particulates. Buffer AL (300 μl; QIAGEN) was then added to the column, followed by centrifugation at 14,000 rpm for 1 min. The DBS disks remaining in the column were colorless after these extraction steps. The filtrate was then mixed with 300 μl of absolute alcohol, and the precipitated DNA was further processed using QIAGEN's Mini DNA kit. DNA was eluted in 65 μl of RNase- and DNase-free water at 6,000 rpm for 1 min. Approximately 50 μl of DNA solution was recovered.

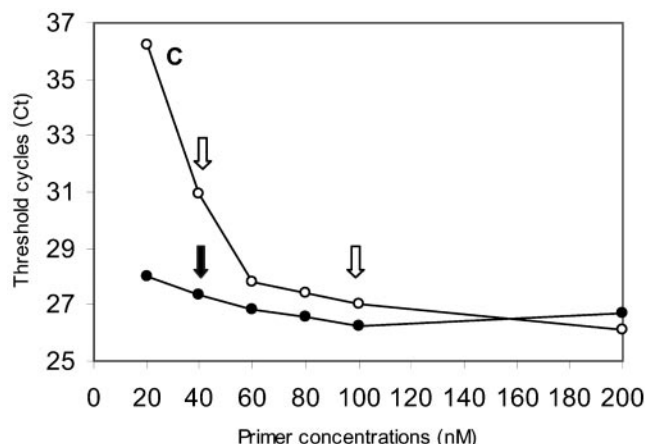

FIG. 1—*Continued*.

Real-time PCR measurement of HIV and RNase P. The extracted DBS DNA (20 μl) was used in a 50-μl-volume real-time PCR assay. Prior to the establishment of the duplex assay, RNase P primer concentrations were examined so that the amplification of RNase P would be limited. The established assay contained 200 nM HIV long terminal repeat (LTR) forward primer (TGCTTAAGCCTC AATAAAGCTTGCCTTGA); 400 nM HIV reverse primer (TCTGAGGGATC TCTAGTTACCAG); 400 nM fluorescent probe (Fam-AAGTAGTGTGTGCC CGTCTGT-Qsy-7); 40 nM RNase P forward primer (AGATTTGGACCTGCG AGCG); 100 nM RNase P reverse primer (GAGCGGCTGTCTCCACAAGT); 200 nM RNase P fluorescent probe (Hex-TTCTGACCTGAAGGCTCTGCGC G-Qsy-7); 1× PCR buffer (Roche, Indianapolis, IN); 5 mM $MgCl_2$; 200 nM each of dATP, dTTP, dGTP, and dCTP; and 400 nM dUTP; 200 nM Rox dye (Standard II; Synthegen, Houston, TX), 0.2 units of uracil DNA glycosylase (Roche), and 4 units of Amplitaq Gold DNA polymerase (Roche). Real-time PCR was performed in a model MX4000 instrument (Stratagene, La Jolla, CA). HIV DNA amplification was detected at 516 nm, and RNase P amplification was detected at 555 nm. Since the reporter oligonucleotides (i.e., probes) for HIV-1 and RNase P genes were labeled with different fluorescent probes, the synthesis of HIV and RNase P DNA sequences during PCR amplification could be measured in the same reaction vessel in real time. Activation of the Amplitaq Gold DNA polymerase was first carried out at 95°C for 10 min, followed by 60 cycles of PCR amplification at 95°C for 15 seconds and 52°C for 1 min.

RESULTS

Use of RNase P DNA gene as the internal control to monitor DNA extraction and the quality of extracted DNA. First, we compared the amplification efficiencies of RNase P DNA and HIV-1 LTR DNA in 8E5 cells, which contain one HIV-1 proviral DNA copy per cell (8). Since there are two LTRs per HIV-1 provirus, the copy numbers of RNase P DNA and HIV LTR in 8E5 cells are the same. We had previously optimized PCR conditions to detect HIV-1 LTR sequences (C.-Y. Ou, unpublished results), and we used the same conditions for RNase P amplification. As shown in Fig. 1A, the 8E5 cellular DNA yielded an HIV-1 LTR threshold cycle (C_T) of 20.75 ± 0.25 (average of three assays). The same 8E5 cellular DNA sample yielded an average RNase P C_T of 23.15 ± 0.03 (Fig. 1B).

Since only a small proportion of white blood cells in the HIV-infected person harbor HIV-1, there are many more RNase P copies than HIV copies in the blood. When a duplex assay with RNase P primers at high concentrations (200 nM each) is applied to DNA isolated from patients, HIV amplification will be diminished or abolished (data not shown). Thus, the amplification of RNase P has to be attenuated. This can be achieved by lowering one or both of the primer concentrations so that the deoxynucleoside triphosphate concentration and DNA polymerase activity will remain high for the late-emerging HIV DNA amplification.

As shown in Fig. 1C, when the forward RNase P primer concentration was lowered from 200 nM to 40 nM, the RNase P C_T increased slightly by 0.6 cycles. In contrast, when the reverse primer concentration was also lowered from 200 nM to 40 nM, the RNase P C_T increased markedly by 4.8 cycles; the C_T only increased by 0.9 cycles if the reverse primer was adjusted to 100 nM. To maintain RNase P amplification at a low but reproducible level, we chose the combination of 40 nM and 100 nM for the forward and reverse primers, respectively. As shown in Fig. 1B, with this combined reduction of both primer concentrations, RNase P amplification (C_T = 26.02 ± 0.29) was delayed by 2.9 cycles, and the fluorescence output was reduced by 50% compared with the PCR using both primer concentrations at 200 nM.

TABLE 1. Recovery and consistency of DNA isolation from DBS and whole blood[a]

Specimen	No. of samples	Avg C_T[b]	
		HIV LTR	RNaseP
Whole blood	4	34.0 (0.25)	22.4 (0.26)
DBS	4	33.6 (0.09)	21.6 (0.35)

[a] Venous blood from a seronegative person was spiked with 8E5 cells, and a DBS was prepared. DNA were extracted from the DBS (one circle = 50 μl of blood) and the blood (50 μl) directly in quadruplicate. The extraction procedure of whole-blood DNA was the same as that for its DBS counterpart but without the use of DBS extraction buffer and 90°C incubation. In both cases, DNA was recovered in 50 μl of water, and 20 μl was used in the duplex assay.

[b] One standard deviation is given in parentheses.

Consistency of DNA recovery from DBS. With the exception of the first few steps, where we used in-house reagents and QIAGEN′s Qiashredder column, we used QIAGEN′s whole-blood kit to extract DNA from the DBS. We isolated the DNA specimens from DBS and from whole blood in parallel and examined them by the duplex assay. We spiked whole blood obtained from a healthy HIV-1-seronegative person with a known number of 8E5 cells to prepare DBS (200 8E5 cells per 50 μl of blood per DBS). We then extracted DNA from the DBS and its whole blood counterpart in quadruplicate. The RNase P C_T value reflects the amount and the quality of the isolated chromosomal DNA. As shown in Table 1, samples isolated from the DBS and the whole blood yielded similar RNase P C_T values. The spiked 8E5 cells constitute only a small portion of the total white blood cell population. Like RNase P C_T values, HIV-1 C_T values from DBS and whole blood are nearly identical. The small intrarun variations of the C_Ts of both HIV (one standard deviation = 0.09) and RNase P (0.35) indicate that our overall procedure of DBS DNA isolation and duplex assay is highly reproducible.

Intra- and interassay variability of the duplex assay. We applied the above-described optimized duplex assay to a standard panel of DBS specimens. 8E5 cells were threefold-serially diluted with the whole blood of a seronegative person and spotted to give a panel with 0, 494, 1,482, 4,444, 13,333, and 40,000 HIV copies per ml (or 0, 25, 74, 222, 667, and 2,000 HIV copies per spot). We selected this range of HIV-1 copies be-

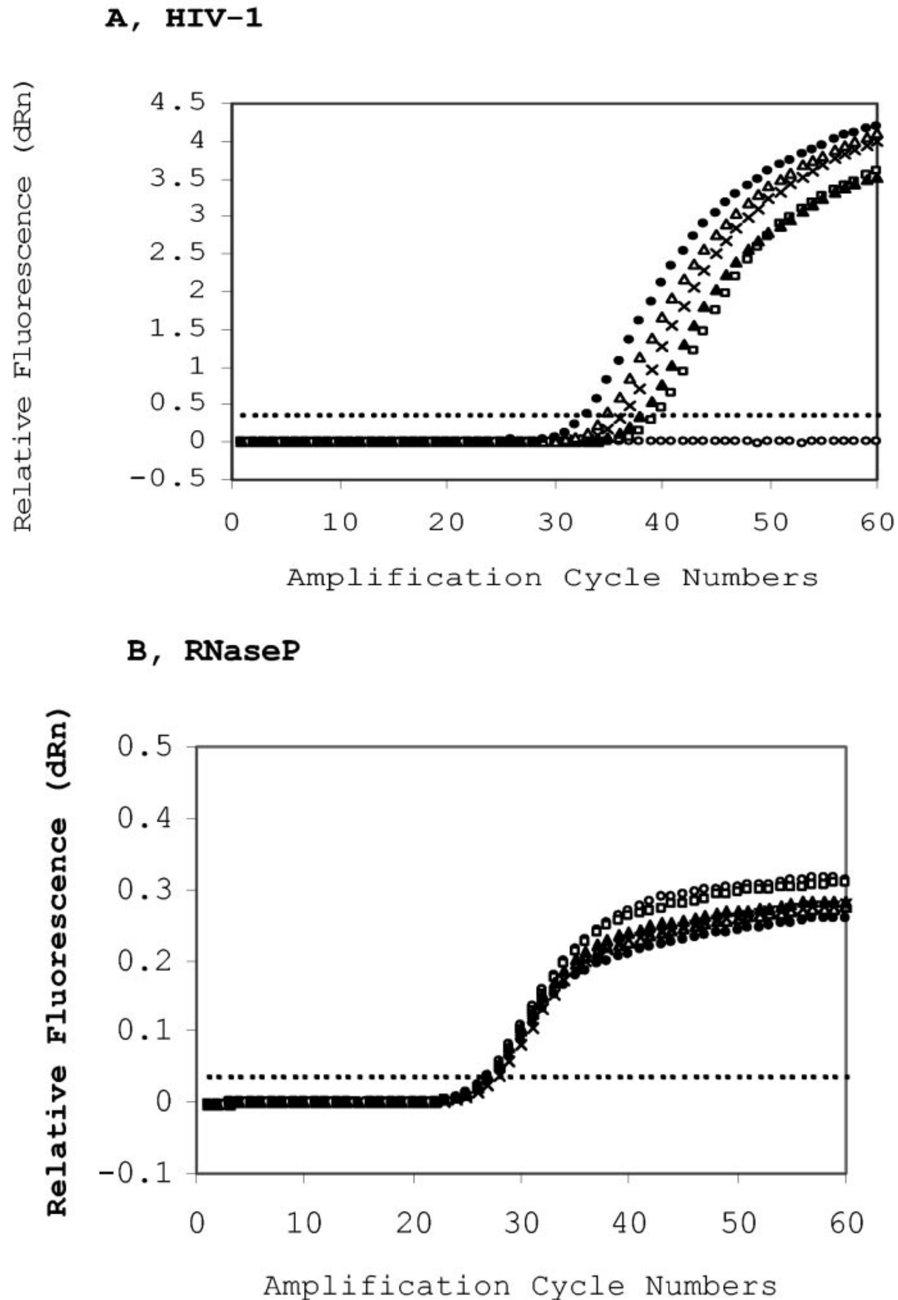

FIG. 2. Quantitative measurement of HIV-1 proviral DNA content. 8E5 cells were diluted with the whole blood of a healthy HIV-1-seronegative person and spotted to generate five spots with decreasing HIV-1 DNA contents and a constant whole-blood background. A spot with only the seronegative blood was used as the negative DBS control. Extraction of DBS in quadruplicate was carried out in a single run, and the extracted DNA samples were assayed in a single real-time run to determine the intrarun variability (see also Table 2). DBS DNA was extracted and eluted in 50 μl of water, and 20 μl of the eluted DNA was used in the duplex assay. (A) Real-time HIV-1 amplification profiles. The HIV-1 copy numbers per ml of DBS (50 μl of whole blood per circle) are 0 (open circles), 494 (squares), 1,482 (solid triangles), 4,444 (crosses), 13,333 (open triangles), and 40,000 (solid circles). The threshold (cutoff) is 0.354. (B) Real-time RNase P amplification profiles to determine the human chromosomal DNA background of the same dried blood spots as in (A). The threshold is 0.034. (C) HIV-1 content quantification in DBS panel based on threshold cycles (C_Ts) or normalized threshold cycles (dC_Ts). The C_Ts (HIV LTR [diamonds] and RNase P [squares]) were plotted against their respective proviral copy numbers. Each datum was derived from the average of four determinations. Vertical bar: one standard deviation of C_T or dC_T. The dC_T (triangle) is the difference between the HIV C_T and its corresponding RNase P C_T.

cause it is the range found in most seropositive persons (on the basis of data shown later in this report).

The intra-assay variability experiment (Fig. 2 and Table 2) was performed in quadruplicate. As shown in the HIV-1 amplification plots in Fig. 2A, the higher the HIV content of the DBS, the earlier the appearance of the HIV-specific fluorescent signal. Since only 40% of the isolated DNA was used for the real-time assay, the numbers of HIV copies used in the

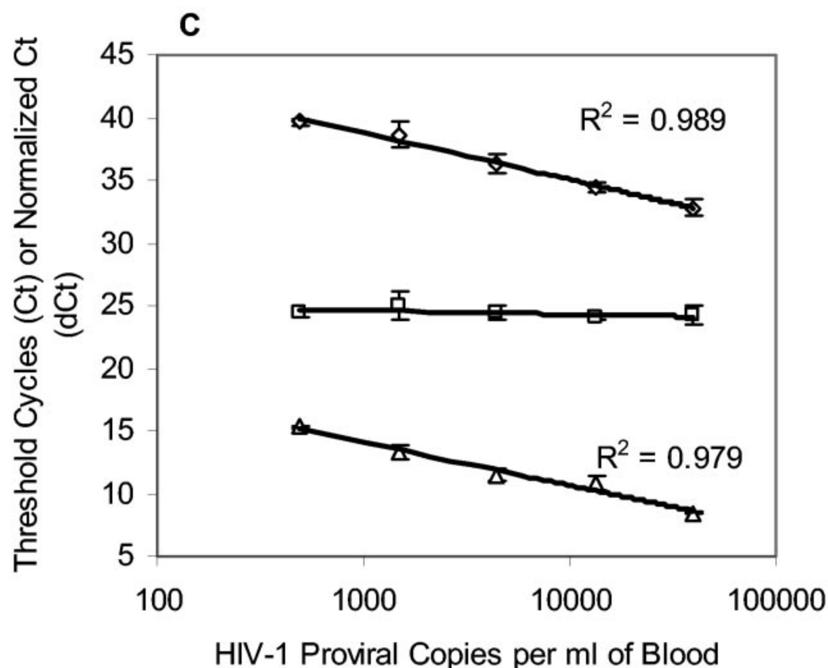

FIG. 2—*Continued*.

assay were from 9.9 to 800 copies per assay. The RNase P amplification plot of each panel member is almost identical, with an average of 24.41 ± 0.7 (Fig. 2B). The DBS sample without 8E5 cells does not show any HIV fluorescent signal over the entire 60 amplification cycles (Fig. 2A). Since this HIV-negative sample contains the same whole-blood background, it yields the same RNase P C_T as other DBS panel members (Fig. 2B).

The linearity of the standard curve of HIV C_T in Fig. 2C is high, with an R^2 value of 0.989. Since the chromosomal DNA content measured by RNase P C_T is consistent (squares in Fig. 2B), RNase P C_T can be used to normalized the HIV content to generate a normalized standard curve, the dC_T plot, as shown in Fig. 2C (triangles). The dC_T value is the difference between the C_Ts of HIV C_T and RNase P C_T of each sample (dC_T = HIV C_T minus RNase P C_T). This normalized standard curve also has a high linearity ($R^2 = 0.979$). The coefficients of variation of the RNase P-normalized intra-assay determination range from 5 to 38% (Table 2).

TABLE 2. Real-time PCR intra- and interassay variability[a]

Input copy no. per ml of blood	Observed copy no.	
	Intra-assay ($n = 4$)[b]	Interassay ($n = 4$)[c]
0	0	0
494	458 ± 22 (5)	521 ± 118 (23)
1,482	1,481 ± 426 (29)	1,445 ± 398 (28)
4,444	4,495 ± 1,226 (27)	4,914 ± 1,032 (21)
13,333	12,128 ± 4,594 (38)	11,776 ± 1,928 (16)
40,000	40,549 ± 3,462 (9)	44,309 ± 8,433 (19)

[a] A standard panel of DBS with the numbers of 8E5 cells from 0 to 40,000 copies per ml of HIV-1-negative blood (3× serial dilutions) was prepared and extracted as described in Materials and Methods. For the intra-assay determination, DNAs from 24 samples (4 panels each with 6 spots with different 8E5 content) were extracted and then run in the same real-time assay. For the interassay determination, DNA samples from the panel were isolated in four separate extraction runs and then assayed in four separate real-time assays. Results are given as means ± one standard deviation; coefficients of variation (%) are given in parentheses.

[b] Proviral DNA number was determined by the dC_T standard curve as described in the legend for Fig. 2C.

[c] dC_T-based standard curve was constructed for each interassay run.

We also examined the interassay variability using the same standard panel samples, performed in quadruplicate (Table 2). The calculation of the observed HIV-1 copy numbers is based on the individual dC_T plot generated from each interassay run. The interassay coefficients of variation are similar (16 to 28%) to those in the intravariation assays.

Evaluation of the duplex assay with U.S. adult specimens. Although our method is quantitative in nature, the specific aim of this report is to use it as a qualitative assay to identify HIV infection using DBS. To evaluate the performance of our duplex assay, we used DBS samples derived from 103 HIV-seropositive and 56 seronegative adults residing in the United States (Table 3). Of the 103 seropositive samples tested, HIV-1 LTR sequences were detected in 101 samples (sensitivity = 98.1%, 95% CI; 95.5% to 100%), with an average C_T of 36.04 (range from 28.6 to 39.95; standard deviation = 2.06). All DBS samples from the 56 seronegative persons were negative for HIV LTR (specificity = 100%, 95% CI; 99% to 100%). The positive and negative predictive values are 100% and 96.6%, respectively. The average RNase P C_T value in the 103 seropositive persons is 25.99 ± 2.49, while the corresponding RNase P C_T value in the 56 seronegative persons is 23.52 ± 2.22.

DISCUSSION

In this article we present the design and performance of a real-time PCR duplex assay to qualitatively identify HIV infection using DNA derived from a circle of DBS (50 μl of

TABLE 3. Detection of HIV LTR and RNase P DNA using DBS from seropositive and seronegative persons residing in the United States

HIV sero-status (n^d)	HIV-1 DNA detection[a]		C_T range[b]	
	No. (%) positive	No. (%) negative	HIV LTR	RNase P
Positive (103)	101 (98.1)	2 (1.9)	28.6–39.95 (36.04 ± 2.06)	20.55–31.46 (25.99 ± 2.49)
Negative (56)	0 (0)	56 (100)	NA[c]	20.58–28.07 (23.52 ± 2.22)

[a] Sensitivity, 98.1% (95% CI, 95.5 to 100%); specificity, 100% (95% CI, 99% to 100%).
[b] The C_T values of 2 HIV PCR-negative samples were not included in the HIV LTR range, average, and 1-standard-deviation calculations. However, their respective RNase P C_T values were included in the RNase P column. Average and one standard deviation are given in parentheses.
[c] NA, not applicable.
[d] No. of samples.

whole blood). The simultaneous detection of a cellular gene (RNase P) and HIV-1 DNA is an essential component in our assay. The RNase P C_T value reflects the quantity and the quality of the extracted DNA from DBS. If the DNA from a given sample is not adequately recovered or if there is copurification of a PCR-inhibitory substance, RNase P C_T would either increase significantly or become undetectable. Thus, if the recovery or the quality of the DNA from an HIV-positive sample was poor, this sample would have a poor RNase P C_T value (high C_T or no amplification at all). Samples with poor RNase P C_T values should be considered invalid, and their DNA extractions have to be repeated.

It is also possible that there are nucleotide polymorphisms in the RNaseP DNA of some persons. Such a polymorphism may lead to a suboptimal amplification of RNase P. We do not know if such polymorphisms existed in our tested population. To guard against this, a second internal chromosomal DNA control should be incorporated in the future improvement of this assay.

The average RNase P C_T values in the seropositive and negative groups are 26.05 ± 2.45 and 23.52 ± 2.22, respectively. The RNase P C_T difference between these two groups is statistically significant ($P < 0.01$). One possible explanation is that the white-cell counts in the HIV-infected persons were lower than those in the healthy seronegative persons.

Among the 103 seropositive samples tested, only 2 failed to yield HIV DNA amplification. These 2 DBS samples had respective RNase P C_T values of 25.11 and 27.16 and thus were considered good DNA specimens. The DBS of the first PCR-negative person was extracted and assayed three additional times and found to be negative. The second DBS specimen was examined two more times and found positive once, with an RNase P C_T of 28.8 and a high HIV C_T of 42.8. The high HIV C_T suggested that the proviral copy number in this sample was very low and thus rendered the detection of HIV inconsistent. We thus considered the detection of HIV in this patient a failure (Table 3).

Among the 101 HIV-1 DNA-positive samples, there was one specimen with an RNase P C_T ($C_T = 31.46$) that exceeded the high end of the 97.5% confidence interval (i.e., 25.99 plus two standard deviations, or 30.97) (Table 3). This sample had an HIV-1 C_T of 37.1 and thus was clearly HIV positive. The highest HIV-positive C_T found in this study was 39.95. Since the assay contains measures to prevent PCR carry-over contamination, samples with a positive HIV C_T are considered positive. Samples with a negative HIV C_T and an RNase P C_T exceeding the 97.5% confidence interval are considered invalid, and the DNA should be prepared and tested again. If the same invalid result is reproduced again upon the second DBS DNA preparation and PCR test, the samples should be considered indeterminate. If a repeated sample showed an improved RNase P C_T value (i.e., better DNA recovery or removal of impurities during isolation) and a positive HIV C_T, it should be considered positive. On the other hand, if the repeated sample showed an improved RNase P C_T but negative HIV PCR, then it should be called "HIV-1 not detected."

The HIV sequence that we targeted for real-time detection is in the well-conserved portion of the LTR. We have used these LTR primers and probe on specimens derived from several countries, including the United States, Uganda, Thailand, Malawi, the Ivory Coast, and Cameroon and commercial standard HIV-1 subtype panels (consisting of eight HIV-1 subtypes, A through H; Boston Biomedica, Inc.) with satisfactory results (Ou, unpublished data). Thus, our assay appears to be able to identify most of the HIV-1 subtypes. In addition to the one-step and closed-system format, our assay also uses dUTP/uracil DNA glycosylase to prevent potential false-positive results due to PCR contamination (13). Several published methods developed by others require a labor-intensive gel electrophoresis to visualize HIV amplified products and use two-step nested amplifications to increase their detection sensitivity (7, 17). Currently, there is not an effective contamination control that can be incorporated in the nested amplification system, and thus, these assays are highly susceptible to false-positive errors. Zhao et al. used real-time technology to measure HIV proviral content in whole-blood specimens but did not employ cellular internal controls (20). Some assays used cellular genes as an internal control, but the cellular gene is tested in a different tube (7, 17), adding to labor and reagent costs. Panteleeff et al. (17) showed that there was a high concentration of PCR inhibitors copurified with DBS DNA. They could only use 5 μl of DBS DNA in a 50-μl PCR volume, and they had to perform nested amplification in the absence of dUTP/uracil DNA glycosylase control. They tested 69 samples in duplicate to achieve a sensitivity of 88% and in quadruplicate to achieve a sensitivity of 96%. The uncertainty of the determination of a positive test based on one positive finding among two or four tests is extremely high. The source of the inhibitor was thought to be a heme compound (1). Our isolation procedure apparently removes most, if not all, of the inhibitors, and thus, we could use 20 μl of DBS DNA in a 50-μl PCR without inhibitory effects.

The total cost of this duplex assay is approximately 5 U.S. dollars. All the components of the assay are commercially available from several sources. To maintain the consistency and the quality of the assay, it is recommended that a large

batch of the primers and probes be synthesized, calibrated, and stored in multiple aliquots. Although this assay was initially established with an MX4000 (Stratagene) instrument, it works essentially identically in MX3000P (Stratagene) and in Applied Biosystem's 7000 and 7700 instruments (data not shown). It is also advisable that this assay be performed in a centralized facility with laboratory personnel with proper training in molecular biology and a real-time instrument to maintain the work quality.

Our assay, upon further optimization and validation, may be useful in quantifying HIV proviral DNA content (Fig. 2). Most of the adult samples tested in this report appear to have a proviral DNA content between 10 and 2,000 copies per DBS (or 200 to 40,000 copies per ml of venous blood). In this study, we only explore the potential of this assay to identify HIV infection status qualitatively using DBS.

One of the major potential uses of this assay is in diagnosing infected infants born to HIV-infected mothers. DBS specimens are easier to collect than venous blood specimens in the pediatric settings, and there is no other reliable test to identify HIV infection in the first few months after birth. It has been reported that the proviral DNA load in infected mothers may be strongly correlated with vertical transmission (15, 18). Thus, there may be a need to have an accurate and reliable assay for proviral DNA measurement for such a population. Further evaluation of this duplex assay using DBS for HIV detection in infants born to HIV-infected mothers is in progress.

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