A novel RealTime HIV-1 Qualitative assay for the detection of HIV-1 nucleic acids in dried blood spots and plasma

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ABSTRACT

Abbott RealTime HIV-1 Qualitative is an in vitro real-time PCR assay for detecting HIV-1 nucleic acids in human plasma and dried blood spots (DBS). The assay was designed to be used in diagnosis of HIV-1 infections in pediatric and adult patients, with an emphasis on the applicability in resource-limited settings. Use of DBS facilitates specimen collection from remote areas and transportation to testing laboratories. Small sample input requirement facilitates testing of specimens with limited collection volume. The Abbott RealTime HIV-1 Qualitative assay is capable of detecting HIV-1 group M subtypes A–H, group O and group N samples. HIV-1 virus concentrations detected with 95% probability were 80 copies/mL of plasma using the plasma protocol, and 2469 copies/mL of whole blood using the DBS protocol. The assay detected HIV-1 infection in 13 seroconversion panels an average 10.5 days earlier than an HIV-1 antibody test and 4.9 days earlier than a p24 antigen test. For specimens collected from 6 weeks to 18 months old infants born to HIV-1 positive mothers, assay results using both the DBS and plasma protocols agreed well with the Roche Amplicor HIV-1 DNA Test version 1.5 (95.5% agreement for DBS and 97.8% agreement for plasma).

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1. Introduction

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of the Acquired Immunodeficiency Syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Popovic et al., 1984). The virus can be transmitted by sexual contact, exposure to infected blood or blood products, prenatal infection of a fetus, or perinatal/postnatal infection of a newborn (Curran et al., 1988; Nduati et al., 2000; Schochetman and George, 1994). Accurate diagnosis of HIV-1 is essential for the prevention and treatment of infections and education of disease management.

Nucleic acid testing (NAT) has been considered generally more accurate and reliable than conventional serologic tests for detecting HIV-1 infection (Wong and Hewlett, 2010). Several NAT methods have been developed, and have been used widely for monitoring therapy and screening blood donations. Two areas with underserved clinical need where NAT is essential for accurate diagnosis are HIV-1 infections in infants born to sero-positive mothers and in acute infections (Creek et al., 2007; Daar et al., 2008; Read et al., 2007; Wong and Hewlett, 2010).

There are more than two million children infected with HIV-1 (<15 years old) worldwide. The majority of the infections (90%) are found in resource-limited settings, where testing and treatment are infrequent and the morbidity and mortality are high (WHO, 2010b). Early diagnosis and treatment have been shown to improve significantly the clinical outcome and life expectancy (Violari et al., 2008). The predominant mechanism of infections in infants is the vertical transmission from mother to child (WHO, 2010b). Because maternal antibodies are transferred passively to infants and may be detectable for up to 18 months (i.e., potentially false positive results by an antibody test), early diagnosis of HIV-1 in infants requires direct detection of the virus or its components (Rakusan et al., 1991). As a result, HIV-1 NAT that targets directly the viral genome has been recommended for detecting infections in children 18 months of age or younger (Panel on Antiretroviral Therapy and Medical Management of HIV-Infected Children, 2010; Read et al., 2007; WHO, 2010a,b).

In the case of acute HIV-1 infection in adults, patients usually present signs and symptoms such as acute febrile illness that start within days to weeks after initial exposure and last typically for less than 14 days (Kahn and Walker, 1998). Diagnosis of HIV-1 infections based on clinical presentations without laboratory testing is not specific and cannot distinguish HIV-1 infections from other illnesses (Daar et al., 2008). It can take weeks to months before antibodies reach a detectable level (i.e., seroconversion) (Busch and Satten, 1997). The acute HIV-1 infection represents a time period associated with high levels of viremia prior to a detectable immune response (Kahn and Walker, 1998). Detection of acute infections is potentially important for HIV-1 prevention due to high transmissibility of the virus, and may also offer clinical benefit for the patients.

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(Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011; Daar et al., 2008). Because the low level of immune response may lead to false negative results by standard serologic testing for antibodies, HIV-1 NAT may be a more sensitive method in detecting acute infections. It has been demonstrated that HIV-1 NAT can lead to earlier detection of the virus and can identify significant number of antibody negative acute infections that would have been missed by antibody test alone (Daar et al., 2008).

Despite recent progress in disease prevention and treatment, there remains a need to expand further the accessibility of HIV-1 testing, particularly in populations (adult and children) within resource-limited settings (WHO, UNAIDS and UNICEF, 2009). Multiple factors related to healthcare infrastructure and testing logistics hinder the effective implementation of HIV-1 testing in these settings, including difficult access to clinics and central testing laboratories by large populations in remote rural areas, low laboratory capacity in molecular testing, poor systems for specimen transportation and result reporting, lack of diagnostic systems with international regulatory approval, and difficulties in maintaining required technical training (WHO, 2007). In addition, the high overall testing volume due to disease load, variable laboratory processing capability, and the significant genetic diversity of the virus, all critically important in resource-limited settings, pose challenges for the development of highly flexible, accurate and reliable HIV-1 diagnostic methods suitable for these settings.

The Abbott RealTime HIV-1 Qualitative (RealTime HIV-1 Qualitative) assay was designed as an in vitro real-time PCR assay that can be used in the diagnosis of HIV-1 infections in pediatric and adult patients, with an emphasis on the technology’s applicability in resource-limited settings. RealTime HIV-1 Qualitative assay was designed to detect HIV-1 nucleic acids in human plasma and dried blood spots (DBS). Use of DBS facilitates specimen collection, storage and transportation, allowing specimens to be collected from remote areas and sent to central testing laboratories. The RealTime HIV-1 Qualitative assay provides flexible workflow in sample processing to address different throughput requirements: a manual method for low throughput laboratories and a fully automated method (up to 94 patient samples per run) for high throughput laboratories. Both sample processing methods utilize a magnetic micro-particle technology that purifies viral RNA in addition to DNA if present in the specimen. Furthermore, the HIV-1 primer and probe sequences of the RealTime HIV-1 Qualitative assay allow reliable detection of diverse HIV-1 variants. In this study, key performance characteristics of the RealTime HIV-1 Qualitative assay were evaluated, including analytical sensitivity, diagnostic sensitivity in seroconversion panels, specificity, reproducibility, and detection of diverse HIV-1 groups and subtypes. Performance of the assay using both DBS and plasma protocols in detecting HIV-1 infections in infants born to HIV-1 positive mothers was also evaluated in comparison with the Roche Amplicor HIV-1 DNA Test version 1.5.

2. Materials and methods
2.1. Samples and sample panels

The two main sample types evaluated were human plasma and DBS, both processed from whole blood collected with anticoagulant EDTA (ethylene diamine tetraacetic acid) or ACD (acid citrate dextrose). Panel members for the limit of detection (LOD) studies were prepared by diluting a viral standard obtained from the Virology Quality Assurance (VQA) Laboratory of the AIDS Clinical Trial Group (Rush-Presbyterian-St. Luke’s Medical Center, Chicago, IL, USA) in HIV-1 negative plasma or pooled freshly collected whole blood. Panel members for the detection of WHO 2nd International Standard were prepared by diluting the standard obtained from National Institute for Biological Standards and Control (NIBSC Code 97/650, Hertfordshire, UK) in HIV-1 negative plasma or pooled freshly collected whole blood. HIV-1 seroconversion panels were purchased from BBI Diagnostics (SeraCare, West Bridgewater, MA, USA), which contain serial bleeds from individual plasma donors during seroconversion. Panel members for the reproducibility studies were prepared by diluting the VQA standard (for low panel member) or HIV-1 LAV 8E5 Virus (SeraCare, West Bridgewater, MA, USA; for medium and high panel members) in negative whole blood. HIV-1 infected plasma samples tested for the subtype/group study were collected mainly from individual blood donors between December 1998 and September 2004 by (1) Dr. Leopold Zekeng, Laboratoire de Santé Hygiène Mobile, Yaoundé, Cameroon & UNAIDS, Accra, Ghana, (2) Dr. Lazare Kaptué, Université de Yaoundé, Yaoundé, Cameroon & Université des Montagnes, Bangangté, Cameroon, (3) Dr. Lutz Gürtler, Max von Pettenkofer Institute, Ludwig Maximilian University, Munich, Germany, (4) Dr. Roberto Badaro, Federal University of Bahia, Bahia, Brazil, (5) Dr. Carlos Brites, Federal University of Bahia, Bahia, Brazil, (6) National Blood Center, Thai Red Cross Society, Bangkok, Thailand, and (7) Dr. Phillip Hay, The Courtyard Clinic, St. George’s Hospital, London, United Kingdom. All these subtype/group specimens were collected per local regulations in the country of origin at the time of collection. Four group O isolates were purchased from BBI Diagnostics. Subtype/group specimens were aliquotted and stored at –70 °C until testing. Subtype designations were based on sequence/phylogenetic analysis of gag p24, pol integrase, and env gp41 immunodominant region as previously described (Swanson et al., 2003). Specimens tested in the specificity study were collected from sero-negative subjects and provided by ProMedDx, LLC (Norton, MA, USA) as a matched set of whole blood and plasma samples. Analytical specificity samples were obtained from ProMedDx for anti-HCV, anti-HTLV-I, flu vaccinee, HBsAg, and HBV vaccinee, from Bioreclamation, LLC (Westbury, NY, USA) for rheumatoid factor, from ProteoGenex, Inc (Culver City, CA, USA) for multiple sclerosis and multiple myeloma, from BocaBiologics, LLC (Coconut Creek, FL, USA) for systemic lupus erythematosus, and from SLR Research Corporation (Carlsbad, CA, USA) for anti-nuclear antibodies. The samples tested in the cross-reactivity study were prepared by adding purified nucleic acids (for EBV, CMV, Herpes simplex virus 1, Herpes simplex virus 2, human herpesvirus 6B, human herpesvirus 8, Varicella-zoster virus, Vaccinia virus, BK human polyomavirus, Neisseria gonorrhoeae, Chlamydia trachomatis, Staphylococcus aureus, Staphylococcus epidermidis, Mycobacterium gordonae, Mycobacterium smegmatis, and Candida albicans), viral lysate (for HIV-2 and HTLV-I), clinical specimen (for HCV), or cloned plasmid DNA (for HBV, HIV16 and HIV18) to HIV-1 negative samples and samples that contained HIV-1 LAV 8E5 Viruses at 1000 copies/mL. For the study evaluating performance for detecting HIV-1 infections in infants, specimens were collected at random from the WITS (University of the Witwatersrand and the National Health Laboratory Services)-associated clinics and hospitals (Johannesburg, South Africa) for infants approximately 6 weeks to 18 months old who were born to HIV-1 positive mothers. The study was conducted under the approval of local human research ethics committee. DNA isolated from HIV-1 infected cell line (Cat 08–707–000, Advanced Biotechnologies Inc, Columbia, MD, USA) was used to evaluate the assay’s performance in purifying and detecting proviral HIV-1 DNA. This cell DNA did not contain HIV-1 RNA targets detectable by the assay (data not shown).

2.2. Blood sample collection and handling

Whole blood was drawn into blood collection tubes, and was processed further to prepare plasma or DBS samples.
Plasma was prepared by centrifuging blood and separating plasma from cells prior to immediate testing or storage. DBS were prepared by spotting whole blood onto a minimum of 2 one-half-inch (12-mm) circles on a Whatman 903 filter paper card (or equivalent) while ensuring that the entire circle is covered (approximately 50 μL blood for each circle), followed by air drying the card at room temperature, and packaging each card in a bag with desiccant packs.

2.3. Assay control and internal control

The RealTime HIV-1 Qualitative positive control and internal control (IC) contain Armored RNA sequences encapsulated within an MS2 bacteriophage particle (Tang et al., 2007). The RNA is protected from RNase digestion within the bacteriophage-like complexes and is released during the lysis stage of the sample preparation procedure. HIV-1 positive control and IC reagents are manufactured by diluting Armored RNA to appropriate concentrations in HIV-1 negative human plasma. The negative control consists of HIV-1 negative human plasma.

2.4. Sample preparation

2.4.1. Pre-treatment for DBS

Prior to nucleic acid extraction, DBS are pre-treated with Abbott mLyis Buffer. Specifically, two DBS for each specimen are cut out from the card and placed in a tube containing 1.7 mL mLyis Buffer (ensure that DBS are fully submerged). After incubation at room temperature for 20 min with intermittent gentle mixing, all liquid is transferred into a sample input tube.

2.4.2. Sample extraction and master mix assembly

The RealTime HIV-1 Qualitative assay uses magnetic micro-particle technology to capture target nucleic acids and washes the particles to remove unbound sample components. The bound nucleic acids are eluted from the micro-particles and then ready for amplification. The sample extraction chemistry is based on the Abbott Sample Preparation System DNA reagents that are used widely for microbial DNA detection or quantification (Huang et al., 2009; Marshall et al., 2007; Thibault et al., 2007). The hydrophobic property of the extraction reagents has been further modified to achieve efficient capture of RNA from plasma or other sample types in addition to DNA, i.e., total nucleic acid purification.

At the completion of sample extraction, an amplification master mix is created with thermostable RTth polymerase enzyme, activation reagent (manganese chloride), and oligonucleotide reagent containing primers, probes and dNTPs. 50 μL aliquots of the master mix and 50 μL aliquots of the extracted eluates are dispensed to a 96-well optical reaction plate. The plate is sealed and transferred to the m2000rt for real-time PCR.

Sample preparation was performed using one of four protocols, an automated and a manual protocol for DBS samples, or an automated and a manual protocol for plasma samples. Sample extraction procedures for the plasma protocols are the same as those for the DBS protocols, with the following exceptions: (1) prior to sample extraction, plasma samples are centrifuged at 2000 × g for 5 min whereas DBS samples are pre-treated as described in Section 2.4.1; and (2) processed sample volume for plasma is 0.2 mL compared with 1.0 mL (mLyis Buffer) for the pre-treated DBS samples (50% of the 1.7 mL pre-treatment volume). Steps and conditions for sample extraction and master mix assembly are the same between automated and manual procedures.

2.5. Amplification and detection

Amplification and real-time fluorescence detection are performed using the m2000rt instrument. Reverse transcription and PCR amplification are achieved by RTth DNA polymerase in the presence of MnCl2, as described previously (Tang et al., 2007). The real-time PCR assay conditions were designed to amplify and detect both RNA and DNA.

Amplification and detection of HIV-1 and IC targets take place simultaneously in the same reaction. The target sequence for the RealTime HIV-1 Qualitative assay is the highly conserved pol integrase region of the HIV-1 genome (Johanson et al., 2001). Partially double-stranded DNA probe design is used for HIV-1 detection (Huang et al., 2007). The IC sequence is derived from the hydroxypyruvate reductase gene from the pumpkin plant, Cucurbita pepo, and is detected with a short single-stranded DNA probe (Tang et al., 2007).

2.6. Sample validity and results

A patient specimen tested by the RealTime HIV-1 Qualitative assay has a result of “HIV-1 Detected” when a valid HIV-1 threshold cycle (Ct) is obtained or “Not Detected” when no valid PCR signal for HIV-1 is observed. A minimum of one negative control and one positive control are required in each run and processed along with patient specimens serving as run controls. The HIV-1 Ct value is used to assess the validity for the positive control. The negative control with a “Not Detected” result verifies that HIV-1 contamination of the negative control does not occur during the sample extraction and set-up of the amplification reaction. An invalid result with either of the run controls invalidates the run. The IC is taken through the sample extraction procedure within each reaction for controls and specimens and acts as a control for sample extraction recovery, sample inhibition and amplification efficiency. The IC Ct value is used to assess the validity of results for each sample.

2.7. Comparator assay results

The Roche Amplicor HIV-1 DNA Test Version 1.5 (Roche Molecular Systems, Branchburg, NJ, USA) was performed according to the manufacturer’s specifications at University of the Witwatersrand and the National Health Laboratory Services, Johannesburg, South Africa. Test results of the Abbott HIVAB HIV-1 HIV-2 (rDNA) EIA and Coulter HIV-1 p24 Ag Assay were obtained from the SeraCare’s product inserts for the sernoconversion panels.

3. Results

3.1. Analytical sensitivity

Sensitivity of the RealTime HIV-1 Qualitative assay was determined using both the plasma and DBS protocols by testing dilutions of a viral standard from the Virology Quality Assurance (VQA) Laboratory of the AIDS Clinical Trial Group. Dilution panels were made in HIV-1 negative human plasma for evaluation with the plasma protocol or in pooled human whole blood that was further spotted on filter paper cards to create DBS for evaluation with the DBS protocol. Forty-five replicates of each panel member were distributed across three assay runs, three amplification lots, and three Abbott m2000 systems. The percentage of replicates detected at each concentration is shown in Tables 1 and 2. By probit analysis (Finney, 1971), the HIV-1 RNA concentration detected with a probability of 95% was determined to be 80 copies/mL (95% CI 65–118 copies/mL) with the plasma protocol, and 2469 copies/mL (95% CI 1939–4040) with the DBS protocol.
Table 1  
Sensitivity using plasma samples.

<table>
<thead>
<tr>
<th>Concentration (copies/mL)</th>
<th>Number tested</th>
<th>Number detected</th>
<th>Percent detected</th>
</tr>
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<tbody>
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<td>30</td>
<td>45</td>
<td>32</td>
<td>71</td>
</tr>
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<tr>
<td>200</td>
<td>45</td>
<td>45</td>
<td>100</td>
</tr>
</tbody>
</table>

By probit analysis, the HIV-1 RNA concentration detected with a probability of 95% was determined to be 80 copies/mL (95% CI 65–118 copies/mL).  
* Based on virus concentration assigned by VQA.

Table 2  
Sensitivity using DBS samples.

<table>
<thead>
<tr>
<th>Concentration (copies/mL)</th>
<th>Number tested</th>
<th>Number detected</th>
<th>Percent detected</th>
</tr>
</thead>
<tbody>
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<td>31</td>
<td>69</td>
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<td>1000</td>
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<td>41</td>
<td>91</td>
</tr>
<tr>
<td>2500</td>
<td>45</td>
<td>42</td>
<td>93</td>
</tr>
<tr>
<td>3000</td>
<td>45</td>
<td>44</td>
<td>98</td>
</tr>
</tbody>
</table>

By probit analysis, the HIV-1 RNA concentration detected with a probability of 95% was determined to be 2460 copies/mL (95% CI 1939–4040 copies/mL).  
* Based on virus concentration assigned by VQA.

Table 3  
Sensitivity using plasma samples.

<table>
<thead>
<tr>
<th>Concentration (IU/mL)</th>
<th>Number tested</th>
<th>Number detected</th>
<th>Percent detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>45</td>
<td>27</td>
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<td>80</td>
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<td>160</td>
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<td>320</td>
<td>45</td>
<td>45</td>
<td>100</td>
</tr>
</tbody>
</table>

By probit analysis, the HIV-1 RNA concentration detected with a probability of 95% was determined to be 149 IU/mL (95% CI 120–217 IU/mL).  
* Based on virus concentration assigned as WHO 2nd International Standard for HIV-1.

Assay performance was also evaluated by testing dilutions of WHO 2nd International Standard for HIV-1 (NIBSC 97/650) (Tables 3 and 4). The HIV-1 RNA concentration detected with a probability of 95% was determined to be 149 IU/mL (95% CI 120–217) with the plasma protocol, and 3085 IU/mL (95% CI 2644–4199) with the DBS protocol.

The capability of the DBS protocol to purify and detect HIV-1 DNA was evaluated further with DNA isolated from HIV-1 infected cells containing proviral sequence (Table 5). DNA was spiked directly into the sample extraction reaction at the lysis/capture step. Data showed that the assay can detect as low as 20 copies of HIV-1 DNA 100% of the time.

3.2. Diagnostic sensitivity in seroconversion

The diagnostic sensitivity of the RealTime HIV-1 Qualitative assay was evaluated by testing plasma specimens collected sequentially from 13 HIV seroconversion panels. These panels are available commercially and pre-characterized for HIV infection. As shown in Table 6, the RealTime HIV-1 Qualitative assay detected HIV-1 in 57 out of 82 total number of bleed samples, with 11 detected by an HIV-1 antibody test (Abbott HIVAB HIV-1/HIV-2 [rDNA] EIA) and 38 detected by a p24 antigen test (Coulter HIV-1 p24 Ag Assay). Compared to the Abbott HIVAB HIV-1/HIV-2 [rDNA] EIA, the first reactive bleed (i.e., panel member) detected by the RealTime HIV-1 Qualitative assay occurred earlier in all 13 panels with a median of 10.0 days (and a mean of 10.5 days) in earlier detection. Compared to Coulter HIV-1 p24 Ag Assay, the RealTime HIV-1 Qualitative assay detected HIV-1 infection earlier in 11 of 13 panels and at the same bleed date in the remaining two panels with a median of 5.0 days (and a mean of 4.9 days) in earlier detection.

3.3. Detection of HIV-1 infection in infants

The performance of the RealTime HIV-1 Qualitative assay in detecting HIV-1 infection was evaluated by testing specimens collected randomly from infants approximately 6 weeks to 18 months old who were born to HIV-1 positive mothers. Blood was collected and portions were processed into plasma and DBS. The plasma samples from 367 subjects and the DBS samples from 288 subjects were tested with the RealTime HIV-1 Qualitative assay. The blood sample from each corresponding subject was tested with Roche Amplicor HIV-1 DNA Test version 1.5. The overall agreement between the RealTime HIV-1 Qualitative results and the Roche Amplicor HIV-1 DNA Test version 1.5 results was 95.5% (275/288, 95% CI 92.40–97.57) using the DBS protocol, and 97.8% (359/367, 95% CI 95.75–99.05) using the plasma protocol (Table 8). The 13 discordant results using the DBS protocol consist of twelve detected by RealTime HIV-1 Qualitative and one detected by Amplicor HIV-1 DNA. The eight discordant results using the plasma protocol consist of eight detected by RealTime HIV-1 Qualitative.

3.4. Detection of HIV-1 groups and subtypes

The performance of the RealTime HIV-1 Qualitative assay with HIV-1 subtypes/groups was evaluated by testing 10 clinical specimens of group M subtypes A, B, C, D, CRF01-AE, F, CRF02-AG, and G, 10 clinical specimens of group O, and 10 replicates each of purified RNA transcripts of group M subtype H and group N. All the samples in each group/subtype category tested (viral loads between 2.5 and 6.1 log copies/mL) were detected by the RealTime HIV-1 Qualitative assay.

Table 4  
Sensitivity using DBS samples.

<table>
<thead>
<tr>
<th>Concentration (IU/mL)</th>
<th>Number tested</th>
<th>Number detected</th>
<th>Percent detected</th>
</tr>
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By probit analysis, the HIV-1 RNA concentration detected with a probability of 95% was determined to be 3085 IU/mL (95% CI 2644–4199 IU/mL).  
* Based on virus concentration assigned as WHO 2nd International Standard for HIV-1.

Table 5  
Sensitivity using HIV-1 DNA isolated from infected cells.

<table>
<thead>
<tr>
<th>Concentration (copies/assay)</th>
<th>Number tested</th>
<th>Number detected</th>
<th>Percent detected</th>
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* The concentration is expressed in number of HIV-1 copies spiked in each sample preparation reaction at the lysis/capture step.
Table 6
Sensitivity with seroconversion panels.

<table>
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<tr>
<th>Panel ID</th>
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<th>Number of reactive panel members</th>
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<th>Difference in days to first reactive result (based on bleed date) between Abbott RealTime HIV-1 Qualitative and</th>
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<td>Abbott HIV-1 Qualitative Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA&lt;sup&gt;a&lt;/sup&gt; Coulter HIV-1 p24 Ag assay&lt;sup&gt;a&lt;/sup&gt; Abbott HIV-1 Qualitative Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA&lt;sup&gt;a&lt;/sup&gt; Coulter HIV-1 p24 Ag assay&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>6</td>
<td>3</td>
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<tr>
<td>PRB966</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>3</td>
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<tr>
<td>Total</td>
<td>82</td>
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<td>16</td>
<td>38</td>
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<td>Mean</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on data from the seroconversion panel vendor.
<sup>b</sup> The dates of the first reactive test results were compared between the Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA and the Abbott RealTime HIV-1 Qualitative assay.
<sup>c</sup> The dates of the first reactive test results were compared between the Coulter HIV-1 p24 Ag assay and the Abbott RealTime HIV-1 Qualitative assay.
<sup>d</sup> All bleeds in these panels were detected with the Abbott RealTime HIV-1 Qualitative assay. Zero was used as the “Days to First Reactive Result”.
<sup>e</sup> All bleeds in these panels were non-reactive with the Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA. The last bleed day was used as the “Days to First Reactive Result”.
Table 7: Agreement between Abbott RealTime HIV-1 Qualitative assay (DBS protocol) and Roche Amplicor HIV-1 DNA Test Version 1.5.

<table>
<thead>
<tr>
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<th>Abbott RealTime HIV-1 Qualitative assay testing DBS samples</th>
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<tr>
<td>Roche Amplicor HIV-1 DNA Test Version 1.5 testing blood samples</td>
<td>54</td>
</tr>
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<td></td>
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</tbody>
</table>

3.5. Specificity

The specificity of the RealTime HIV-1 Qualitative assay was evaluated with both the plasma and DBS protocols by testing plasma and whole blood specimens collected from 550 HIV-1 seronegative subjects. For each assay protocol, the specimens were tested on two Abbott m2000 systems with four lots of amplification reagents. HIV-1 was not detected in any of the 550 samples for both specimen types, resulting in 100.0% specificity (95% CI 99.33–100.00%) for both the plasma and DBS assay protocols in this representative study.

The specificity of the RealTime HIV-1 Qualitative assay was evaluated further by testing plasma specimens from individuals either diagnosed or screened for an autoimmune disorder or serologically characterized as positive for the following markers: systemic lupus erythematosus (n = 10), anti-nuclear antibodies (n = 10), rheumatoid factor (n = 10), multiple sclerosis (n = 7), multiple myeloma (n = 10), HBsAg (n = 10), anti-HTLV-I (n = 10), anti-HCV (n = 10). Also tested were subjects who received flu vaccine (n = 10) and HBV vaccine (n = 10). HIV-1 was not detected in any of the specimens tested. In addition, HIV-1 was detected in all of these specimens that had been spiked with HIV-1 RNA.

The specificity of the assay design was also evaluated by testing potential cross-reactants listed in Table 9. Each potential cross-reactant (purified nucleic acids, viral lysate, clinical specimen, or cloned plasmid DNA) was added to HIV-1 negative samples and samples that contained 1000 copies/mL HIV-1 RNA. No interference in the RealTime HIV-1 Qualitative results (HIV-1 Detected or Not Detected) was observed in the presence of the potential cross-reactants for all positive and negative samples tested.

3.6. Reproducibility

The reproducibility of the RealTime HIV-1 Qualitative assay was evaluated for the automated protocol using the Abbott m2000sp. A four-member HIV-1 RNA panel was prepared consisting of one negative member and three positive members at 1,000,000, 100,000 and 10,000 copies/mL. The panel was tested by three operators. Each operator, using a unique combination of reagent lot and instrument system, tested 10 replicates of each panel member once per day for 5 days, for a total of 50 replicates per operator (i.e., 150 total replicates per panel member across three operators). The RealTime HIV-1 Qualitative assay detected HIV-1 in all 450 HIV-1 positive samples (150 samples at each high, medium and low concentration). The assay results of all negative samples were “Not Detected”. The overall agreement for 600 results compared with expected results was 100.0% (95% CI 99.39–100.00%).

3.7. Stability of dried blood spots

DBS were made from HIV-1 negative blood and blood spiked with HIV-1 LAV 8E5 virus at three target levels (20,000, 200,000, and 2,000,000 copies/mL), and were stored at 45°C, 37°C, 15–30°C, 2–8°C, or −10°C in cold packs. Four replicates of DBS at each target level (negative or positive) were tested with RealTime HIV-1 Qualitative assay in 1, 2, 4, and 8 weeks for 45°C and 37°C, at 1, 4, 8, and 12 weeks for 15–30°C, at 4, 8, 12, and 24 weeks for 2–8°C, and at 8, 12, and 24 weeks for −10°C or colder. At each time point and storage condition, all HIV-1 negative samples remained not detected and all HIV-1 positive samples remained detected. The Ct values for the HIV-1 signals were stable throughout the time courses for all the storage conditions tested (Fig. 1).

4. Discussion

Serologic tests have been used as standard tests in many areas of HIV-1 diagnosis, including screening of donor blood, diagnosis of new infections, and confirmation of positive screening results. However, detection of HIV-1 antibody has limited utility in several important areas of underserved medical needs such as diagnosis of early infant infections (age 18 months or younger) or acute infections, due to the biological limitations of antibody as a reliable diagnostic marker (Daar et al., 2008; Wong and Hewlett, 2010). Accurate diagnosis in these areas relies upon direct detection of the virus or its components. For early infant infections, it is recommended that molecular assays detecting HIV-1 nucleic acids be used to test exposed infants between the ages of 6 weeks and 18 months (WHO, 2010b). For acute infections, HIV-1 RNA testing can provide early detection of the infections that could otherwise be missed (Daar et al., 2008). Several NAT assays are available currently for diagnosis of HIV-1, but are challenged by lack of regulatory approval, laborious manual procedures, lack of sufficient process control, limited sample types, or well documented performance (Stevens et al., 2008). In addition to HIV-1 NAT and antibody testing, HIV-1 diagnosis can be achieved by measuring immune
complex-dissociated p24 antigen. However, wide application of p24 tests is hindered by lack of assay throughput suitable for scaled up testing and lower sensitivity compared to the HIV-1 NAT methods even for the most sensitive p24 tests (Fiscus et al., 2006; Weber, 2006).

The Abbott RealTime HIV-1 Qualitative assay was developed as a real-time PCR assay for the qualitative detection of HIV-1 nucleic acids from human plasma and dried blood spots (DBS), and is intended to be used as an aid in the diagnosis of HIV-1 infection in pediatric and adult subjects.

It has been proposed that purification and subsequent detection of both viral RNA and DNA may increase the sensitivity in detecting HIV-1 infection (Ou et al., 2007; WHO, 2010b). Analytical sensitivity in this study was performed by testing samples spiked with RNA or DNA of designated concentrations. As shown in Tables 1 and 2, the HIV-1 RNA concentrations detected with a probability of 95% were determined to be 80 copies/mL for the plasma protocol and 2469 copies/mL of whole blood for the DBS protocol. By testing DNA isolated from HIV-1 infected cells without detectable RNA, this study showed that the assay can detect as low as 20 copies of HIV-1 proviral DNA per reaction with 100% probability (Table 5). It should be noted, however, that this sensitivity for DNA cannot be applied directly to DBS samples since it does not take into account the efficiency of the target elution from the filter paper, which can be affected by the physical and chemical properties of the target (e.g. DNA versus RNA, target length, etc.) and its matrix (e.g. cell versus viral particle, etc.). In general, infants not undergoing ART treatment or adults with acute infections have HIV-1 viral loads that are considerably higher than the LOD levels observed in this study, with average RNA viral loads ranging between several hundred to several million copies per milliliter (Daar et al., 2001; Hecht et al., 2002; Leelawiwat et al., 2009; Nesheim et al., 2003; Palumbo et al., 1998; Rouet et al., 2003; Young et al., 2000). Therefore, the RealTime HIV-1 Qualitative assay should be capable of detecting pediatric and acute HIV infections in diagnostic settings. It is important to note that an initial low-positive HIV RNA level in infant and/or acute infections may represent a false-positive result (Daar et al., 2008; Read et al., 2007). Recent guidelines recommend that positive virological tests be confirmed, and for infants, confirmatory test should not delay ART (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011; WHO, 2010a).

In seroconversion panels, the RealTime HIV-1 Qualitative assay detected infections a median 10.0 days earlier than an antibody tests and a median 5.0 days earlier than an antigen tests, suggesting excellent diagnostic sensitivity. This is consistent with previous findings for other HIV-1 NAT methods used in donor screening or HIV-1 diagnosis (Weber, 2006).

The reliability of the RealTime HIV-1 Qualitative assay was demonstrated by specificity, cross-reactivity and reproducibility studies. The assay was 100% specific for matched sero-negative whole blood and plasma specimens. It was also demonstrated that autoimmune diseases and viral, bacterial or fungal pathogens other

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**Fig. 1.** Storage stability of DBS samples at different conditions: (a) 45°C, (b) 37°C, (c) 15–30°C, (d) 2–8°C, and (e) −10°C or colder.
than HIV-1 did not affect the RealTime HIV-1 Qualitative results. Reproducibility across four target levels (negative, low, median and high positive) performed by three operators using three instruments and three reagent lots showed overall agreement of 100% with expected results.

The genetic diversity of existing and emerging HIV-1 variants in the resource-limited settings (Wong and Hewlett, 2010) as well as developed areas (Cartwright, 2006; Lin et al., 2006) may pose significant technical challenges to accurate diagnosis (Bolivar et al., 2009; Brennan et al., 2006). The RealTime HIV-1 Qualitative assay uses a PCR design that targets a highly conserved region in the HIV-1 genome, pol integrase, consequently reducing the number of potential polymorphisms in the target region. Furthermore, the assay uses primers (Johanson et al., 2001), probe (Huang et al., 2007), and cycling conditions that provide reliable HIV-1 detection and quantification (Tang et al., 2007). In this study, the assay was capable of detecting all major groups/subtypes, i.e., group M subtypes A, B, C, D, CRF01-AE, F, CRF02-AG, G, and H, group O, and group N. Other studies also demonstrated that the primer and probe designs in this assay can tolerate mutations selected by integrase strand transfer inhibitor with no impact on assay performance (Hackett et al., 2010; Young et al., 2011).

To be used for both pediatric and adult populations, the RealTime HIV-1 Qualitative assay processes very small sample volumes, i.e., 0.2 ml plasma (0.3 ml if using automated procedure) or 0.1 ml whole blood (2 DBS). Minimal collection volumes (1 ml whole blood) may facilitate testing of infants as well as adults where multiple tests are typically performed on a single specimen.

In addition to liquid plasma, DBS is a suitable sample type for the RealTime HIV-1 Qualitative assay. Use of DBS in an HIV-1 NAT offers the benefits of ease of collection, storage and transportation of samples (Stevens et al., 2008). Long-term stability of HIV-1 RNA and DNA on DBS has been demonstrated (Fiscus et al., 2006). Data obtained in this study also demonstrated that, when tested with the RealTime HIV-1 Qualitative assay, HIV-1 RNA was stable on DBS, after being dried thoroughly and stored with humidity control, for at least 24 weeks at frozen or refrigerated temperatures, for at least 12 weeks at room temperature, and for at least 8 weeks at elevated temperatures such as 37 °C and 45 °C.

The RealTime HIV-1 Qualitative assay provides both manual and automated assay procedures to address various work load scenarios. The automated RealTime HIV-1 Qualitative assay has a throughput of up to 94 specimens in less than 8 h, from sample processing to result reporting.

The performance of the RealTime HIV-1 Qualitative assay was evaluated on clinical specimens collected from HIV-1 exposed South African infants between the ages of approximately 6 weeks and 18 months. Assay results using both the plasma and DBS protocols were in concordance with results from the Roche Amplicor HIV-1 DNA Test version 1.5 that tested on the blood samples.

In summary, this study describes the design and performance of the Abbott RealTime HIV-1 Qualitative assay for accurate and reliable detection of HIV-1 infections in pediatric populations or populations with acute HIV-1 infection. Flexibility of sample types, especially the use of DBS, makes this an alternative attractive for testing in resource limited settings.

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