

Nucleic Acid Assay System for Tier II Laboratories and Moderately Complex Clinics to Detect HIV in Low-Resource Settings

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There is a clear need for an instrument-free molecular diagnostic system for detecting human immunodeficiency virus type 1 (HIV-1) RNA or DNA that can be used in developing countries. Such a test could be used for early diagnosis of HIV-1 infection during infancy and could serve as a surrogate end point for vaccine trials. We developed the IsoAmp HIV-1 assay (BioHelix Corporation), which targets the HIV-1 *gag* gene with use of isothermal reverse-transcription helicase-dependent amplification chemistry. The IsoAmp HIV assay uses a disposable amplicon containment device with an embedded vertical-flow DNA detection strip to detect the presence of HIV-1 amplicons. The vertical-flow DNA detection strip has a control line to validate the performance of the device and a test line to detect the analyte. The analyte is detected by a sandwich immunoassay for reporter moieties on a capture probe and a detection probe. The control line consists of the detection probe reporter moiety conjugated to the vertical-flow DNA detection strip. The preliminary limit of detection of the IsoAmp HIV assay was evaluated by testing serial dilutions of HIV-1 armored RNA (Assuragen). We found that 21 (75%) of 28 assays yielded positive results when 50 copies of HIV-1 armored RNA were input into the IsoAmp HIV reaction.

Early human immunodeficiency virus (HIV) infection is accompanied by a rapid transient decrease in peripheral blood CD4⁺ T cell count and relatively high viral RNA load. For example, Schacker et al [1] reported that the median plasma HIV-1 RNA level within the first 30 days of HIV infection is 235,000 copies/mL. Viral load decreases to an average of 46,000 copies/mL

after 60 days, and after 120 days, viral load is 200–717,000 copies/mL. This acute phase is followed by an asymptomatic period that may last for several years and that begins with a rapid partial recovery of CD4⁺ T cells and a reduction in viral RNA load. If infected asymptomatic patients are left untreated, they will experience a progressive reduction in immune function and a resurgence in viral RNA load [2]. The high risk of death before the age of 2 years among HIV-infected infants and the fact that maternal antibodies complicate diagnosis for the first 18 months of life support the argument for early detection of HIV infection with use of molecular tests [3]. Current rapid assays (eg, OraQuick [OraSure Technologies]) based on antibody detection are relatively insensitive during the early stages of infection (ie, a 14–16-day window must pass before antigen tests can give a positive result, and a 21-day window must pass before antibody tests can yield positive results) [4]. On the other hand, false-negative results of HIV rapid antibody tests have been reported for infants with AIDS [5]. In contrast, molecular tests

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are sensitive and shorten the period of detection to 8–10 days [4].

Implementation of molecular testing for HIV in developing countries can be very challenging because of the high degree of complexity of the current tests used in developed countries and the high cost of the instruments used to perform these tests. For example, the branched HIV DNA test (Quantiplex HIV-1; version 3.0; Abbott Laboratories) is sold in Brazil for US \$9 (C. Pilcher, personal communication), a price in line with estimates for the material costs of real-time reverse-transcriptase polymerase chain reaction (PCR) assays [6]. The Quantiplex HIV-1 and all the other commercial HIV-1 molecular assays (ie, the Roche Amplicor HIV-1 Monitor test [version 1.5], the bioMérieux NucliSENS HIV-1 QT assay, Abbott RealTime, and the Primagen Retina Rainbow/NucliSENS EasyQ) have one major limitation: a need for expensive instrumentation and skilled personnel [7]. There are few affordable systems for performing HIV molecular tests in resource-poor developing countries [7]. Existing low-cost CD4⁺ T cell count monitoring tests [8] cannot replace viral RNA monitoring tests as a means of detection for early HIV infection, because only the central memory pool of CD4⁺ T cells (rep-

resenting <1% of the circulating CD4⁺ T cells) is affected early during HIV infection. Therefore, there is a clear need for an instrument-free molecular diagnostic system for HIV-1 RNA monitoring to support vaccine trials. In addition, the early diagnosis of HIV-1 infection during infancy is not feasible without molecular testing because of the persistence of passively transferred maternal antibodies for up to 18 months after birth [9, 10].

Some nucleic acid detection platforms have potential applications as “instrument-free” means of detecting HIV nucleic acid amplification reaction products. Among these are assays that detect amplification by increases in liquid turbidity caused by the formation of magnesium pyrophosphate [11], colorimetric dot blot hybridization assays [12], and immunochromatographic detection of amplification products [13, 14]. In addition, isothermal amplification technologies are clearly preferable for “equipment-free” HIV molecular diagnostics in developing countries. Unfortunately, most isothermal reactions rely on complicated sets of primers and biochemical manipulations [15–20]. Unlike these other technologies, helicase-dependent amplification (HDA) is relatively simple [21–23]. Indeed, HDA is the system that is most similar to PCR because it uses helicases to separate DNA strands rather than heat and, thus, simply relies on DNA polymerase to amplify DNA rather than on the combinations of polymerases with other enzymes used in other isothermal nucleic acid amplification methods. This greatly simplifies the enzymology involved in the amplification process, yet maintains the advantage of all isothermal amplification technologies. In addition, HDA uses probes for detecting amplification products and can readily accommodate the use of internal controls. For HIV detection, in which accuracy is essential, both of the aforementioned characteristics of HDA are preferable to nonspecific means of detection, such as turbidity caused by the formation of magnesium pyrophosphate.

We developed an HIV-1 assay targeting the HIV-1 *gag* gene with use of reverse-transcription HDA (RT-HDA) [22]. This assay uses an innovative amplicon containment device with an embedded vertical-flow DNA detection strip [13, 14] to detect the presence of HIV-1 amplicons. The vertical-flow DNA detection strip has a control line to validate the performance of the device and a test line to detect the analyte.

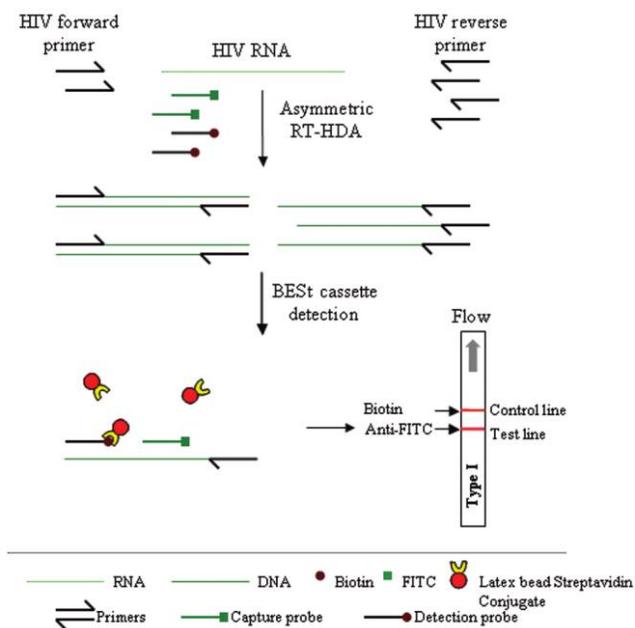


Figure 1. Schematic diagram of the mechanisms of the IsoAmp HIV assay. By using an asymmetric reverse-transcription helicase-dependent amplification (RT-HDA) method, single-stranded DNA is generated from the amplification of the HIV-1 *gag* gene. The biotin-labeled detection probe and the fluorescein isothiocyanate (FITC)-labeled capture probe can bind to the single-stranded DNA after the RT-HDA reaction. These probe-target hybrids then bind to streptavidin-conjugated color particles and are captured by an anti-FITC antibody striped on the DNA detection strip in the BEST cassette detection.

MATERIALS AND METHODS

Oligonucleotides. Multiple-sequence alignment of a 456-base fragment of the HIV-1 *gag* gene was performed by analyzing a set of different HIV-1 subtype sequences that are available from public databases with the Megalign program of Lasergene (DNASTAR). A conserved region was chosen to design the RT-HDA primer pair GagF11 (ACCATGCTAAACACAGTGGGG-

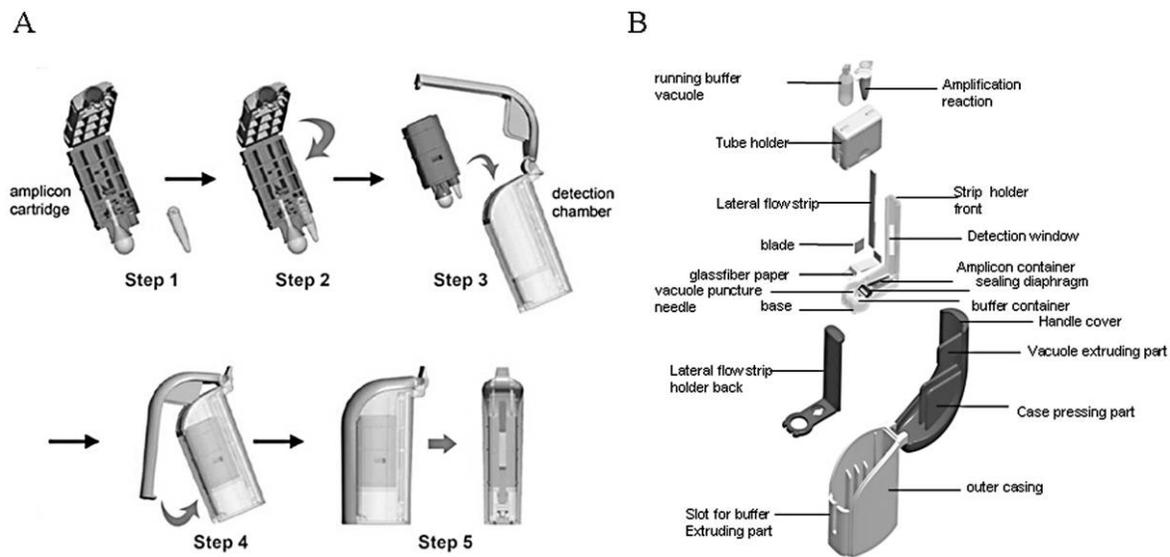


Figure 2. A, Operation of the BEST cassette. The amplification reaction vessel is placed in an amplicon cartridge (step 1), the cartridge is closed to immobilize the reaction vessel (step 2), the amplicon cartridge is inserted into the detection chamber (step 3), the handle of the detection chamber is closed to seal the vessel into the chamber and to cut open the running buffer reservoir and the reaction vessel (step 4), and after 15 min, the detection window of the chamber is read by eye to score the assay result (step 5; reprinted with permission from [14]). B, Detailed view of the BEST cassette.

GGACA), GagR5 (ATCCCATCTGCAGCTTCCTCATGAT), the capture probe GagP4FI (CAAGCAGCCATGCAAATGTTA-fluorescein isothiocyanate [FITC]), and the detection probe GagP4Bio (ACCATGCTAAACACAGTGGG-biotin), with the help of the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The melting temperature of the primers and the probes was selected at 57°–62°C and 50°–55°C, respectively, from the nearest neighbor calculation using the Oligonucleotide Properties Calculator program (<http://www.basic.northwestern.edu/biotools/oligocalc.htm>) to suit RT-HDA and cassette detection. The specificity of the primers and probes was analyzed using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Probes GagP4FI and GagP4Bio were labeled with FITC and biotin, respectively, at their 3' ends. All oligonucleotides were purchased from Operon Biotechnologies.

Sample preparation. Sample preparation was performed in triplicate with use of the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's protocol. We created simulated clinical samples by spiking 140 μL of human plasma with either 1400, 700, or 140 copies of HIV armored RNA (Assuragen). A negative control consisted of 140 μL of HIV-negative, pooled, unspiked plasma. Extractions were performed by adding 560 μL of Viral Lysis Buffer (Qiagen) with carrier RNA to a 1.5-mL microcentrifuge tube and then adding 140 μL of human plasma; the mixture was incubated at room temperature for 10 min. After incubation, 560 μL of 100% ethanol was added to the mixture, and the samples were applied to QIAamp spin columns. Columns were centrifuged at 6000 g

for 1 min, washed using Buffers AW1 by centrifuging at 6000 g for 1 min, and washed with AW2 by centrifuging at 20,000 g for 3 min. RNA was eluted from the column with 60 μL of Buffer AVE by incubating the solution in the column at room temperature for 1 min and then centrifuging at 6000 g for 1 min. After sample preparation, RT-HDA was performed using 5 μL of the eluates in a final volume of 50 μL (according to the QIAamp Viral RNA Mini Kit handbook, this kit can also be performed using the QIAvac 24 Plus [Qiagen] if a centrifuge is not available).

RT-HDA. RT-HDA was performed using HIV-1 armored RNA as a template. RT-HDA conditions and reagent concentrations were optimized to obtain the final parameters described hereafter. RT-HDA was performed using an IsoAmp Rapid HIV Detection Kit (BioHelix Corporation). To generate single-stranded amplicon for probe hybridization, asymmetric RT-HDA was performed. In brief, 25 μL of mix A, containing RNA template, primers, and probes, was mixed with 21.5 μL IsoAmp HIV Reaction Mix and 3.5 μL IsoAmp Enzyme Mix in a 200- μL PCR tube, to provide a final concentration of 20 mmol/L Tris-HCl (pH at 25°C, 8.8), 10 mmol/L KCl, 40 mmol/L NaCl, 3.5 mmol/L MgSO_4 , 400 $\mu\text{mol/L}$ of each of the 4 deoxynucleoside triphosphates, 3 mmol/L dATP, 20 units/mL ThermoScript reverse transcriptase (Invitrogen), 50 nmol/L *gag* gene primer GagF11, 100 nmol/L *gag* gene primer GagR5, 30 nmol/L capture probe GagP4FI, and 30 nmol/L detection probe GagP4Bio in the RT-HDA reaction. After a 75-min incubation period at 65°C in a water bath or heat block, the reaction tube

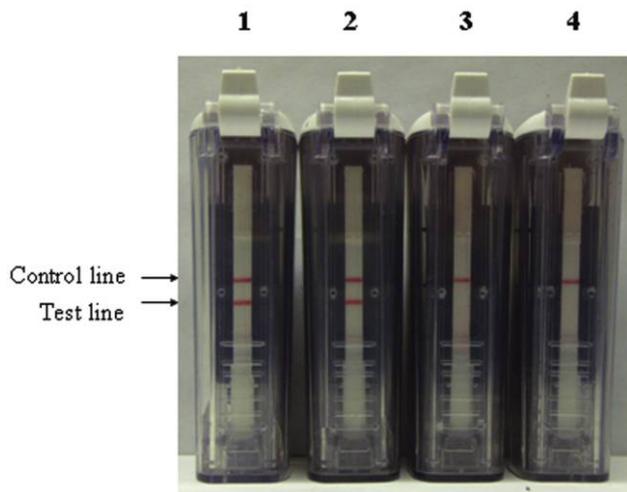


Figure 3. Determination of the detection sensitivity of the IsoAmp HIV assay. Different amounts of HIV-1 armored RNA were first amplified by reverse-transcription helicase-dependent amplification (RT-HDA) and then applied to BEST cassette detection. The amounts of HIV-1 armored RNA per reaction used in the assay were as follows: 1, 500 copies; 2, 50 copies; 3, 5 copies; and 4, no copies.

was placed directly into the BioHelix Express Strip (BEST) cassette (BioHelix Corporation) for amplicon detection.

BEST cassette detection. The dual-labeled probe-amplicon products generated from the asymmetric RT-HDA were detected by using a type I BEST cassette with the test line for capturing FITC-biotin-labeled *gag* gene probe-amplicon and the control line for capturing the excess streptavidin-conjugated color particles. The biotin labels in the detection probe attract streptavidin-conjugated color particles for visualization, and the test results are shown as a colored line visible by the naked eye. In brief, the RT-HDA reaction tube was placed in an amplicon cartridge of a type I BEST cassette immediately after RT-HDA. The cartridge was then closed and inserted into the detection chamber. The handle of the detection chamber was closed to start amplicon detection. The assay result was read by eye to score from the detection window of the chamber after 10–15 min. A positive read was scored when both the test line and the control line were visible through the detection window of the cassette. A negative read was scored when only the control line was displayed. The assay was regarded as invalid when neither the test line nor the control line was displayed.

RESULTS

Development of the IsoAmp HIV assay. A 1-step asymmetric RT-HDA assay was developed to amplify a conserved region in the *gag* gene among HIV-1 subtypes. As shown in Figure 1, an excess of the reverse primer is used to generate single-stranded DNA that can be hybridized to the capture and detection probes, and probes are labeled with different 3' mod-

ifications, to allow for a sandwich immunoassay detection of amplicons. The detection probe of the *gag* gene was labeled with biotin for binding the streptavidin-conjugated color particles, and the capture probe was labeled with FITC for the capture at the test line on the vertical-flow DNA detection strip. The control line in the vertical-flow DNA detection strip is a biotin conjugate that can capture latex beads conjugated to streptavidin. The RT-HDA reaction was performed in 1 step in 1 tube. Single-stranded DNA was generated from the excess primer in the asymmetric RT-HDA, and no denaturation was required to make the single-stranded DNA available for binding by the FITC and the biotin-labeled probes (Figure 1). The concentrations of the primers and the probes were optimized to achieve the best performance of asymmetric RT-HDA and BEST cassette detection.

A type I BEST cassette was used for end point analysis of the RT-HDA products. Figure 2 shows the individual steps involved in the postamplification detection of amplicons with BEST cassettes and the constituent elements of the BEST cassette. The self-contained cassette is composed of 2 individual components: an amplicon cartridge that holds the running buffer and a single 0.2-mL wall PCR tube containing the amplified product and the detection chamber, which houses the amplicon cartridge and a vertical-flow DNA detection strip embedded in the cassette. The DNA detection strip is striped with an FITC antibody and biotin, which serve as the test line and the control line, respectively, in the assay. A razor blade and a plastic pin lodged at the bottom of the detection chamber cut open the PCR tube and the running buffer bulb when the handle of the detection chamber is closed. The mixture flows through a fiberglass paper connected with the DNA detection strip, which is attached with a fiberglass pad preloaded with streptavidin-conjugated color particles for color visualization.

Detection sensitivity test. The detection sensitivity of the IsoAmp HIV assay was evaluated by testing serial dilutions of 500, 50, and 5 copies of HIV-1 armored RNA. Eight (100%) of 8 IsoAmp HIV assays detected 500 copies of HIV-1 armored RNA. When the HIV RNA level was reduced to 50 copies, 21 (75%) of 28 assays yielded positive results. At 5 copies, none of the 3 assays yielded positive results. These test results suggest that our assay has a detection sensitivity of ~50 copies of HIV armored RNA per assay. Assuragen's HIV armored RNA is derived from subtype B of the M clade of HIV-1, and the sensitivity of our assay with other subtypes has not been determined. An example from the detection sensitivity study of the IsoAmp HIV assay is shown in Figure 3.

Integrated assays. HIV-1 armored RNA was spiked in human plasma at concentrations of 1000–10,000 copies/mL. Total RNA was extracted from the spiked plasma samples with use of the QIAamp Viral RNA Mini Kit, and one-twelfth of the eluate (17–117 copies) was input into IsoAmp HIV assays.

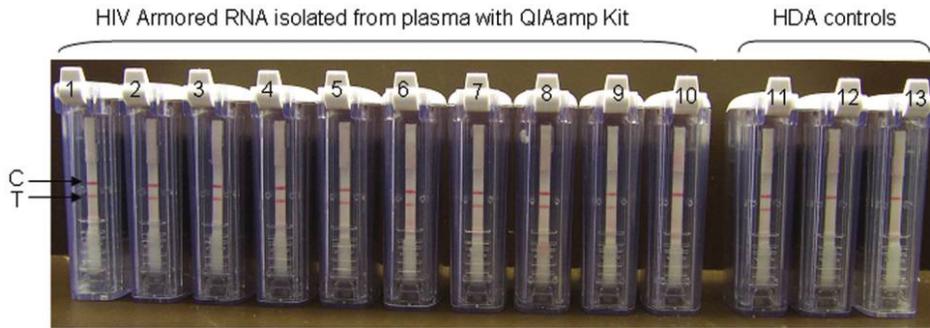


Figure 4. Integrated assays using plasma spiked with armored HIV RNA. Armored HIV RNA was spiked into human plasma at concentrations of 1000–10,000 copies/mL and extracted with a QIAamp Viral RNA Mini Kit, according to the manufacturer’s protocol. Cassettes 1–3 show results obtained with reactions performed with samples containing 10,000 copies HIV armored RNA/mL plasma; cassettes 4–6 show results of amplification with samples containing 5000 copies HIV armored RNA/mL of plasma; cassettes 7–9 show results obtained with amplification of samples containing 1000 copies HIV armored RNA/mL plasma; cassette 10 shows the result of a reaction performed with unspiked plasma; cassette 11 shows a reaction performed with 500 copies HIV armored RNA; cassette 12 shows no template control reaction; and cassette 13 shows no reverse transcriptase control performed with 500 copies HIV armored RNA. C, control line; HDA, helicase-dependent amplification; T, test line.

Figure 4 shows a sample of the data obtained. Three of 3 plasma samples containing 10,000 copies, 5000 copies, and 1000 copies of HIV-1 armored RNA/mL tested positive in the IsoAmp HIV assays (Figure 4).

DISCUSSION

To our knowledge, this is the first report of the use of RT-HDA in combination with a vertical-flow DNA detection strip embedded in an amplicon containment cassette. The vertical-flow DNA detection strip uses streptavidin conjugated to latex beads and antibodies that bind to the reporter groups incorporated in probes (biotin, FITC, or digoxigenin). Because several different reporters can be used in combination with biotin, the device can detect multiple amplicons, provided capture zones with the appropriate antibodies were conjugated to the strip. Takada et al [24] reported using a similar sandwich immunoassay amplicon detection format for PCR assays. Embedding the vertical-flow DNA detection strip in a closed device protects the laboratory from being contaminated with the amplicons that would be released from the amplification reaction vessel if it was opened after the reaction.

In addition, we are currently developing dry reagent formulations for our assays that may facilitate the deployment of IsoAmp DNA amplification chemistry in the field. The primers and probes described in this article can also be used to amplify HIV DNA from dry blood spots, as described by Patton et al [25] and Zhang et al [10]. By combining this device with a simple sample extraction method and dry reagents, technology has the potential to greatly simplify the detection of early HIV infection in patients who have experienced seroconversion (ie, detect HIV in newborns of HIV-infected mothers and in candidates of HIV immunization trials).

Another key requirement for the assay is to couple it with

a moderate-complexity sample preparation. We tested the use of FTA filters (Whatman) as a means of extracting armored RNA from human plasma samples that were spiked with known quantities of virions before extraction. Extraction of RNA with FTA filters is a relatively simple process that requires no precise volumetric measurement before the elution of nucleic acids by boiling. We showed that our assay can detect $\sim 1 \times 10^3$ copies of HIV RNA in 1 mL of plasma; therefore, we anticipate that a relatively low volume of plasma could be used to screen patients for HIV infection if the screening is performed frequently enough to detect early infection (ie, within the first 30 days, when viral titers are in the range of 1×10^5 copies/mL of plasma). A more complete investigation of the sensitivity of the integrated assay will be needed to determine whether this assay is sensitive enough to detect the resurgence of HIV RNA after the asymptomatic period.

The cost of goods for the IsoAmp HIV test is relatively modest, and thus, the test may be in a good price range for developing countries. The BEST cassette is manufactured in China and has a transfer cost of US \$2–\$3.75. RT-HDA reaction kits have a cost of goods of US \$5, and the FTA filter–based sample preparation method has a sale price of US \$1/disc. Therefore, we believe that we can offer a moderate-complexity and instrument-free molecular test for <US \$10. The costs of purchasing controls, performing quality assurance according to Clinical Laboratory Improvement Amendments regulation, and technician time are not factored in this list price estimate.

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