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Research article

Development and evaluation of a high-sensitivity RT-PCR lateral flow assay for early detection of HIV-1 infection

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ABSTRACT

Early diagnosis of HIV-1 is crucial to minimize transmission, morbidity, and mortality, particularly for neonates with developing immune systems. This study aimed to develop and evaluate a simplified, high-sensitivity assay for early HIV-1 detection before seroconversion. The assay utilizes reverse-transcription-polymerase chain reaction (RT-PCR) to amplify the HIV-1 RNA *protease* gene. Digoxigenin (dig)-labeled forward, and biotin-labeled universal reverse primers are used, generating digoxigenin-amplicon-biotin (DAB) products. These products are detected using a lateral flow assay (LFA) containing a conjugated pad with colloidal gold-labeled 6-histidine tag-fused maltose-binding protein-monomeric streptavidin (^{6HIS}MBP-mSA-CGC). Anti-dig monoclonal antibody (mAb) and biotinylated-BSA are immobilized in the test and control line zones, respectively. Five plasma samples with known viral load (VL) were used to simulate the efficacy of early HIV-1 detection. RNA extracted from these samples was amplified by RT-PCR using the labeled primers, and DAB products were examined on agarose gel electrophoresis and LFA. RT-PCR from diluted clinical samples yielded visible DNA bands in agarose gel electrophoresis, consistent with positive LFA results. Conversely, negative samples only displayed the control line on LFA. This assay exhibited a limit of detection (LOD) of 82.29 RNA copies/mL, comparable to other nucleic acid amplification tests (NAATs). This novel technique provides a highly sensitive assay for early HIV-1 diagnosis, even with low VL, making it suitable for resource-limited settings.

1. Introduction

In recent years, concerted efforts through HIV prevention campaigns and the increased accessibility of highly active antiretroviral

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therapy (HAART) have successfully driven down the incidence of HIV infection [1,2]. Nevertheless, new HIV cases continue to occur daily. Therefore, early diagnosis and treatment of HIV infection is vital to reduce the VL as quickly as possible, and thus help reduce virus transmission, morbidity, and mortality [3,4]. Especially in neonates infected with HIV, early diagnosis can directly impact disease progression since their immune system is still developing. Timely interventions with antiretroviral therapy (ART) improve the child's immediate health, ensuring a higher quality of life and a more favorable long-term prognosis [5].

Several immunology-based techniques have been wildly used for HIV diagnosis. The third-generation tests widely used currently may impart false negative results around 2–3 weeks after infection because it detects the presence or absence of HIV IgG and IgM antibodies [6,7]. Fourth-generation FDA-approved HIV diagnostic test kits, which include p24 HIV antigen in the assay, has reduced the test-negative window period to 2 weeks [8]. This delay in detection is particularly significant for neonates, whose immune responses may be less developed, leading to a longer window period.

Consequently, molecular-based techniques have been developed for implementation a sensitivity from the drawbacks of antigen/antibody detection. HIV RNA can be detected approximately 7 days before a p24 antigen raising, and around 12 days before detection of antibodies [9]. Currently, the VL is used to predict the progression of HIV-1 infection, and it has also been used for confirmation of new HIV infection with threshold better than the standard HIV testing pathway [10]. Whereas the use of genomic RNA or NAAT should be widely encouraged for early HIV detection. The only FDA-approved diagnostic NAAT is the Aptima HIV-1 assay (Hologic Inc., San Diego, CA), which can detect RNA with a 100 copies/mL threshold. However, both VL and NAAT are complex, time-consuming, and high cost per test, need well-trained technicians. They cannot be used in small laboratories or community hospitals [11,12]. Other methods have been developed for early diagnostic testing of HIV infection that address the limitations of NAAT, such as reverse-transcription loop-mediated isothermal amplification (RT-LAMP), which requires only a heat block making it a low-cost option with a shorter turnaround time [13,14]. However, the RT-LAMP detection limit was 10^5-10^4 RNA copies/mL, depending on each experiment [15], also rendering it of limited utility in diagnosing for early infection.

LFAs have traditionally been designed for the qualitative and semi-quantitative assessment of various biological markers [16,17]. Here, we propose an assay for detecting the HIV-1 RNA *protease* gene, which plays a crucial role in the maturation of the virus. Our approach combines the use of HIV-1 RT-PCR with a specific forward primer labeled at the 5′-end with dig and a universal reverse primer labeled at the 5′-end with biotin. This allowed us to amplify the DAB that flanks the *protease* genes. Instead of agarose gel electrophoresis, a technique suitable for laboratory settings with limited resources, the amplified products were detected using LFA. The sensitivity of RT-PCR-LFA has been validated in the detection of HIV-1 RNA from clinical samples.

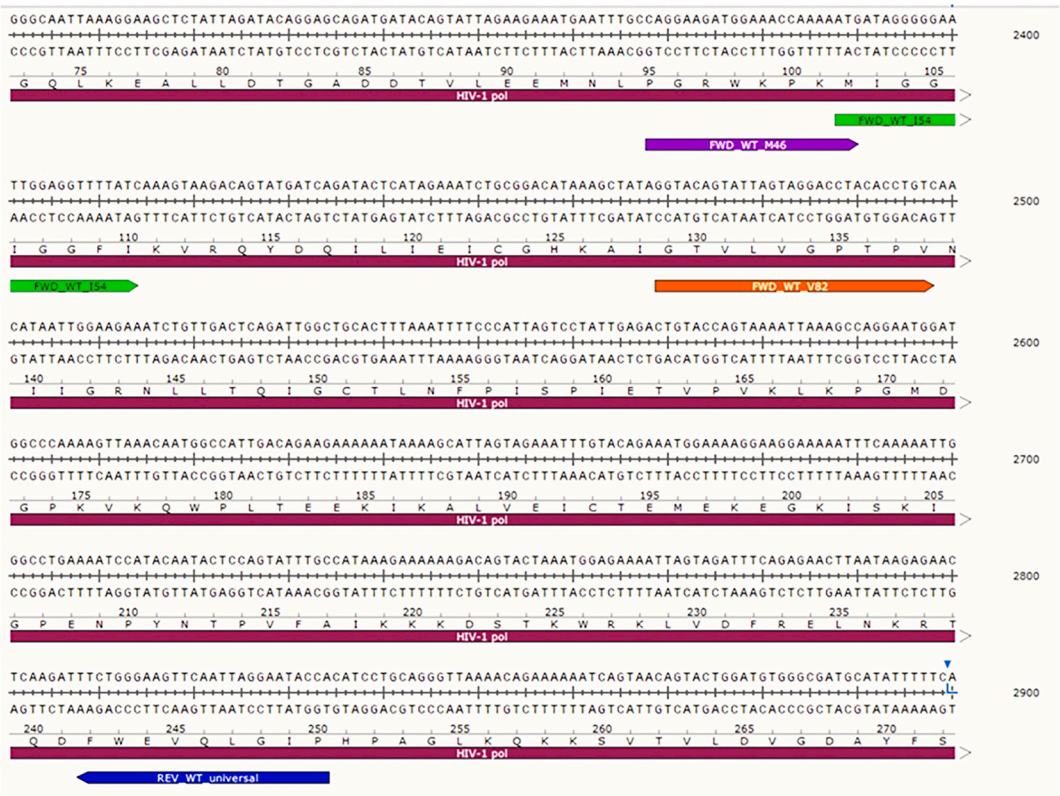

Fig. 1. A schematic representation of the primer design for PCR amplification. Three forward primers are recognized specifically for the HIV-1 wild-type *protease* gene at three points, M46 (purple), I54 (green), and V82 (orange). The reverse primer (blue) is designed for universal amplification. The PCR products of M46, I54, and V82 are 467, 447, and 366 bp, respectively.

2. Materials & methods

2.1. Vectors and samples of study subjects

Plasmid vector pNL4-3 HIV-1 laboratory strain obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) was used as positive control for primer evaluation. Five plasma samples with undetectable VL and plasma samples with known VL of HIV-1 (sample number M458, M525, M554, M771, and M852) were specimens left over from testing of HIV-1 drug-resistant gene and plasma VL using the Abbott RealTime HIV-1 (m2000 platform) and the COBAS AmpliPrep/COBAS TaqMan HIV-1 test (CAP/CTM) at the Clinical Microbiology Service Unit, the PROMT Health Center, Faculty of Associated Medical Sciences, Chiang Mai University. Plasma samples collected between March 2021 and March 2024 were anonymized before use, and consent was not required. To evaluate the LOD of PCR amplification, plasma samples were subjected to a 2-fold dilution before RNA extraction using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). A sample input volume of 140 μL resulted in the extraction of 60 μL of RNA. The extracted RNA in each sample dilution was stored in a −70 °C freezer until further testing. All data collections were processed between March 3, 2022, and April 30, 2024. Ethical review and approval were waived for this study by the ethic committee at Faculty of Medical Technology, Chiang Mai University. The certificate of exemption number: 43/2022 was obtained on February 2, 2022, and will be expired on February 1, 2025.

2.2. Primer design for HIV-1 protease amplification

The sequence of plasmid vector pNL4-3 was used for primer design using the SnapGene® 2.8.3 program. Forward primers were designed to precisely recognize at 3 points, M46, I54, and V82 on the HIV-1 wild-type *protease* gene. A reverse primer was designed only one primer for sharing usage all 3 points as universal amplification. A schematic representation of the primer design used for PCR amplification is shown in Fig. 1. The melting temperatures (Tm), %GC content and annealing temperature of the primers were calculated by Tm Calculator version 1.16.5 available on New England Biolabs websites. The forward primers were labeled 5' terminal with a small tracer as dig, whereas the reverse primer was labeled with biotin at its 5-end. All primers were obtained from Bio Basic Inc., Canada. All the sequences of primers used are summarized in Table 1.

2.3. The optimum conditions PCR for primer evaluation and clinical sample amplification

The amount of plasmid vector pNL4-3 at 50 ng was used as a DNA template for primer evaluation. The PCR condition is performed in Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA) with 2X PCR master Solution [i-Taq TM] (iNtRON Biotechnology, Gyeonggi-do, Korea) with the total reaction volume of 20 μL containing primer 0.5 μM each primer (sense and anti-sense). The cycling parameters were as follows: 1 cycle at 94 °C for 5 min, then 35 cycles at 94 °C for 45 s, 63 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min.

For the clinical sample, RNA was used as a template for the first round PCR using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA). All reactions contained the same maximum volume 10 μL of extracted RNA per reaction and 12.5 μL of 2X Reaction buffer containing 1.2 mM $MgSO_4$, 200 μM each dNTP, 0.4 μM each primer (sense and anti-sense), and 0.5 μL SuperScript™ III RT/Platinum™ Taq High Fidelity Enzyme Mix in total reaction 25 μL. The same reaction mixture, using distilled water (Invitrogen, Grand Island, NY) instead of RNA, was performed as a negative control (NC). For the second round PCR, the reaction contained 2 μL of the first round PCR product in 2X PCR master Solution [i-Taq ™] (iNtRON Biotechnology, Gyeonggi-do, Korea) with the total reaction volume of 40 μL containing primer 0.5 μM each primer (sense and anti-sense). The thermocycling conditions were set the same for both first and second round PCR in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA), but first round PCR of RNA-PCR reverse transcription was set at 1 cycle of 55 °C for 30 min before the routine cycling condition as follow: 1 cycle 94 °C for 5 min, then 35 cycles of 94 °C for 45 s, 63 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 5 min. The PCR products were visualized on a 2 % agarose gel (dissolved in TAE buffer) electrophoresis containing RedSafe™ Nucleic Acid Staining Solution at 95 V for 35 min and was subsequently visualized on a UV transilluminator.

2.4. Production of mSA-colloidal gold conjugates

Colloidal gold was synthesized using Turkevich's method and was prepared in the laboratory as earlier reported [18]. The pET-MBP-mSA2 plasmid mSA gene, 6-histidine tag gene, and maltose-binding protein-monomeric streptavidin (^{6HIS}MBP-mSA) was produced as previously described by Thongkum et al. [19]. In brief, the purified ^{6HIS}MBP-mSA was dialyzed in 5 mM sodium borate

Table 1
primers and modification.

Primer	sequence (5′ to 3′)	Length (bp)	HXB2 location	Modification
FWD_WT_M46	5′-CAGGAAGATGGAAACCAAAAATG-3′	23	2368–2390	5′ dig
FWD_WT_I54	5′-ATGATAGGGGGAATTGGAGGTTTTATC-3′	27	2388–2414	5′ dig
FWD_WT_V82	5′-GGTACAGTATTAGTAGGACCTACACCTGTC-3′	30	2469–2498	5′ dig
REV_WT_Universal	5′-TGGTATTCCTAATTGAACTTCCCAGAA-3′	27	2808–2834	5′ Biotin

buffer, pH 9.0. For the conjugation process, an appropriate concentration of ^{6HIS}MBP-mSA at 500 μg/mL was added to the colloidal gold and incubated for 30 min at room temperature to allow for direct adsorption. Then, the unconjugated gold surfaces were blocked with 5 % BSA (w/v) in 5 mM NaCl and incubated at room temperature for another 30 min. After incubation, the mixer was centrifuged at 4 °C, the supernatant was decanted, and the pelleted nanoparticles were resuspended in 1 % BSA (w/v) in 49 mM Na_2HPO_4. UV–visible spectrophotometry and immunoblotting assay were used to monitor the conjugation of ^{6HIS}MBP-mSA onto gold surfaces.

2.5. Dot blotting immunoassay for amplified HIV-1 protease gene detection

A direct dot blot was used to confirm the quality of ^{6HIS}MBP-mSA-colloidal gold conjugate (^{6HIS}MBP-mSA-CGC). The direct dot blot was performed by dotting biotinylated–IFN–γ (positive control) at concentrations of 10 and 100 μg/mL onto nitrocellulose membranes, along with PBS buffer and biotinylated-BSA at a concentration of 10 μg/mL (test control). To prevent non-specific binding, the strips were subjected to incubation in 5 % (w/v) BSA in PBS buffer (pH 7.4). Then, blocked membranes were washed using PBS buffer. The ^{6HIS}MBP-mSA-CGC (OD 520 nm at 1) was subsequently added to the membrane and incubated on a shaker at room temperature for 30 min. The addition of ^{6HIS}MBP-mSA-CGC resulted in the observation of a positive dot position.

The detection of HIV-1 *protease* gene amplification product containing the DAB at the M46, V82, and I54 positions was first performed by the dot blotting immunoassay. The binding capability between anti-dig mAb (Abcam, UK) to the DAB was tested using a sandwich dot blotting immunoassay. Nitrocellulose membranes were dotted at 1, and 10 μg/mL of anti-dig mAb, 10 μg/mL of anti IFN-γ (B27) mAb, PBS buffer, and 10 μg/mL of biotinylated-BSA, respectively. After washing, the membranes were incubated with a blocking buffer to block non-specific binding for 1 h (h) at 37 °C. The diluted DAB at 1:5, 1:50, and 1:500 was then added into the dotted membranes and incubated for 1 h at room temperature. After 1 h incubation, an excess amount of sample was washed three times with a washing buffer. Then, diluted ^{6HIS}MBP-mSA-CGC at OD 1 was added to the membrane and incubated on a shaker at room temperature for 30 min.

2.6. LFA for HIV-1 protease gene polymerase amplification detection from clinical samples

The principle of the LFA is based on sandwich assays. LFA was developed using ^{6HIS}MBP-mSA-CGC, which was applied onto a glass fiber at conjugated pad zone [19]. Anti-dig mAb and biotinylated-BSA at 1 mg/mL were jetted on laminated nitrocellulose membrane at the test line and control line zone, respectively. LFA assembly was performed as previously described [20]. The conjugated pad and jetted nitrocellulose membrane were subsequently incubated at 37 °C for 4 h and then dried by placing in desiccators at room temperature. The assembly of immobilized nitrocellulose membrane, absorbent pad, conjugate pad, and sample pad was performed, followed by the cutting of the resultant assembly into separate strips by using a strip cutter. The individual LFA was kept in an aluminum package with a small pack of desiccant. Five clinical plasma samples including undiluted and diluted 2-fold for limiting the VL and 5 plasma samples with undetectable VL were extracted for RNA preparation and amplified using primer: 5′ dig_FWD_WT_M46 and 5' Biotin_ REV_WT_Universal with PCR condition as described above. PCR products were visualized on a 2 % agarose gel electrophoresis, and these PCR products were diluted 1: 20 in PBS buffer (200 μL) for detection with an LFA. Then, LFA was dipped into the

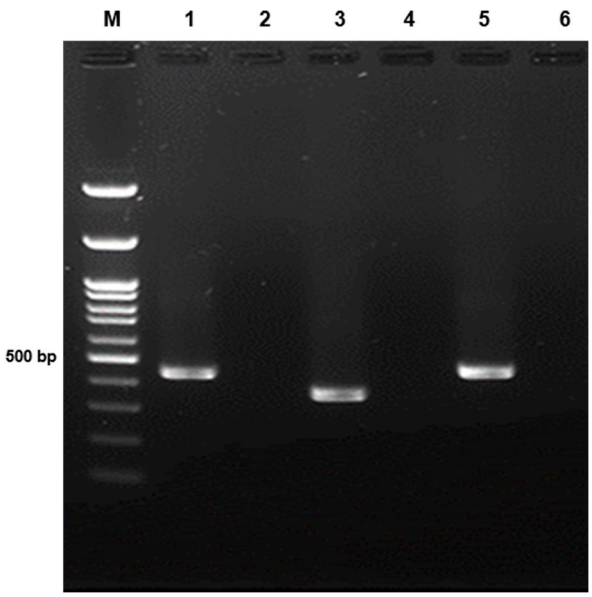

Fig. 2. Analysis of the DAB derived from the *protease* gene regions of HIV-1 pNL4-3, specifically at positions I54, V82, and M46, by 2 % agarose gel electrophoresis. Lane M: Ladder 100 bp, Lane 1: I54 positive PCR product (447 bp), Lane 2: I54 negative PCR product, Lane 3: V82 positive PCR product (366 bp), Lane 4: V82 Negative PCR product, Lane 5: M46 positive PCR product (467 bp), Lane 6: M46 Negative PCR product.

reaction tube containing the PCR products. The interpretation is valid when a band must appear in the control line. Negative result occurs only in control line, whereas two bands at the test and control lines indicate a positive result. The experimental result can be interpreted after red-purple color development in 10 min.

3. Results

3.1. PCR for primer evaluation in detection of HIV-1 gene

For detection of HIV-1 *protease* gene at M46, I54, and V82, three sets of primers were designed with labelling the forward primers at 5′ terminal with dig, whereas the reverse primer was labeled with biotin at 5′-end as shown in Table 1. Plasmid vector pNL4-3 was used as a template for amplification to produce amplified DAB at I54, V82, M46. The PCR products were visualized on 2 % agarose gel electrophoresis containing RedSafe ™ Nucleic Acid Staining Solution at 95 V for 35 min (Fig. 2).

3.2. Verification of ^{6HIS}MBP-mSA-CGC

The conjugation efficiency of ^{6HIS}MBP-mSA-CGC was verified through the generation of red-purple dots at the biotinylated–IFN–γ and biotinylated-BSA positions used as the positive control and test, respectively. No dot was noticed at the position of the dotted PBS buffer (Fig. 3). The intensity of the red-purple dots is associated with the concentration of dotted biotinylated IFN-γ. The presence of red-purple dots at both biotinylated- IFN-γ and biotinylated-BSA positions indicated that ^{6HIS}MBP-mSA had been successfully conjugated.

The binding of anti-dig mAb to target DAB at M46, V82, and I54 positions were performed by sandwich dot blotting immunoassay. The sandwich dot blot assays were comprised of anti-dig mAb, an irrelevant mAb to IFN-γ (anti–IFN–γ (B27) mAb), PBS buffer, and biotinylated-BSA. Purified PCR products at a concentration of 5 ng/mL (Fig. 4) and non-purified PCR products of only the M46 position diluted at 1:5, 1:50, and 1:500 (Fig. 5) was added to each dotted membrane. The results presented that the ^{6HIS}MBP-mSA-CGC formed the complex with amplification positive PCR products which contain DAB, and these complexes were captured with the immobilized anti-dig mAb resulting in generating a red-purple color at the test (anti-dig mAb) and control (biotinylated-BSA) dots as shown in Fig. 4 (strip No. 1,3, and 5). In contrast, in the absence of targeted DAB, ^{6HIS}MBP-mSA-CGC cannot bind to the immobilized anti-dig mAb, but it is only captured at the control dot (Fig. 4, Strip No. 2,4, and 6). Moreover, the results from non-purified PCR products corresponded to those from purified PCR products. All dilutions of amplified DAB (non-purified at dilutions 1:5, 1:50, and 1:500) produced positive dots (Fig. 5, strips No. 1–3) at both anti-dig mAb and biotinylated BSA. Whereas only biotinylated-BSA dots were observed in the non-amplified DAB (Fig. 5, strips No. 4–6). These results confirmed that ^{6HIS}MBP-mSA-CGC specifically binds to target DAB, both purified and non-purified, because of the specific binding capability of the anti-dig mAb to target DAB.

3.3. Comparison of detection of the PCR product at M46 position by agarose gel electrophoresis and LFA in clinical samples

The LFA platform is shown in Fig. 6. The LFA of integrated design is dipped into the reaction mixture. The ^{6HIS}MBP-mSA-CGC

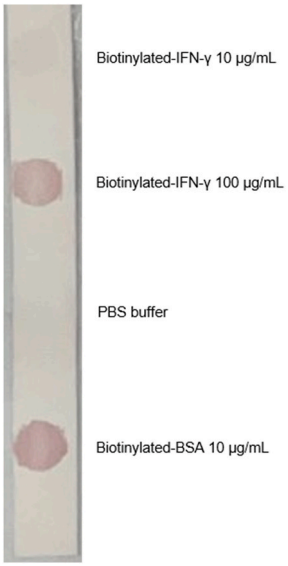

Fig. 3. Verification of ^{6HIS}MBP-mSA-CGC, biotinylated-IFN-γ, and biotinylated-BSA using a dot blotting immunoassay. The binding activity of ^{6HIS}MBP-mSA-CGC was tested by direct interaction with dotted biotinylated–IFN–γ, PBS, and biotinylated-BSA, respectively. The red-purple dot was further observed.

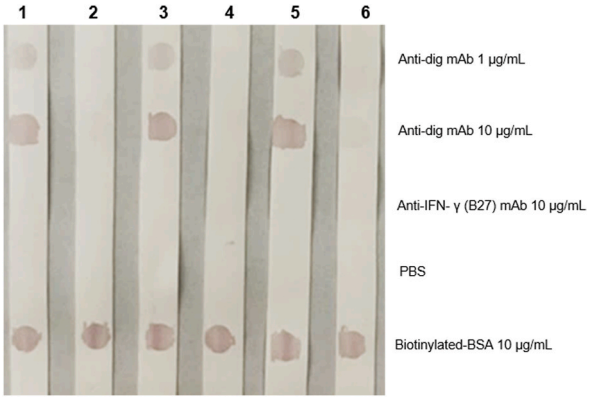

Fig. 4. The binding capability of anti-dig mAb to target purified PCR products at M46, V82, and I54 positions. The dotted membranes were incubated with amplified DAB at M46, V82, and I54 positions (strip No. 1, 3, and 5, respectively) and non-amplified DAB (strip No. 2, 4, and 6 respectively). Then, ^{6HIS}MBP-mSA-CGC were added into the membranes to develop the red-purple dot.

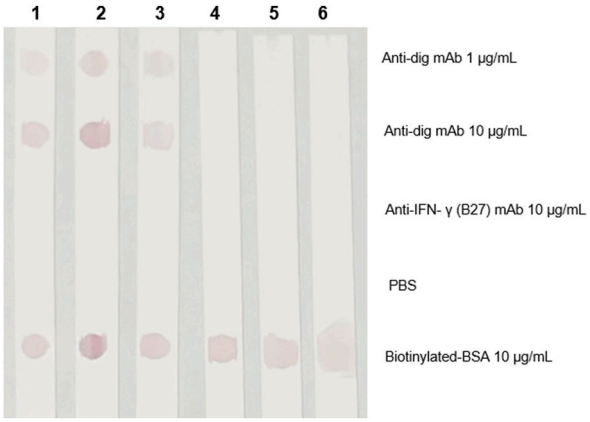

Fig. 5. The binding capability of anti-dig mAb to target non-purified PCR products at M46. The dotted membranes were incubated with amplified DAB (strip No. 1–3) and non-amplified DAB (strip No. 4–6) at dilution of 1:5, 1:50, and 1:500, respectively. Then, ^{6HIS}MBP-mSA-CGC were added into the membranes to develop the red-purple dot.

adsorbing to the conjugated pad reacts with the amplified DAB and migrates upward via capillary action. At the test and control lines, the complex is captured by immobilized anti-dig mAb and biotinylated-BSA respectively, resulting in a red-purple dot color (Fig. 6A). In contrast, in the presence of a non-amplified DAB, an excess of ^{6HIS}MBP-mSA-CGC passes through the test line and is only captured at the control line (Fig. 6B).

To determine the potential of RT-PCR-LFA for early detection of HIV-1 infection in clinical samples, all extracted RNA from serial dilution of plasma samples representing various VL were amplified by PCR using M46-specific labeled primers mentioned above. The detection of agarose gel electrophoresis analysis and LFA for the PCR product at M46 position (*467 bp) were evaluated in 5 clinical samples (M458, M525, M554, M771, and M852) as shown in Fig. 7 (A-E). Agarose gel electrophoresis of M458 sample (VLs at 17450.10 to 8.52 copies/mL), M525 sample (VLs at 3861.00 to 60.33 copies/mL), M554 sample (VLs at 1807.70 to 7.06 copies/mL), M771 sample (VLs from 7189.70 to 56.17 copies/mL), and M852 sample (VLs from 6890.00 to 6.72 copies/mL) and NC were showed in Fig. 7 (1A-1E).PCR products were visualized by agarose gel electrophoresis comparing with LFA. The result showed the consistency of both detection methods that positive results on agarose gel electrophoresis can be detected 2 bands (test and control band), while negative results can be observed only control band by LFA (Fig. 7, 2A-2E). The LOD of amplification and detection on LFA can be observed at VLs: 68.17, 120.66, 56.49, 112.34, and 53.80 copies/mL (mean 82.29 copies/mL) in sample M458, M525, M554, M771, and M852, respectively. The consistent results were obtained from duplicate LFA tests, with the last dilution showing a positive test line, and the subsequent dilution showing a negative test line (data not shown). This confirms the reliable sensitivity of the developed LFA. To verify assay specificity, samples No. 1–5 with undetectable VL were examined. Agarose gel electrophoresis of both first and second round PCR products showed no amplified DAB in these samples, while the sample M771 only displayed a band at 467 bp in the second round. Additionally, LFA was performed on all second round PCR products in duplicate. Only the control band appeared in the negative samples, while the sample M771 displayed both the control and test bands. These results confirm the specificity and consistency of the assay (Supplementary Fig. 1).

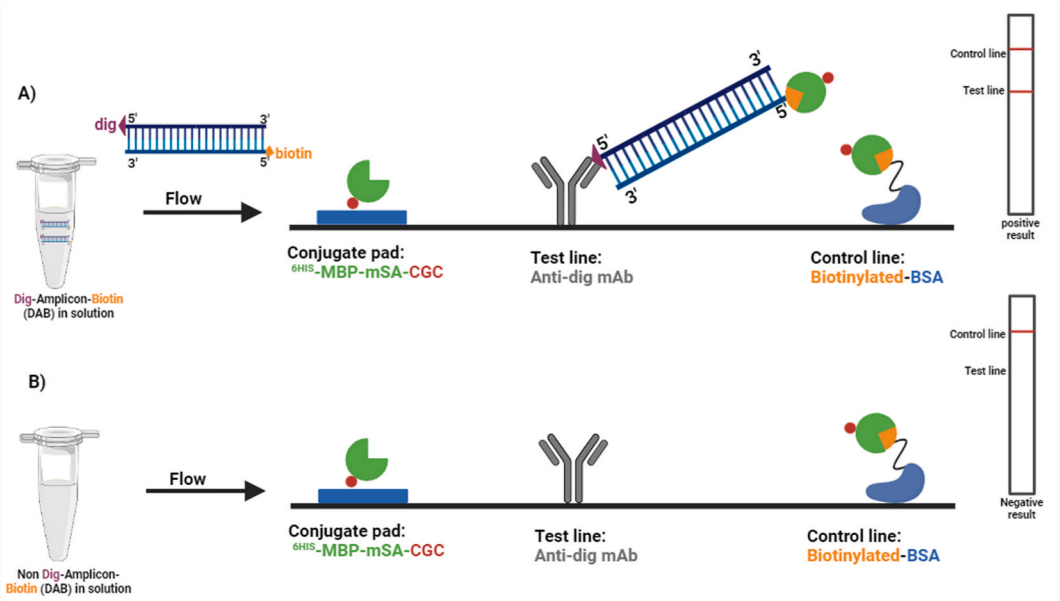

Fig. 6. The LFA platform. Reagent components for detection via LFA, embedded in the flow assay device conjugate pad is colloidal gold conjugated with ^{6HIS}MBP-mSA (^{6HIS}MBP-mSA-CGC). The test and control line are sprayed with anti-dig mAb and biotinylated-BSA, respectively. **(A)** In the presence of an amplicon (Positive result), DAB is bound first by the ^{6HIS}MBP-mSA-CGC and then accumulates at the test band via capture of the anti-dig mAb by its ligand. **(B)** In the absence of an amplicon (Negative result), the conjugated ^{6HIS}MBP-mSA flows to and accumulates at the control line position, where they are captured by biotinylated-BSA.

4. Discussion

The impact of early HIV diagnosis, especially among neonates, can help to identify them for receiving the potential of timely intervention and mitigation of disease progression [4,21] Neonates possess developing immune systems rendering them exceptionally vulnerable to severe disease outcomes [22]. Impounding of p24 by host anti-p24 antibody introduces the immune complex, which may lower the sensitivity of the antibodies of the kit to bind p24 in the sample [23]. Moreover, subtype diversity including HIV-2, non-M and mutation of p24 failed to detect the infection with fourth-generation tests [24,25]. NAAT is employed for routine testing algorithm in some developed worlds by advanced diagnostic laboratories [26,27].The study in South Africa, NAAT has showed a significant number of HIV-infected individuals which are misdiagnosed at points-of-care by follow-up rapid tests [28]. However, all newly HIV infected individuals were identified in pooled NAAT samples to reduce the expense. The RT-PCR-LFA was developed with 10 times lower cost. According to the comparable LOD as NAAT, it should be more applicable for routine diagnosis, particularly in hyper-endemic with resource-limited settings. To mimic the early detection, 5 clinical samples with known VL were serial diluted to access the LOD of RT-PCR-LFA. This RT-PCR-LFA can show the efficiency to detect the surrogate VL at an average of 82.29 copies/mL. Since the detection sensitivity of RT-PCR-LFA is comparable to VL, it should be further validated in the population with high-risk of HIV infection.

Formerly RT-LAMP, were designed to target the sequence of HIV-1 *protease* and *p24* genes [14]. The amplified products was observed by turbidity or color [15]. Since the LOD of RT-LAMP is approximately 10^4 RNA copies/mL, it was not as sensitive as a PCR based assay. Although, it would be improved to enhance the sensitivity by increasing the whole reaction volume [14], and concentrated the sample volume through the capture membrane [29]. Consequently, this disadvantage limits the widespread adoption of RT-LAMP. Therefore, RT-PCR was proposed for amplification of HIV-1 *protease* gene resulting in the remarkable improvement of the detection sensitivity 100 times. In addition, the LFA was introduced to simplify the detection of amplicons without processing agarose gel electrophoresis and gel docking apparatus. LFA has been formerly applied to directly detect HIV-1 *gag* RNA using gold nanoparticle probes (GNPs). However, the detection sensitivity is rather low $11\log_{10}$ copies/mL [30]. More recently, quantum dots-based fluorescent LFA has been employed to detect a little of HIV-DNA remaining in plasma at picomolar level [31,32]. However, techniques are poorly suited for use in low resource laboratories as they require such as fluorescent test strip reader and Raman microscope system. While RT-PCR-LFA offers greater simplicity compared to real-time PCR, it comes with an increased risk of aerosol contamination. This disadvantage is offset by the significant cost and maintenance burden associated with real-time PCR machines, particularly in resource-limited settings. Additionally, although real-time PCR propositions a relatively fast workflow and run time, the overall turnaround time in such regions can be significantly prolonged due to the necessity of sample transportation to central laboratories. The primary objective of this study is to achieve early detection of HIV infection in newborns born to mothers with HIV. In small laboratories, beginning with RNA extraction followed by two steps of PCR and then interpreting the results using a LFA at the point of care may improve screening compared to fourth-generation kits. Accordingly, this developed LFA should facilitate timely intervention

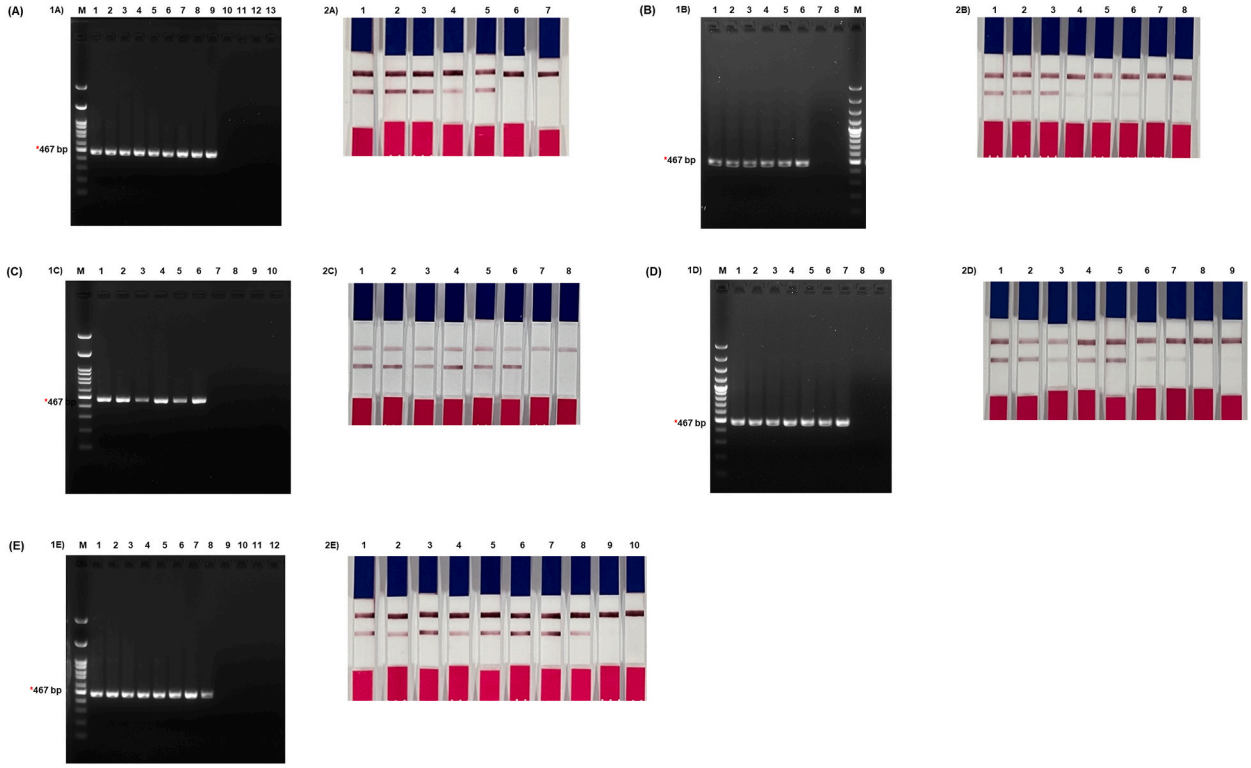

Fig. 7. Detection limit of agarose gel electrophoresis analysis and LFA for the PCR product at M46 position (*467 bp) in 5 clinical samples (M458, M525, M554, M771, and M852). PCR products were analyzed by agarose gel electrophoresis **(1A-1E)** and LFA **(2A-2E)**. **(A)** M458; **1A)**: Lane M: Ladder 100 bp, Lane 1 to 12 (2-folds VLs from 17450.10 to 8.52 copies/mL), Lane 13: NC. **2A)**: No. 1–7 at VL 17450.10, 1090.63, 272.60, 136.33, 68.17, 34.01 copies/mL and NC. **(B)** M525; **1B)**: Lane 1 to 7 (2-folds VLs from 3861.00 to 60.33 copies/mL), Lane 8: NC, Lane M: Ladder 100 bp. **2B)**: No.1-8 at VL 3861.00, 1930.50, 965.25, 484.63, 241.31, 120.66, 60.03 copies/mL and NC. **(C)** M554; **1C)**: Lane M: Ladder 100 bp, Lane 1 to 9 (2-folds VLs from 1807.70 to 7.06 copies/mL), Lane 10: NC. **2C)**: No.1-8 at VL 1807.40, 903.85, 451.92, 225.96, 112.98, 56.49, 28.25 copies/mL and NC. **(D)** M771; **1D)**: Lane M: Ladder 100 bp, Lane 1 to 8 (2-folds VLs from 7189.70 to 56.17 copies/mL), Lane 9: NC. **2D)**: No. 1–9 at VL 7189.70, 3594.85, 1797.42, 898.71, 449.36, 224.67, 112.34, 56.17 copies/mL and NC. **(E)** M852; **1E**): Lane M: Ladder 100 bp, Lane 1 to 11 (2-folds VLs from 6890.00 to 6.72 copies/mL), Lane 12: NC. **2E)**: No. 1–10 at VL 6890.00, 3445.00, 1722.50, 861.25, 430.62, 215.30, 107.60, 53.80, 26.90 copies/mL and NC.

with ART.

In addition to the sensitivity of the LFA developed in this study being comparable to real-time PCR because the amplification of two-round PCR assay increases the PCR product which improves the sensitivity of the LFA for detection by the naked eye. It can be observed that the PCR product of positive control from clinical sample showing positive band only in the second round of PCR amplification. The entire assay, including sample extraction, PCR amplification, and LFA interpretation, took approximately 4 h. It offers key advantages such as significantly lower cost and suitability for settings with limited laboratory infrastructure. Due to the simplified detection process of LFA, it is also suitable for monitoring therapeutic interventions.

In this study, the efficiency of HIV-1 RNA detection in clinical samples was performed only at the M46 position of *protease* gene. However, the other 2 couple primers for amplification encompassing the V82 and I54 position can be applied with our RT-PCR-LFA. Since M46, V82, and I54 are the most common mutation points, the primer design should be further validated its potential for discriminating the mutant strains in clinical samples. This assay platform could be adapted to simultaneously target other HIV-1 genes for increasing the test sensitivity using multiple primer sets.

5. Conclusions

This study describes the development and validation of a high-sensitivity molecular LFA for detecting HIV-1 RNA in clinical plasma samples. The simplified RT-PCR-LFA emerges as a promising tool to enhance access to early diagnosis and treatment for individuals suspected of HIV infection. With LOD comparable to NAATs, the RT-PCR-LFA has the potential to revolutionize HIV diagnosis in resource-limited settings to enhance global efforts in the fight against HIV/AIDS.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Supachai Sakkhachornphop: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Weeraya Thongkum:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Kanokporn Sornsuwan:** Methodology. **On-anong Juntit:** Methodology. **Kittaporn Jirakunachayapisan:** Methodology. **Natedao Kongyai:** Resources. **Chatchai Tayapiwatana:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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