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Optimisation of a Diagnostic LAMP Assay for HIV Monitoring

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Abstract

This study aimed to optimise a colorimetric Loop-mediated Isothermal Amplification (LAMP) assay for sensitive and specific HIV detection in resource-limited, point-of-care settings. Optimisation focused on simplifying the assay for use without professional training, reducing costs, and reducing the inhibitory effects of whole blood components on nucleic acid optimisation. Erythrocyte separation methods were evaluated to enhance colour change observation when using whole blood, while investigating strategies to reduce amplification inhibition in both LAMP and qRT-PCR. The ultimate goal was to develop a rapid, robust, and accessible HIV diagnostic assay suitable for use by individuals with minimal laboratory experience, improving HIV monitoring.

Introduction

Human Immunodeficiency Virus (HIV) is a single-stranded RNA retrovirus with 2 types: HIV-1 and HIV-2, with HIV-1 more prevalent worldwide and HIV-2 more confined to West Africa (Gottlieb et al. 2008, as cited in Hope et al., 2018). HIV infects CD4⁺ T cells through interactions between gp120 glycoprotein on the viral envelope and CD4 receptor on the host cell surface, followed by conformational changes that allow binding to a co-receptor, typically CXCR4 (or CCR5 in early infection) (Hope et al., 2018). The virus then enters the cell through a membrane fusion process and initiates reverse transcription of its RNA genome into DNA, which is then integrated into the host cell's genome. This integration results in a chronic infection that progressively depletes CD4⁺ T cells, compromising the immune system and resulting in Acquired Immunodeficiency Syndrome (AIDS) at the last stage of HIV infection.

Currently, there is no cure for HIV, and it is only treatable via antiretroviral therapy (ART) and broadly neutralising antibodies. Accurate diagnosis of HIV infection is crucial for ART initiation, which can significantly improve the lifespan of infected individuals and reduce further transmission of the virus. In practice, HIV is usually diagnosed via antibody, antigen/antibody, and NATs, with NATs being the gold standard. (Williams et al., 2023).

NATs can identify an infection much earlier, through detecting the virus's genetic material (RNA or DNA) directly, while antibody tests rely on the body producing antibodies, taking weeks or months. Early diagnosis is difficult in infants born to HIV-positive mothers, since they will possess maternal antibodies against HIV from their infected mothers. Nucleic acid tests (NATs) can directly detect the virus in infants, allowing for prompt diagnosis and treatment. Since the RNA load in plasma of infected infants is low, resulting from maternal antibodies, DNA detection would be needed. In addition, NATs can identify an infection much earlier, through detecting the virus's genetic material (RNA or DNA) directly, while antibody tests rely on the body producing antibodies, taking weeks or months. NATs can also be used for virus titre monitoring in case of rebound infection under ART.

While q(RT)-PCR methods are currently used as Gold Standard in HIV diagnosis, among NATs, Loop-mediated Isothermal Amplification (LAMP) emerges as a promising tool for viral infection diagnosis, especially in point-of-care (POC) settings. LAMP is a nucleic acid amplification technique that amplifies DNA and RNA with high specificity and efficiency under isothermal conditions. There are several types of LAMP assays, including fluorescence LAMP and those measuring the degree of turbidity. However, colorimetric LAMP remains advantageous over traditional PCR and other LAMP assays in terms of simplicity, speed, and reduced equipment requirements. H⁺ ions are released through incorporation of dNTPs to synthesised DNA, acidifying the reaction solution as more DNA is synthesised. The presence of Phenolred, a pH indicator, turns the reaction from yellow before nucleic acid amplification

(when the solution is alkaline) to pink after nucleic acid amplification (when the solution is acidic). It is difficult to use blood in LAMP, since erythrocytes make it difficult to see any colour change, with components in the plasma also impairing LAMP. Hence, different methods and additives to improve the reaction have been tested in this project.

Materials and Methodology

Samples

Previously extracted HIV DNA and RNA from an HIV+ cell line were diluted in nuclease-free water.

Colorimetric LAMP Assay

We followed the protocol from the New England Biolab (NEB, Ipswich, USA) colourimetric LAMP assay (M1800) with brief changes. All reagents were thawed and pipetted at room temperature, with 1 μ L of sample being pipetted per 25 μ L reaction, and the reaction was incubated for 20-60 minutes at 63 °C using Applied Biosystems™ SimpliAmp™ Thermal Cycler. The reaction was determined as positive if there was a change from pink to yellow colour after incubation. Aspirated plasma samples are boiled at 95 °C for 5 minutes before analysis.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

qRT-PCR was carried out using the BIO-RAD CFX96 C1000 Touch Real-Time PCR with Takara PrimeDirect RT-PCR kit according to the manufacturer's protocol. The reaction mix included 12.5 μ L of 2x Takara PrimeDirect RT-PCR mix, 0.625 μ L of both forward and reverse primers LTRTaql and LTRTaqr (10 μ M), 0.31 μ L of LTRTaqp probe (FAM, 10 μ M), 8.94 μ L of nuclease-free water, and 2 μ L of sample, with a total volume of 25 μ L. The reaction was initially incubated at 90°C for 3 minutes, followed by 60°C for 5 minutes. Then, 45 cycles were performed, each with a denaturation step at 95°C for 5 seconds and an annealing/extension step at 60°C for 30 seconds.

Erythrocyte Removal Studies

Whole blood was collected from healthy controls in K2 EDTA tubes. Dextran, a polysaccharide, is added for the sedimentation of erythrocytes, while phytohemagglutinin-E (PHA-E) is added for erythrocyte aggregation.

Whole blood (undiluted and blood diluted 1:1 with PBS) was added to dextran solutions to make up final dextran concentrations of 1.5mg/mL and 3mg/mL. The solution is then allowed to stand at room temperature for 15 to 30 minutes. Plasma is then aspirated for use in LAMP and qRT-PCR.

Whole blood was added to phytohaemagglutinin-E (PHA-E) solutions to make up final dextran concentrations of 20 μ g/mL, 30 μ g/mL, 40 μ g/mL, and 50 μ g/mL. The solution is then allowed to stand at room temperature for 5 to 40 minutes.

Erythrocytes Removal via PHA-E

To remove red blood cells, undiluted blood and blood diluted 1:1 with PBS were treated with concentrations of PHA-E of 0, 20, 30, 40, and 50 $\mu\text{g}/\text{mL}$ in a round-bottom 96-well plate (Figure 2). Visual assessment of erythrocyte aggregation was performed at 5, 15, and 40 minutes. The results indicated that a PHA-E concentration of 30, 40, and 50 $\mu\text{g}/\text{mL}$ in undiluted blood yielded aggregation at 40 minutes, as observed by accumulation of aggregated erythrocytes at the bottom of the plate.

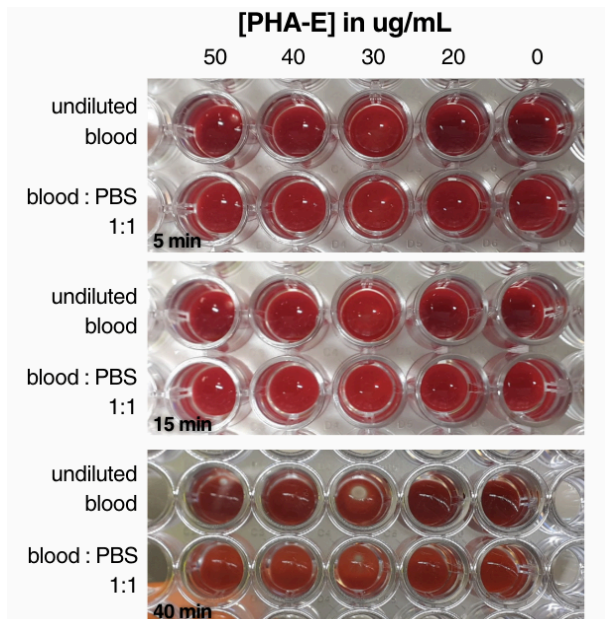


Figure 2. Whole blood incubated at room temperature conditions with PHA-E of 50 $\mu\text{g}/\text{mL}$, 40 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$, 20 $\mu\text{g}/\text{mL}$, 0 $\mu\text{g}/\text{mL}$ (from left to right), PHA-E for 5, 15, 40 minutes. No HIV nucleic acids are added.

A subsequent experiment was conducted to further optimize the blood-to-PHA-E ratio (Figure 3). Since the volume of reagents used could dilute the sample containing DNA and RNA, we would like to test whether using diluted blood would make a difference in erythrocyte aggregation. On the other hand, diluted blood could also make it easier for supernatant aspiration.

Since PHA-E is relatively expensive, the solutions were made up to a final PHA-E concentration of 30 $\mu\text{g}/\text{mL}$ and undiluted blood and blood diluted 1:1 with PBS were mixed with PHA-E at different ratios (PHA-E to blood volume ratio of 25:25, 20:30, 10:40, and 5:45 μL). Smaller and narrower tubes were used for clearer separation of plasma and erythrocytes. Visual assessment of erythrocyte aggregation was performed at 5 and 35 minutes. The results demonstrated that PHA-E effectively aggregated erythrocytes at a PHA-E to blood volume ratio of 25:25, 20:30 μL at 35 minutes, resulting in a visible separation of erythrocytes from the plasma.

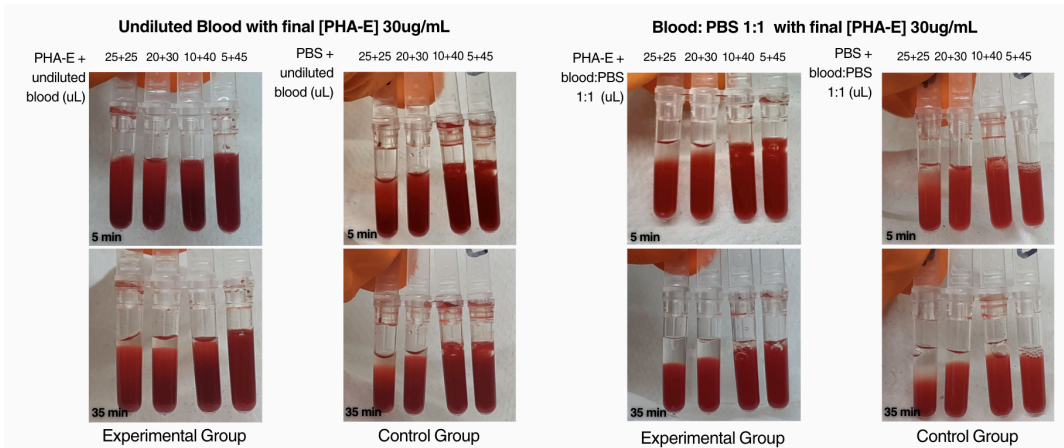


Figure 3. Whole blood incubated at room conditions with PHA-E of 30 µg/mL for 5 and 35 minutes

Erythrocyte Sedimentation via Dextran

Whole blood was spiked with HIV of different dilutions (1:10, 1:100, 1:1000), then mixed with dextran to make final dextran concentrations of 1.5 mg/mL and 3mg/mL (Figure 5). Undiluted blood and dextran are added at ratios of 50+50 µL, 75+25 µL, and 90+10 µL (Figure 5). Observations at 15 minutes revealed similar sedimentation efficiency with both dextran concentrations (Figure 4). Plasma aspiration was feasible after 15 minutes for all wells. The undiluted blood + dextran ratio of 90+10µL was deemed optimal, as it effectively minimized blood dilution while achieving adequate erythrocyte sedimentation.

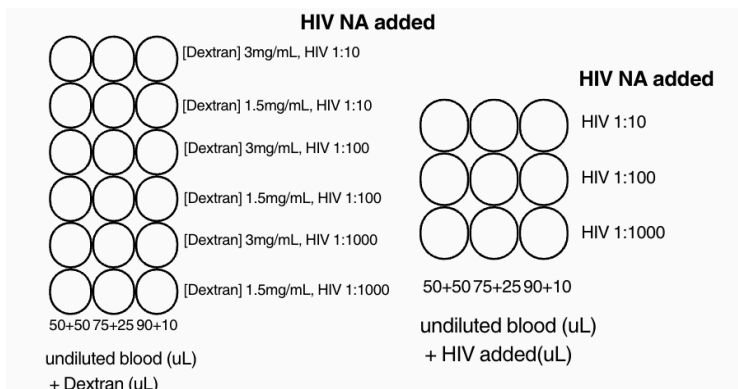
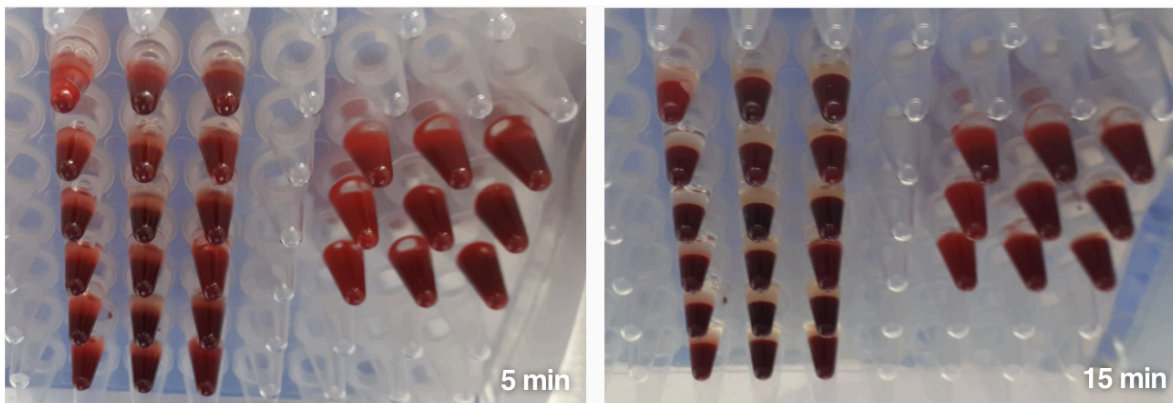


Figure 4 (up) & 5 (down). Sedimentation of HIV-spiked whole blood using dextran. Dextran concentrations and HIV dilutions are illustrated in Figure 5.

LAMP with Plasma Spiked with HIV Nucleic Acids

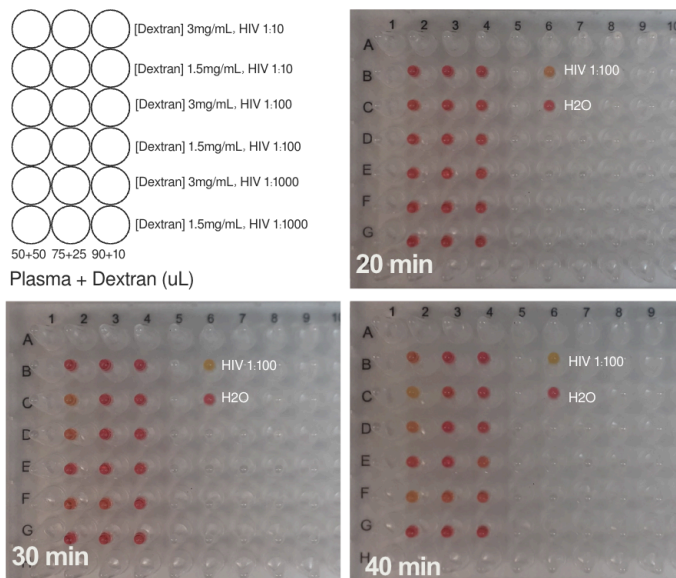


Figure 6. LAMP results for plasma spiked with HIV and various dextran concentrations, HIV dilutions, and plasma/dextran volumes at 20, 30, and 40-minute time points.

LAMP was performed on samples spiked with extracted HIV nucleic acids obtained after Dextran sedimentation. The experiment tested various concentrations of dextran (3mg/mL and 1.5mg/mL) and HIV dilutions (1:10, 1:100, and 1:1000) in combination with different volumes of plasma and dextran mixtures (50+50 μ L, 75+25 μ L, and 90+10 μ L). Visual assessment of the LAMP reactions was conducted at 20, 30, and 40-minute time points.

As shown in Figure 6, the results obtained were unreliable. Positive reactions, indicated by a color change, were observed in some wells, but the pattern was inconsistent across different dextran concentrations, HIV dilutions, and plasma/dextran ratios.

The presence of high-density dextran may be interfering with the LAMP assay. It is also possible that the combination of dextran and plasma components is inhibiting the LAMP reaction. Further optimization of the assay conditions, such as adjustments of dextran concentration and plasma volume, may be necessary to improve the reliability and sensitivity of the LAMP assay for HIV detection in plasma samples.

Reversal of Plasma Inhibition in LAMP of Dextran-treated Samples

Subsequent experiments focused on mitigating plasma inhibition and optimizing reagent concentrations. This involved testing the effects of several additives – MgCl₂, DMSO, and BSA – that could enhance LAMP amplification or counteract plasma inhibition. MgCl₂ provides essential magnesium ions (Mg²⁺) as a cofactor for DNA polymerase activity and proper primer binding (Gunilla et al., 2014). DMSO reduces secondary structures in DNA templates, especially GC-rich regions, facilitating primer annealing (Hardjasa et al., 2010). Furthermore, BSA stabilizes PCR and LAMP reactions by binding inhibitors and protecting DNA polymerase from degradation (Islam & Koirala, 2022).

Hence, combinations of different concentrations of DMSO, MgSO₄, and MgCl₂ were tested to identify optimal concentrations for robust amplification of nucleic acid in the presence of plasma (Figure 7,8).

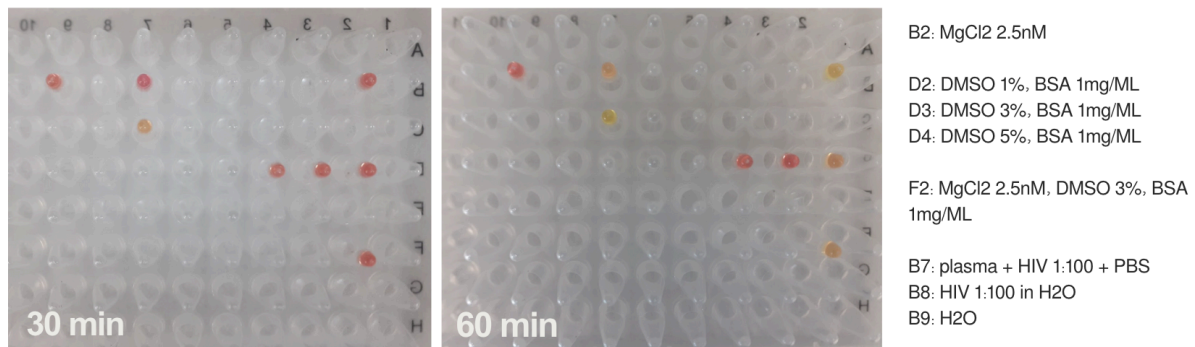


Figure 7. LAMP assay results for HIV detection in HIV-spiked plasma with additives DMSO, BSA and MgCl₂. The experiment included HIV 1:100 dilutions as a sample and various combinations of DMSO, BSA and MgCl₂ concentrations. The figure shows reaction wells at 30 and 60 minutes of incubation at 63 °C.

Visual assessment of the LAMP reactions at 30 and 60 minutes revealed that the addition of MgCl₂, DMSO, and BSA could partially reverse the plasma inhibition observed in the initial experiments. Wells containing MgCl₂, particularly at a concentration of 2.5 nM, exhibited a more pronounced color change indicative of amplification compared to wells without these additives. While DMSO and BSA also showed some improvement in amplification, their effect appeared less pronounced than that of MgCl₂. Specifically, wells containing DMSO 3%, BSA 1mg/mL and DMSO 5%, BSA 1mg/mL remained pink with no nucleic acid amplification.

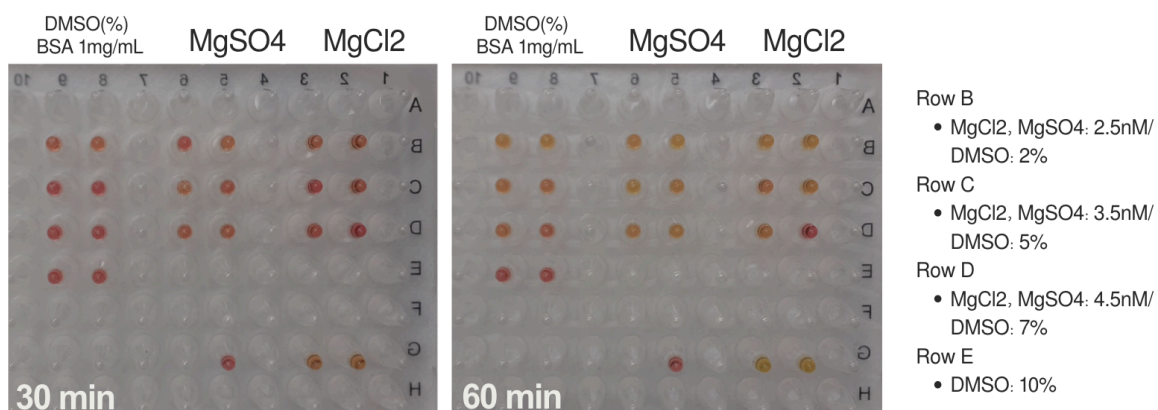


Figure 8. LAMP assay results for HIV detection in HIV-spiked plasma with additives DMSO, BSA, MgCl₂, and MgSO₄. The experiment included HIV 1:100 dilutions as a sample and various

combinations of DMSO, BSA, MgCl₂ and MgSO₄ concentrations. The figure shows reaction wells at 30 and 60 minutes of incubation at 63 °C.

Further optimization of reagent concentrations indicated that a combination of 2% DMSO and 1 mg/mL BSA, along with 2.5 nM MgSO₄ and MgCl₂, yielded the most consistent and robust amplification signals. Generally, MgSO₄ outperformed MgCl₂ in amplification, suggesting that sulfate ions may have enhanced Mg²⁺ performance as a cofactor for DNA polymerase activity and proper primer binding.

Discussion

Dextran sedimentation offers a simple and cost-effective method for erythrocyte sedimentation and plasma aspiration, requiring minimal equipment and technical expertise. The 15-minute sedimentation time is advantageous for rapid plasma aspiration while the cost-effectiveness of dextran also makes it attractive for resource-limited settings. However, the RT-LAMP studies revealed a significant drawback: dextran inhibits amplification. This inhibition, observed through inconsistent color changes in our assays, suggests that dextran must be removed or neutralized before LAMP can be reliably used with dextran-prepared plasma, potentially due to interference with polymerase activity or nucleic acid binding.

PHA-E aggregation effectively removes erythrocytes. However, PHA-E is more expensive than dextran and requires a longer incubation time of 35 minutes. LAMP on HIV+ samples should be performed with PHA-E to explore whether PHA-E impacts LAMP and increases the limit of detection.

Given the observed inhibitory effects of dextran and the potential for similar inhibition from PHA-E or other blood components, further optimization of the LAMP assay is crucial. Specifically, future research should focus on systematically evaluating the optimal concentrations of MgCl₂, MgSO₄, DMSO, and BSA in combination with both dextran and PHA-E-prepared samples to identify conditions that maximize amplification efficiency and minimize inhibition in LAMP. Until such optimization is complete, caution should be exercised when interpreting LAMP results obtained with plasma prepared using erythrocyte removal methods, and appropriate controls should be included to monitor for potential inhibitory effects.

Future Directions

Having assessed the suitability of Dextran sedimentation for erythrocytes removal in LAMP, future studies should focus on evaluating the efficacy of PHA-E aggregation for erythrocytes removal before conducting LAMP, and compare both methods

The next step would be spiking blood with non-extracted HIV+ cells with or without cell culture supernatant. Furthermore, the limit of detection (LOD) of the optimized LAMP assay should be determined through conducting digital PCR in plasma samples.

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