Rapid Detection of HIV-1 Proviral DNA for Early Infant Diagnosis Using Recombinase Polymerase Amplification

David S. Boyle,[a] Dara A. Lehman,[b] Lorraine Lillis,[a] Dylan Peterson,[b] Mitra Singhal,[a] Niall Armes,[c] Mathew Parker,[c] Olaf Piepenburg,[c] Julie Overbaugh[b]

Program for Appropriate Technology in Health, Seattle, Washington, USA[a]; Fred Hutchinson Cancer Research Center, Seattle, Washington, USA[b]; TwistDx Ltd., Cambridge, United Kingdom[c]

D.S.B. and D.A.L. contributed equally to this work.

ABSTRACT Early diagnosis and treatment of human immunodeficiency virus type 1 (HIV-1) infection in infants can greatly reduce mortality rates. However, current infant HIV-1 diagnostics cannot reliably be performed at the point of care, often delaying treatment and compromising its efficacy. Recombinase polymerase amplification (RPA) is a novel technology that is ideal for an HIV-1 diagnostic, as it amplifies target DNA in <20 min at a constant temperature, without the need for complex thermocycling equipment. Here we tested 63 HIV-1-specific primer and probe combinations and identified two RPA assays that target distinct regions of the HIV-1 genome (long terminal repeat [LTR] and *pol*) and can reliably detect 3 copies of proviral DNA by the use of fluorescence detection and lateral-flow strip detection. These *pol* and LTR primers amplified 98.6% and 93%, respectively, of the diverse HIV-1 variants tested. This is the first example of an isothermal assay that consistently detects all of the major HIV-1 global subtypes.

IMPORTANCE Diagnosis of HIV-1 infection in infants cannot rely on the antibody-based tests used in adults because of the transfer of maternal HIV-1 antibodies from mother to child. Therefore, infant diagnostics rely on detection of the virus itself. However, current infant HIV-1 diagnostic methods require a laboratory setting with complex equipment. Here we describe the initial development of an HIV-1 diagnostic for infants that may be performed at the point of care in rural health clinics. We utilize a method that can amplify and detect HIV-1 DNA at an incubation temperature within the range of 25 to 42°C, eliminating the need for thermocycling equipment. HIV-1 diagnostics are challenging to develop due to the high diversity seen in HIV-1 strains worldwide. Here we show that this method detects the major HIV-1 strains circulating globally.

Address correspondence to David S. Boyle, dboyle@path.org.

Despite the increase in effective methods to prevent mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1), there were an estimated 390,000 new pediatric HIV-1 infections in 2010, the majority of which occurred in resource-limited settings (1). Without treatment, ~52% of HIV-infected infants die by 2 years of age (2). Fortunately, early infant diagnosis (EID) and treatment programs can substantially improve survival rates (3), and as a result, there has been a >6-fold increase in the number of children enrolled in antiretroviral (ART) programs between 2005 and 2010. However, overall treatment coverage of children remains poor, as only 23% of the HIV-infected children estimated to need antiretrovirals currently have access to treatment (1). The barriers to early identification of ART-eligible infants include technical issues such as affordable and effective point-of-care diagnostics, in addition to access and other social issues. Overcoming these barriers is key to improving access to early treatment interventions that can reduce HIV-1 disease progression and infant mortality (4).

Improved access to EID and treatment depends in part on reliable and low-cost HIV-1 detection methods that can be effec-tively performed in rural health clinics in resource-limited settings. Serology-based HIV-1 assays are inappropriate for the diagnosis of infants under 18 months of age due to the transfer of maternal HIV-1 antibodies (5). Instead, infant diagnostic tests, including a variety of commercial and laboratory-developed assays, have focused on HIV-1-associated biomarkers, including host cell integrated proviral DNA (6–10), cell-free viral RNA (11–17), and the viral capsid protein antigen p24 (18, 19). Of the currently available diagnostic technologies, PCR-based methods predominate, as they typically have a higher degree of sensitivity across HIV-1 subtypes than p24-based tests (20, 21). However, PCR-based diagnostics require complex instrumentation, cold chain-dependent reagents, a reliable electricity supply, and highly skilled laboratory technicians. This complexity requires significant infrastructure, and therefore most samples are sent to centralized facilities in urban areas. A recent review of EID programs shows that the time to HIV-1 test results varies widely (from 9 days to 5 months) and that in the majority of studies only half of families/caregivers return for test results (22). For example, a recent study of EID in Nigeria noted a median time to result of 47 days,

after which only 25% of 125 infants diagnosed with HIV-1 were successfully enrolled in ART programs (23). A similar study in rural Kenya had a median turnaround time of 1.7 months, with almost half of caregivers not returning for the test result (24). Thus, although laboratory testing is typically rapid in high-volume laboratories, the logistics of testing in centralized labs can lead to loss of the caregivers of infected infants to follow up and prevent subsequent treatment (25–28). An EID assay that provides results during the initial visit to a clinic may significantly reduce this loss to follow up. To date, there is no infant HIV-1 diagnostic that can be reliably used at the point of care in rural health clinics (29).

In the past decade, several new methods that have the potential to amplify nucleic acids outside the typical molecular biology laboratory have been described (30). These assays require only a uniform incubation temperature (i.e., are isothermal), which offers a significant reduction in complexity of instrumentation compared to that of PCR-based methods. In addition, many of the test formats can employ a wide range of detection methods, including real-time analysis (31), bioluminescence (32), fluorescent/visual dyes (33, 34), endpoint analysis via turbidimetry (35), or immunochromatographic strips (ICS) that recognize hapten-labeled amplicons (36). A further advantage of isothermal assays is that they appear to be less inhibited by confounding substances in blood, permitting the use of more-crude preparations of nucleic acids before amplification and thus simplifying sample preparation while reducing reagent requirements, cost, user training, and time to result (37–39).

To date, several isothermal HIV-1 assays using helicase-dependent amplification (HDA) (40, 41), loop-mediated amplification (LAMP) (37, 41–43), nucleic acid sequence-based amplification (NASBA) (44, 45), and recombinase polymerase amplification (RPA) (46) have been described. However, it is unclear if these newer methods are capable of detecting the major circulating HIV-1 subtypes, which differ by ~30% in envelope sequences between HIV-1 subtypes within the dominant circulating group (46). Distinctions between subtypes are less pronounced when other viral genes are examined, such as *pol*, which differs by 10 to 20% between subtypes (47, 48). For global use, an HIV-1 diagnostic must be designed and validated on multiple subtypes. Importantly, subtypes A, C, and D need to be detected by diagnostics used in resource-limited areas, since they are the dominant subtypes found in Africa, where the majority of new infant infections occur. Only one study has shown isothermal amplification of multiple HIV-1 subtypes (43); however, this assay was tested only on RNA and was not tested on subtype C or D, which together account for ~50% of global HIV-1 infections (49). In addition, both LAMP and HDA require precise incubation temperatures in the range of 55 to 65°C for 60 to 75 min (50).

In this study, we present the application of RPA as a rapid and highly sensitive isothermal amplification method with which to detect HIV-1 proviral DNA (36). RPA utilizes a recombinase to facilitate the insertion of oligonucleotide primers into their complement in a double-stranded DNA molecule. This can occur at a relatively low and constant temperature in the range of 25 to 42°C (36). A strand displacing DNA polymerase can then extend from the primer-bound complex to synthesize a new complementary DNA strand. As with PCR, the use of two opposing primers allows exponential amplification of the target sequence. RPA probes are designed to hybridize to a target sequence within the amplicon.

TABLE 1 Initial screen of candidate RPA primer combinations

HIV-1 target region	No. of forward primers	No. of reverse primers	No. of primer combinations tested	No. of primer sets amplified	No. of primer sets for further testing
gag	3	6	18	0	0
LTR	6	4	24	5	1
pol	5	4	21	6	1

Each probe contains a specific abasic nucleotide analogue which is cleaved via a specific enzyme (e.g. exonuclease III [ExoIII], formamidopyrimidine-DNA glycosylase [Fpg], or endonuclease IV [Nfo]) only when the probe is hybridized to its complementary target and forms double-stranded DNA (36). Detection of the resultant amplicon can be via real-time fluorescence (Fpg or Exo probes) or by ICS detection (Nfo probes).

RPA has great potential as a nucleic acid-based diagnostic for a variety of reasons: all critical reagents can be provided in a single lyophilized pellet, which simplifies assay preparation and may allow long-term storage without refrigeration; amplification occurs over a broad range of incubation temperatures (25 to 42°C), which makes the assay robust to inadvertent temperature changes; and reaction times are considerably shorter than those of many isothermal technologies (typically <15 min) (36, 51). The ICS assay format requires only a basic heat source for reaction incubation and a simple ICS for RPA amplicon detection, making it suitable for use in low-resource settings and requiring minimal training and equipment (51).

Piepenburg et al. demonstrated that RPA could detect as few as 4 copies of methicillin-resistant *Staphylococcus aureus* DNA in <30 min, representing the rapid and accurate amplification of target DNA (36). A recent study also demonstrated the potential of RPA to amplify a particular HIV-1 sequence using exactly matched primer and probe sequences, indicating the potential for RPA to rapidly and accurately detect fully complementary sequences (51). However, the challenge of detecting HIV-1 in a diagnostic setting is the virus's extraordinary genetic diversity (52). In this work, we describe the initial development and performance of an RPA assay to detect HIV-1 proviral DNA across multiple HIV-1 subtypes.

RESULTS

Candidate RPA primer/probe screen. As the optimal primer/probe combinations for RPA cannot be predicted *a priori*, an initial screening was performed to identify primers and probes that are compatible with RPA and can amplify HIV-1 DNA. Sixty-three primer/probe combinations (see Table S1 in the supplemental material) targeting HIV-1 *gag*, LTR, and *pol* regions were screened for their ability to amplify 40 copies per RPA reaction mixture with an exact sequence-matched HIV-1 DNA template derived from ACH-2 cells. The screening of each primer pair was performed using an Fpg probe designed to target a conserved region within the putative amplicon, which can be detected in real-time via fluorescence.

None of the combinations of the three forward and six reverse primers designed to target *gag* amplified 40 copies of exact sequence-matched DNA template above the cutoff of >200 RFU used to define a positive result (Table 1), while control reaction mixtures supplied with the RPA reagent kit were positive. Of the six forward primers and four reverse primers designed to target
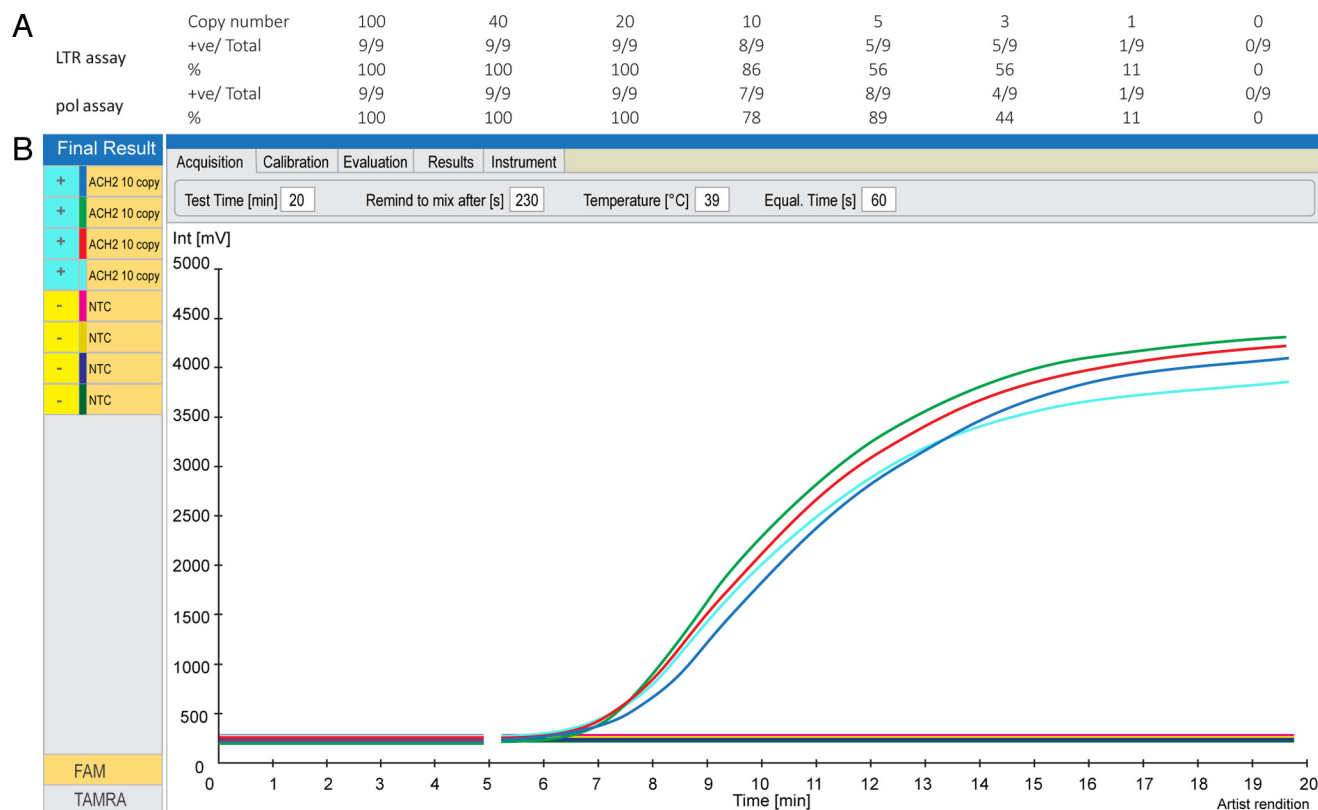
FIG 1 (A) Limit of detection. *pol* and LTR primer/probe sets were tested on samples of 100, 40, 20, 10, 5, 3, 1, and 0 copies of an exact sequence-matched HIV-1 DNA template. NTC, no-template control. (B) Fluorescent detection of low-copy-number DNA with the *pol* primer/probe set. Fluorescent detection of 10 copies of exact sequence-matched DNA. Reactions were scored positive when the change in fluorescence exceeded 200 units. No false positives were detected with HIV-negative genomic DNA alone (NTC controls).

the LTR, five primer combinations successfully amplified 40 copies of the exact sequence-matched ACH-2 DNA template (Table 1). Five forward primers and four reverse primers that target a region in *pol* were screened, and six of these primer combinations successfully amplified with RPA. All of the primer combinations that were positive were individually screened with HIV-negative human genomic DNA to rule out nonspecific amplification. None of the reaction mixtures produced an amplicon with HIV-negative human genomic DNA only. Based on the primer/probe combinations that consistently gave the greatest change in fluorescence, one *pol* assay and one LTR assay were chosen for further development efforts.

RPA detects low levels of exact sequence-matched HIV-1 DNA. To determine whether the candidate primer/probe sets were able to detect lower levels of HIV-1 DNA, we tested their ability to amplify low copy numbers of HIV-1 DNA in which the primer and probe binding sites were an exact sequence match to the template using the RPA Exo format, here called real-time HIV-1 RPA. Replicates of 100, 40, and 20 copies of ACH-2 HIV-1 DNA were amplified in 100% of RPA reaction mixtures with both the LTR and *pol* primer/probe sets (Fig. 1A). Both sets of primers successfully amplified <10 copies of exact sequence-matched HIV-1 DNA. With the LTR primers, 10 copies were amplified in 8 of 9 (89%), 5 copies in 5 of 9 (56%), 3 copies in 5 of 9 (56%), and 1 copy in 1 of 9 (11%) replicate RPA reaction mixtures. With the *pol* primers, 10 copies were amplified in 7 of 9 (78%), 5 copies in 8

of 9 (89%), 3 copies in 4 of 9 (44%), and 1 copy in 1 of 9 (11%) replicate RPA reaction mixtures. Both primer sets were highly specific, with no false positives (0/9 each) using HIV-negative human genomic DNA (Fig. 1A and B).

RPA primers amplify diverse viral sequences across HIV-1 subtypes. To test whether the primers/probes used in the LTR and *pol* real-time HIV-1 RPA assays were able to amplify across HIV-1 subtypes, we utilized a panel of 16 genomic DNAs derived from HIV-1 primary isolates. This panel included diverse variants of subtypes A, C, D, and G, as well as recombinants (Table 2). In quadruplicate testing of an estimated 100 HIV-1 proviral DNA copies based on HIV-1 real-time PCR, the *pol* primers amplified all 16 DNAs in 100% of the tests. The LTR primers amplified 15 of 16 (94%) variant DNAs tested in 4 of 4 replicates ($n = 14$) or in 3 of 4 replicates ($n = 1$). In one instance, a subtype C/A recombinant, there was no amplification in any of the quadruplicate reaction mixtures (Table 2). Sequencing of this C/A recombinant virus revealed 6 nucleotide changes and one insertion in the primer/probe binding sites, which could explain the inability to amplify this variant (data not shown).

Based on the ability of the *pol* assay to detect all 16 variants, including subtypes A, C, and D, we chose to further test the ability of RPA to amplify diverse HIV-1 sequences. Real-time HIV-1 RPA with the *pol* primers was performed on an estimated 100 copies of each of 56 *pol* DNA sequences subcloned from the NIH International Panel of HIV-1 viruses (53). This panel included 8 to 10

TABLE 2 Detection of multiple subtypes by the LTR and *pol* HIV-1 RPA assays

| Variant identification no. | Subtype | No. of replicates with DNA amplified/total no. (%) by RPA assay of: | |
		LTR	*pol*
QC168	A	4/4	4/4
MA399	A	4/4	4/4
QF495	A	4/4	4/4
MK879	A	4/4	4/4
QA842	A	4/4	4/4
QG393	A	4/4	4/4
MA462	A	4/4	4/4
QH301	C	4/4	4/4
MM898	C/A	0/4	4/4
QH343	D/A	4/4	4/4
MQ415	D	4/4	4/4
MP417	D	4/4	4/4
MO742	D	4/4	4/4
MP275	D	3/4	4/4
MP731	D	4/4	4/4
MP241	G	4/4	4/4
Total amplified		15/16 (94)	16/16 (100)

variants each of subtypes A, B, C, and D, as well as CRF AE and AG (53). In addition, to ensure specificity of these *pol* primers, simian immunodeficiency virus (SIV) was used as a template and was not amplified with the *pol* RPA assay (data not shown). Among HIV-1 templates tested in triplicate, the *pol* primers amplified 51 of the 56 viral sequences in all 3 replicates (Fig. 2). For 4 of 56 templates, *pol* amplification was above background in only 2 of the 3 tests, including 2 subtype B and 2 subtype D templates. There was one *pol* variant in which amplification was detected in only 1 of 3 tests. Therefore, overall, there were 55 of 56 (98%) viral variants that were successfully amplified with our *pol* RPA primers in at least two replicate RPA reaction mixtures.

RPA with ICS allows noninstrumented detection. The primary goal of this work was to develop a sensitive, rapid, simple-to-use, and minimally instrumented assay that can accurately diagnose HIV-1 infection in infants in low-resource settings. Therefore, we modified the *pol* RPA HIV-1 assay for use with an ICS detection format using the TwistAmp Nfo reagents (see methods). Replicates of eight samples each of 20, 10, 5, 3, 1, or 0 copies of exact sequence-matched DNA template per reaction mixture were screened with the *pol* ICS RPA assay. Two types of ICS devices were used. Four reaction mixtures for each HIV-1 copy number were analyzed with a traditional ICS (Milenia GenLine strips), while another four were analyzed with the enclosed ICS format (the BESt cassette II). Overall, 8 of 8 (100%) ICS replicates were amplified in the 20- and 10-copy-number reaction mixtures, 7 of 8 (88%) were amplified in the 5-copy-number reaction mixtures, 8 of 8 (100%) were amplified in the 3-copy-number reaction mixtures, and 6 of 8 (75%) were amplified in the 1-copy-

FIG 2 Assessment of the real-time HIV-1 RPA *pol* assay to amplify and detect diverse viral variants. The *pol* RPA primer and probe sequences, shown at the bottom, are aligned with the 56 *pol* sequences tested to highlight the exact location and number of polymorphisms relative to the primer and probe sequences.

TABLE 3 Limit of detection of the HIV-1 *pol* RPA assay using ICS detection

	No. of positive replicates/total no. tested (%)		
Copy no.	Milenia ICS	BESt ICS cassette	Pooled ICS data
20	4/4 (100)	4/4 (100)	8/8 (100)
10	4/4 (100)	4/4 (100)	8/8 (100)
5	4/4 (100)	3/4 (75)	7/8 (87.5)
3	4/4 (100)	4/4 (100)	8/8 (100)
1	3/4 (75)	3/4 (75)	6/8 (75)
0	0/4 (0)	0/4 (0)	0/8 (0)

number reaction mixtures, while 0 of 8 (0%) were positive in the no-copy controls (Table 3). Results appeared similar regardless of which format of ICS we used (Table 3).

DISCUSSION

In this study, we describe the initial development of an infant HIV-1 diagnostic using RPA, which can be performed rapidly without complex equipment and may have the potential for use in a basic laboratory setting or rural health clinic. We have identified and designed two primer/probe sets and a test methodology that can reliably amplify as few as 1 to 3 copies of an exact sequence-matched DNA template. In HIV-infected children <2 years of age, there are on average 75 (range, 1 to 382) copies of HIV proviral DNA in 1 μl of blood (54–56), with limited decay in the presence of antiretrovirals (57–59). Therefore, the ability to amplify <10 copies of proviral DNA provides the sensitivity to detect HIV in a drop of blood (~50 μl) from infants even with low viral loads and with exposure to ARTs for prevention of mother-to-child transmission.

We show that both primer sets can effectively cross-subtype, amplifying variants that represent the diversity exhibited in circulating HIV-1 strains worldwide. Indeed, the LTR primer set amplified 93% of 15 diverse viruses, while in a larger screen, the *pol* primer set amplified 71 of 72 (98.6%) variants tested. These results are promising, as they suggest that RPA, which utilizes longer primers/probes and lower incubation temperatures than both PCR and other isothermal methods, may be very tolerant to polymorphisms in the primer/probe binding sites.

Part of the advantage of PCR methods for HIV-1 detection has been that they can tolerate some diversity in the template sequence. The ability of RPA to detect sequences with some diversity was unknown. In this study, we found that RPA can occur even with up to 9 changes across the primer and probe binding sites (Fig. 2). The one variant (isolate AF484502) that amplified in only 1 of 3 tests had five polymorphisms, one of which was within 3 nucleotides of the 3′ end of the reverse primer. While the HIV-1 RPA assays are able to amplify diverse viral variants, the *pol* primers/probes did not result in amplification of an SIV plasmid DNA, suggesting that they are both sensitive and specific to HIV-1.

Interestingly, only a subset of the primers and probes assessed in our initial screen, 13 of 63 (21%), successfully amplified an exact sequence-matched template, and these were limited to the LTR and *pol* target regions. None of the primers targeting *gag* amplified effectively in the initial screen. In retrospect, this is surprising, given recently published data describing a *gag*-based HIV-1 RPA assay (51). However, the *gag* primers used here did not directly overlap primers used in the earlier study and the dif-

ferences in primer or probe sequences likely explain this discrepancy. The *gag* RPA assay described by Rohrman and Richards-Kortum is able to amplify 10 copies of an exactly matched template but has not been demonstrated to cross-subtype (51).

Certain groups of plasmid preparations that were processed in parallel did not amplify with repeated RPA testing, in some cases with different primers (see methods). In all cases, a second plasmid stock, prepared at a later date, was amplified by RPA. This suggests that a step in processing could have led to a residual component that was inhibitory to RPA. We are currently investigating these plasmid preparations to determine if there are specific inhibitors or confounders of these DNA preparations that limit RPA. Inhibition was never observed with any of the genomic DNA stocks, which represent the type of DNA sample we anticipate will be tested when patient specimens are used.

The ultimate goal of this research was to develop a highly sensitive and specific HIV-1 diagnostic test for infants that is minimally instrumented and that can be used at the point of care in rural health clinics. ICS is a common detection format in point-of-care assays, as it does not require complex equipment, is low cost, and is relatively simple to use; here we demonstrate sensitive detection with the ICS RPA assay format. One major drawback of traditional ICS methods to detect amplified DNA is the requirement that reaction tubes be opened for application of the test reaction mixture to the ICS to obtain the result. This can result in amplicon contamination of the test area, which is a significant concern for quality control in a point-of-care diagnostic performed by minimally trained users. A device that permits ICS detection without opening the tubes with amplified product has been developed (the BESt cassette II), and this substantially reduces the risk of contamination of the test site (55). Our data suggest that detection of RPA amplicons with this device is identical in performance to traditional ICS strips.

In this study, we describe an HIV-1 RPA assay that has a very low limit of detection and rapid time to result (<20 min) and that can detect diverse virus variants—an important requirement for an effective HIV-1 diagnostic. Our identification of RPA primers that target two distinct genome regions and that can amplify diverse subtypes may allow for the development of a multiplexed RPA method, improving the highly sensitive detection of all HIV-1 variants, including newly identified recombinant strains (60). RPA can be performed at a temperature anywhere between 25°C and 42°C, without a precise incubation temperature as required by other isothermal or PCR-based assays. With this flexibility in reaction mixture incubation temperature, it is feasible that RPA could be performed at ambient temperature or with heat sources that do not require a power supply, for example, chemical heaters using phase-change materials (61). Limitations of our study include that to date, development and testing have been performed only in-house. A comparison of this RPA assay to a gold standard PCR-based assay in a real-world setting remains to be done. Additional challenges will be streamlining the specimen processing and limiting the consumables; methods to achieve these goals are available and potentially could be combined with the RPA assay described here (62–64). We are confident that there is an opportunity to create a simple-to-prepare, rapid, and yet highly accurate test method for EID of HIV-1 infection in low-resource settings.

MATERIALS AND METHODS

Preparation of target DNA. The ACH-2 cell line, containing a full-length single integrated copy of HIV-1 Bru (subtype B; GenBank accession number K02013.1) DNA per cell (65), was used to create quantified proviral DNA standards (66). ACH-2 cells and noninfected CEM-174 cells were cultured in parallel in RPMI medium and quantified with a hemocytometer prior to mixing them at ratios ranging from 1 to 1e2 infected cells in a background of 1e4 uninfected cells. DNA was extracted from cell mixtures using phenol-chloroform extraction, and HIV-1 DNA was quantified by real-time PCR as described previously to verify the estimated copy number per microliter (5, 67).

A panel of HIV-1 isolates was utilized to make a cross-subtype reference panel of 72 viruses in total, including subtypes A, B, C, D, and G, as well as circulating recombinant forms (CRFs). These include 16 full-length primary isolates from several cohorts in Kenya with diverse HIV-1 subtypes that were obtained by coculturing HIV-infected peripheral blood mononuclear cells (PBMCs) from an HIV-positive sample with uninfected donor PBMCs (68, 69). DNA was extracted from these cells using the Qiagen DNeasy 96 blood DNA extraction kit, and the HIV-1 DNA copy number was quantified by real-time PCR (5, 67). The subtype was defined based on *pol* sequences as previously described (68, 69). An additional 56 viral isolates were obtained from the International Reference Panel from the NIH AIDS Research and Reference Reagent Program and used to generate HIV-1 *pol* clones (53). The *pol* gene was subcloned from each of the 56 viruses by amplifying a 1.96-kb region of *pol* encoding amino acids 340 through 994 via reverse transcription (RT)-PCR using viral RNA. PCR products were cloned into PCR4-TOPO (Invitrogen, Carlsbad, CA) and sequenced, and the subtype based on this *pol* sequence was determined. Plasmids were purified using a Qiagen plasmid minikit according to the manufacturer's instructions, and concentrations of the resulting DNA plasmids were quantified by spectrophotometry. Plasmid dilutions were quantified by real-time PCR to make samples with specific HIV-1 copy numbers (5, 67). There were instances in which a group of plasmid preparations from the same day were all negative when tested in the RPA assay, suggesting the existence of an issue with the processing on that day that may have led to inhibition of RPA. In these cases, new DNA stocks were prepared and rescreened and the results from the new DNA templates were used. Of note, this issue was not encountered for any of the genomic DNA preparations described above.

Identification of conserved target regions for RPA assay design. Over 700 sequences of *gag*, LTR, and *pol* of HIV-1 subtypes A to E were aligned using Sequencher software (Gene Codes Corp., Ann Arbor, MI). RPA primers are ideally 30 to 35 nucleotides long and form an amplicon of 100 to 200 bp with intervening probe sequences of 35 to 52 nucleotides depending on the probe chemistry used (70). The regions with highest conservation were screened for >35-nucleotide stretches that differ from the consensus by fewer than 4 nucleotides (11% to 13%) in any sequence in the alignment. To identify these conserved regions, the alignments were analyzed with a Perl script (provided by Janet Young; unpublished) that quantifies the percent conservation at each nucleotide position in the alignment. All primer and probe sequences screened for performance with RPA are shown in Table S1 in the supplemental material.

RPA conditions and detection. RPA reaction mixtures were supplied by TwistDx Ltd., Cambridge, United Kingdom. Detection of RPA amplicons is via one of three endonuclease formats (Exo, Fpg, or Nfo), as described previously (70). TwistAmp Fpg reagents were used for the initial primer screening of *gag*, LTR, and *pol* targets, and TwistAmp Exo or Nfo reagents were used for all other testing as noted. All TwistAmp reaction mixtures were prepared according to the manufacturer's instructions (70). Preincubation was performed at 39°C for 1 min, followed by incubation at 39°C for a further 20 min, with brief mixing of the reaction mixtures after 5 min. Real-time detection of RPA via 6-carboxyfluorescein (FAM) fluorescence was measured using a Twista real-time instrument (TwistDx Ltd., United Kingdom) every 20 s. The RPA fluorescence data were assessed by taking a baseline relative fluorescence unit (RFU) mea-

surement after 6 min of incubation and subtracting this from the final fluorescence reading after 20 min of incubation. Any changes in fluorescence (ΔRFU) that were greater than either 200 RFU or a 3-fold increase over the background number of RFU in the negative controls consisting of either H_2O or HIV-negative human genomic DNA were considered positive results.

For the endpoint detection of RPA via ICS, the RPA Nfo reactions were immediately terminated after incubation by the addition of 5 μl EDTA (250 mM). Detection of the hapten-labeled RPA amplicons was assessed with either a conventional ICS (Milenia GenLine; TwistDx Ltd., United Kingdom) or an ICS fully enclosed in a cassette (BESt cassette II; BioHelix Corp., Beverly, MA) (71). For detection with the Milenia GenLine strips, 2 μl of a terminated RPA Nfo reaction mixture was placed on the capture pad, which was followed by the immediate immersion of the capture pad in 100 μl of ICS running buffer. Test results were scored after 5 min of incubation, with a positive reaction indicated by development of both the test stripe and the control stripe and a negative result indicated by development of only the upper control stripe. The BESt cassette II was used according to the manufacturer's instructions. Briefly, the entire 200-μl reaction tube containing the entire terminated RPA reaction mixture was placed, unopened, in the loading cartridge, which was then inserted into the cassette; the lid was then closed to rupture the reaction tube and sample buffer reservoir and to activate the ICS detection process. The result was read 5 min after the device was sealed. A positive result was scored if a stripe developed next to the region of the cassette marked "T." A negative result was scored if the entire test strip remained blank.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00135-13/-/DCSupplemental.

Table S1, DOCX file, 0 MB.

ACKNOWLEDGMENTS

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