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Development and validation of a multienzyme isothermal rapid amplification and lateral flow dipstick combination assay for HTLV-1 proviral DNA detection

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Abstract

Background Human T-cell lymphotropic virus-1(HTLV-1) infection may lead to adult T-cell leukemia, HTLV-associated myelopathy/tropical spastic paraplegia, and various neurological diseases. Therefore, developing a rapid and accurate detecting method is crucial for preventing and controlling HTLV-1 infection. This study aims to develop and validate a detection assay for HTLV-1 proviral DNA by combining multienzyme isothermal rapid amplification(MIRA) and lateral flow dipstick(LFD) techniques.

Methods Primers and probes were designed based on the conserved sequence of the HTLV-1 Tax gene, and the MIRA-LFD method was established and optimized. Different concentrations of HTLV-1 plasmids were tested by the MIRA-LFD assay to verify the limit of detection(LOD) of the method. To evaluate the cross-reactivity, viral pathogens were detected by this assay, including hepatitis B virus(HBV), hepatitis C virus(HCV), hepatitis E virus(HEV), cytomegalovirus(CMV), herpes simplex virus-1/2(HSV-1/2), Epstein-Barr virus(EBV), Parvovirus B19(B19V), and human T-cell lymphotropic virus-2(HTLV-2). 500 clinical samples were simultaneously detected using real-time PCR(qPCR) and MIRA-LFD methods, and the consistency between the two methods was statistically analyzed. The qPCR was used as the reference method to determine the diagnostic sensitivity and specificity of the MIRA-LFD method.

Results The MIRA-LFD method can detect HTLV-1 within 20 min at 37℃. The LOD for HTLV-1 using this method was 200 copies/μL. This method had no cross-reaction with the other eight viruses, with good specificity. Using qPCR as the standard, the diagnostic sensitivity and specificity of the MIRA-LFD method for 500 clinical samples were 100%. The MIRA-LFD and qPCR methods had 100% consistency(kappa value = 1.00).

Conclusions This study established a method based on MIRA-LFD for detecting HTLV-1 proviral DNA, which has the advantages of fast, accurate, good sensitivity, strong specificity, simplicity, and portability. This method meet the needs of rapid on-site detection and is easy to promote and use in grassroots medical institutions or blood stations.

Keywords Multienzyme isothermal rapid amplification, Lateral flow dipstick, Human T-cell lymphotropic virus-1, Rapid detection

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Background

Human T-lymphotropic virus(HTLV) is the earliest discovered human retrovirus, including four main types: HTLV-1 to 4, with HTLV-1 and HTLV-2 being the most common [1, 2]. HTLV infects almost 10–20 million individuals in the world [3]. HTLV-1 is endemic in the Caribbean, northeastern South America, southwestern Japan, and certain regions of Africa. There are also small outbreaks or sporadic outbreaks of HTLV-1 in southeastern China [4–6]. Human infection with HTLV-1 often presents as asymptomatic carriers with an incubation period of over 20 years. In the later stages, it may cause adult T-cell leukemia, HTLV-associated myelopathy/tropical spastic paraplegia, and various neurological diseases. In severe cases, it can lead to death [7, 8]. The main transmission pathways of HTLV-1 include sexual transmission, mother-baby transmission, and blood transmission [9, 10]. The transmission of HTLV-1 through blood is mainly due to the infusion of blood products containing lymphocytes infected with HTLV-1 [11]. At present, blood transfusion has become the main effective transmission route of HTLV-1, which has made HTLV-1 one of the factors threatening the health of blood recipients [12]. Therefore, developing a simple, fast, and accurate HTLV-1 laboratory detection method is particularly important for screening blood donors and clinical patients.

HTLV-1 antibodies are serological responses produced by the human body stimulated by the HTLV-1 virus. At present, enzyme-linked immunosorbent assay(ELISA) is mainly applied to screen for HTLV-1 antibodies, but it has some shortcomings. Firstly, the window period for antibody detection is relatively long, making it easy to miss detection. The second issue is the existence of more false positive results [13, 14]. Thus, for specimens with positive HTLV antibody screening, further validation is performed using western blot(WB), line immunoassay(LIA), or nucleic acid testing. WB and LIA are expensive and generally provide uncertain results, while nucleic acid testing has become a more suitable method for confirming HTLV-1 [15–17]. At present, there is no commercial reagent kit approved by National Medical Products Administration(NMPA) for detecting HTLV-1 nucleic acid. Therefore, it is necessary to develop HTLV-1 nucleic acid screening reagents with good sensitivity and specificity.

Molecular diagnostic techniques represented by real-time PCR(qPCR) are considered powerful diagnostic tools due to their high sensitivity and strong specificity. However, they have strict requirements for experimental instruments, environment, and the technical level of operators, and are not suitable for promotion and use in grassroots medical institutions [18]. Moreover, qPCR testing is time-consuming and complex [19]. Recently, many isothermal amplification techniques have been developed for detecting pathogens, such as recombinase polymerase amplification(RPA), loop mediated isothermal amplification(LAMP), multiple cross displacement amplification(MCDA), strand displacement amplification(SDA), polymerase spiral response (PSR), and others [20–24]. Multienzyme isothermal rapid amplification(MIRA) technology is a new nucleic acid rapid isothermal amplification technique, similar to RPA. This technology realizes rapid nucleic acid amplification relying on a variety of functional proteins(single-stranded binding(SSB) protein, recombinase, DNA polymerase, etc) in synergy [25]. This method can be finished within 5–20 min under isothermal conditions of 25–42℃, with simple operation and no need for precise instruments [26]. It can be implemented with one water bath or constant temperature instrument. In addition, by combining MIRA technology with lateral flow dipstick(LFD), the detection results can be visualized by observing the bands with the naked eye [27]. The working principle of MIRA-LFD technology is shown in Fig. 1 [28, 29]. Firstly, the complex formed by the combination of primer and recombinase searches for complementary regions on the target sequence. Then SSB opens the dsDNA, and the complex invades the dsDNA template, forming a D-Loop region at the invasion site. When the primer-recombinase complex finds the target region complementary to the primer, the recombinase is removed, and the DNA polymerase binds to the 3' end of the primer. Simultaneously, probe binds to complementary DNA template. Nucleic acid exonuclease IV(nfo) recognizes and cleaves THF on the probe, and DNA polymerase binds to the exposed 3'-OH end of the probe, initiating the extension of the strand to form the amplicon labeled with both 6-carboxyfluorescein(6-FAM) and biotin simultaneously. Since the test line of the LFD strip is marked with antibody against biotin, and the control line is marked with anti-rabbit antibody, as well as antibody against 6-FAM on colloidal gold particles, when using the LFD strip to detect the MIRA products, a complex of antibody against biotin/biotin-amplicon-6-FAM/antibody against 6-FAM-colloidal gold is formed on the test line of the LFD strip to visualize the results of gene amplification products detection.

Compared with conventional PCR methods, MIRA-LFD technology has the advantages of being fast, easy to operate, result visualization, strong specificity, good sensitivity, low cost, no need for complex equipments and trained technicians, and suitable for on-site rapid detection [29, 30]. Therefore, it is widely used in the rapid detection of pathogens [30–32]. To date, there have been no reports of applying this method to HTLV-1 detection. In this study, we established a novel method for detecting HTLV-1 based on a combination of MIRA and

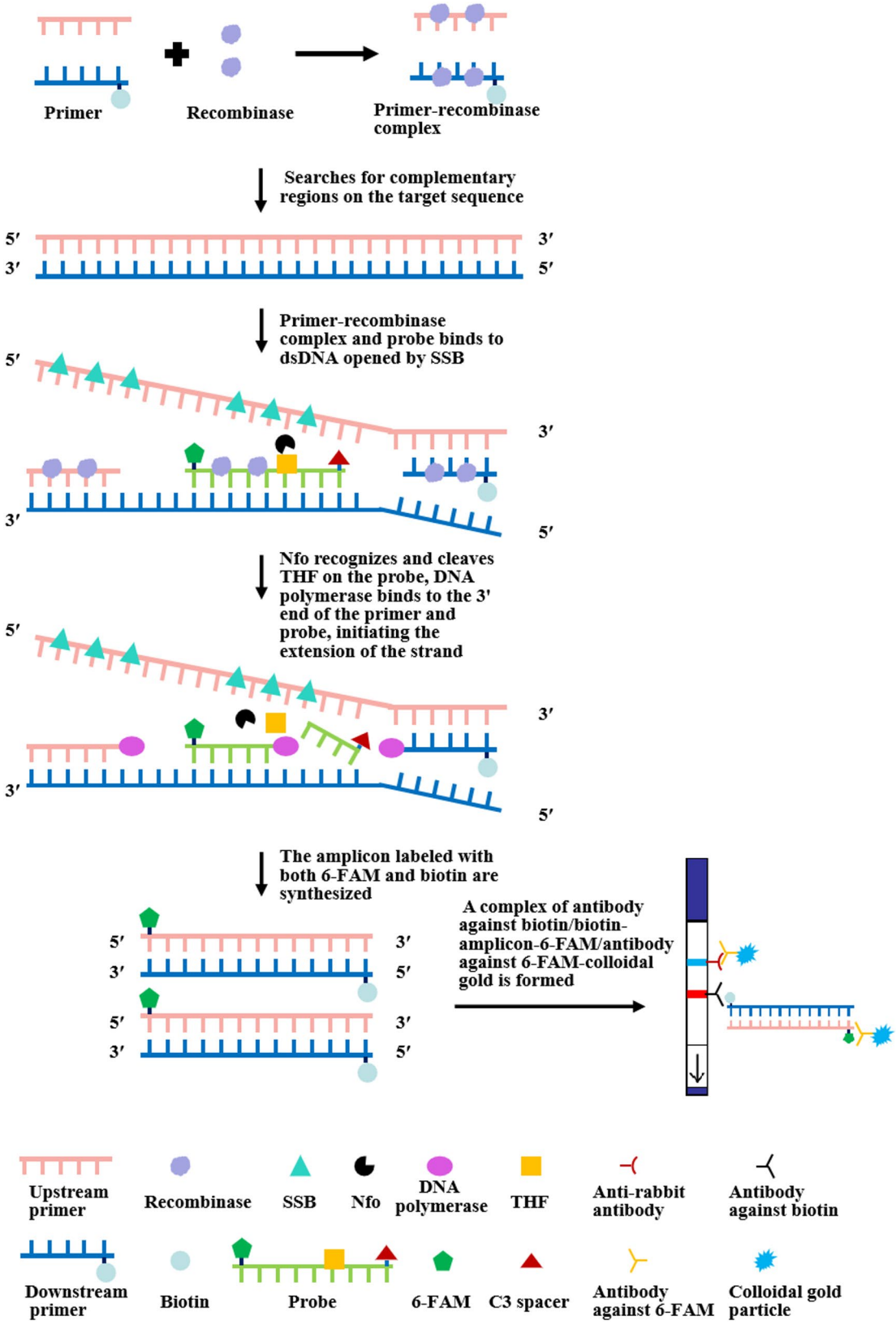

Fig. 1 The working principle of MIRA-LFD technology

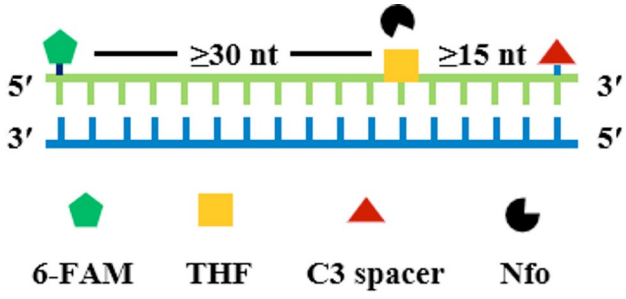

Fig. 2 Schematic diagram of probe for the MIRA-LFD assay

LFD techniques, which meets the requirements of efficient and convenient detection and can provide technical support for the prevention and treatment of HTLV-1 infection.

Methods

Clinical samples and nucleic acid preparation

From 2022 to 2023, whole blood samples from 500 inpatients were collected from The First Affiliated Hospital, Zhejiang University School of Medicine. The positive whole blood samples for hepatitis B virus(HBV), hepatitis C virus(HCV), hepatitis E virus(HEV), cytomegalovirus(CMV), herpes simplex virus-1/2(HSV-1/2), Epstein-Barr virus(EBV), and Parvovirus B19(B19V) were obtained from The First Affiliated Hospital, Zhejiang University School of Medicine. Viral nucleic acids from the whole blood samples were extracted using DNA/RNA Extraction Kit II(Geneaid Biotech, Taiwan, China) in accordance with the manufacturer's instructions. The extracted nucleic acids were stored at − 80 °C until the assays were performed.

Primers and probes design

By comparing the full-length gene sequences of HTLV-1 available in GenBank, it was found that the Tax region of this gene is highly conserved. Thus, according to the MIRA-LFD technique primer/probe design principle, primer pairs and probes for the MIRA-LFD assay were designed targeted the Tax sequence of HTLV-1(accession no.:J02029). The primer length is 30–35 bp(ideally no less than 25 bp), the GC content is between 30% and 70%, and a biotin group needs to be labeled at the 5 'end of downstream primers. As shown in Fig. 2, the probe was designed between the upstream and downstream primers. 6-FAM was added to the 5′-end of the probe sequence, with tetrahydrofuran(THF) labeled in the center(as an identification site for nfo), and a C3 spacer was added to the 3′-end. The sequences of all primer pairs and probes were listed in Table 1 and were synthesized by Sangon Biotech Co., Ltd.(Shanghai, China).

Plasmid standard synthesis

A gene fragment containing the Tax region of HTLV-1 was cloned into the pUC57 plasmid. According to the formula(copies/μL=[6.02×10^{23} x plasmid concentration (ng/μL) x 10^{-9}]/[plasmid length x 660]), the plasmid concentration was converted to copy number, then stored at -80 °C.

Establishment of the MIRA-LFD assay

DNA Constant Temperature Rapid Amplification Kit(Anpu Future, Nanjing, China) was used to perform the MIRA-LFD assay. A 50 μL reaction mixture was prepared by mixing 29.4 μL of A buffer, 2.5 μL of B buffer, 2 μL(10 mM) of forward primer, 2 μL(10 mM) of reverse primer, 0.6 μL(10 mM) of probe, 5 μL of DNA template, and 8.5 μL of double distilled water(ddH_2O). Then, the reaction tube was shaken and mixed evenly, and immediately incubated in a water bath. 8 μL of the product was collected after incubation and mixed uniformly with 152 μL of ddH_2O. The LFD strip is then inserted into the above mixture for interpreting result within 5 min. Each

Table 1 Primer pairs and probes for detecting HTLV-1 used in this study

Primers/probes	Sequence (5′–3′)	Positions*	Product size(bp)
HTLV-1-1-Forward	AGACCCCGGACTCCGGCCCCAAAACCTG	7722–7749	363
HTLV-1-1-Reverse	Biotin-GGCGTGCCATCGGTAAATGTCCAAATAA	8084 – 8057	
HTLV-1-1-Probe	6-FAM-GCCAGCTCGGGGCCTTCCTCACCAATGTTC[THF] CTACAAGCGAATAGA-C3 Spacer	7850–7895	
HTLV-1-2-Forward	TCCTCCTTTATATTTCACAAATTTCAAACC	8140–8169	171
HTLV-1-2-Reverse	Biotin-GTCATTGTCATCTGCCTCTTTTTCGTTA	8310 – 8283	
HTLV-1-2-Probe	6-FAM-TCCTTTCATAGTTTACATCTCCTGTTTGAA[THF]AATACACCAACATCCC-C3 Spacer	8221–8267	
HTLV-1-3-Forward	CTAAGACCCTCAAGGTCCTTACCCCGCCAATC	7571–7602	326
HTLV-1-3-Reverse	Biotin-TTCTATTCGCTTGTAGGGAACATTGGTGAG	7896 – 7867	
HTLV-1-3-Probe	6-FAM-CCCTTCCGAAATGGATACATGGAACCCACC[THF]TTGGGCAGCACCTCC-C3 Spacer	7660–7705	
HTLV-1-4-Forward	GGACATTTACCGATGGCACGCCTATGAT	8063–8090	213
HTLV-1-4-Reverse	Biotin-GAGAAATGGGGATGTTGGTGTATTCTTC	8275 – 8248	
HTLV-1-4-Probe	6-FAM-TCCTCCTTTATATTTCACAAATTTCAAACCA[THF]GGCCTACCACCCCTC-C3 Spacer	8140–8186	

*Nucleotide positions according to HTLV-1 sequence(J02029)

test was conducted with ddH_2O as the negative control and HTLV-1 nucleic acid as the positive control.

Optimization of temperature and time

To determine the appropriate temperature and time, gradient optimization was conducted at different reaction temperatures of 30 °C, 37 °C, and 42 °C, and times of 5, 10, 15, 20, and 25 min.

LOD analysis

To determine the limit of detection(LOD) of the MIRA-LFD reaction, the HTLV-1 plasmids were diluted to 10^7, 10^6, 10^5, 10^4, 10^3, 500, 200, and 100 copies/μL and used as templates for detection using this method.

Specificity analysis

The specificity of the MIRA-LFD assay was determined by detecting nucleic acids of HBV, HCV, HEV, CMV, HSV-1/2, B19V, and EBV, as well as HTLV-2 plasmid DNA. Since the nucleic acids of HCV and HEV are RNA, RNA Constant Temperature Rapid Amplification Kit(Anpu Future, Nanjing, China) was used to perform the MIRA-LFD assay during specificity validation.

Detection of clinical samples and statistical analysis

To determine the practical application potential of the MIRA-LFD assay for detecting HTLV-1, 500 clinical samples were detected. To compare the MIRA-LFD method to qPCR, clinical sensitivity, specificity, and consistency were evaluated by SPSS 26.0(IBM, New York, NY). The concordance rate of MIRA-LFD and qPCR in detecting HTLV-1 was analyzed by kappa test.

Results

Schematic mechanism of the MIRA-LFD assay

The workflow of this assay for testing HTLV-1 is shown in Fig. 3. In short, HTLV-1 DNA was extracted from the whole blood samples, placed in the same test tube as MIRA reagents, and shaken and mixed. The above mixture was amplified in a water bath at 37 °C for 15 min. After amplification, the amplification product is detected by the LFD strip. Determining the test result by observing the color change of the LFD strip. A blue strip located on the quality control line and a red strip located on the test line simultaneously appear on the LFD strip, indicating a positive result. A blue band appears on the quality control line of the LFD strip, while there is no band on the test line, indicating a negative result. If there is no strip on both the control line and the test line, it indicates that the result is invalid and needs to be retested.

Validation of primers and probes

To screen suitable primers/probes for the MIRA-LFD assay, four pairs of primers/probes(HTLV-1-1,

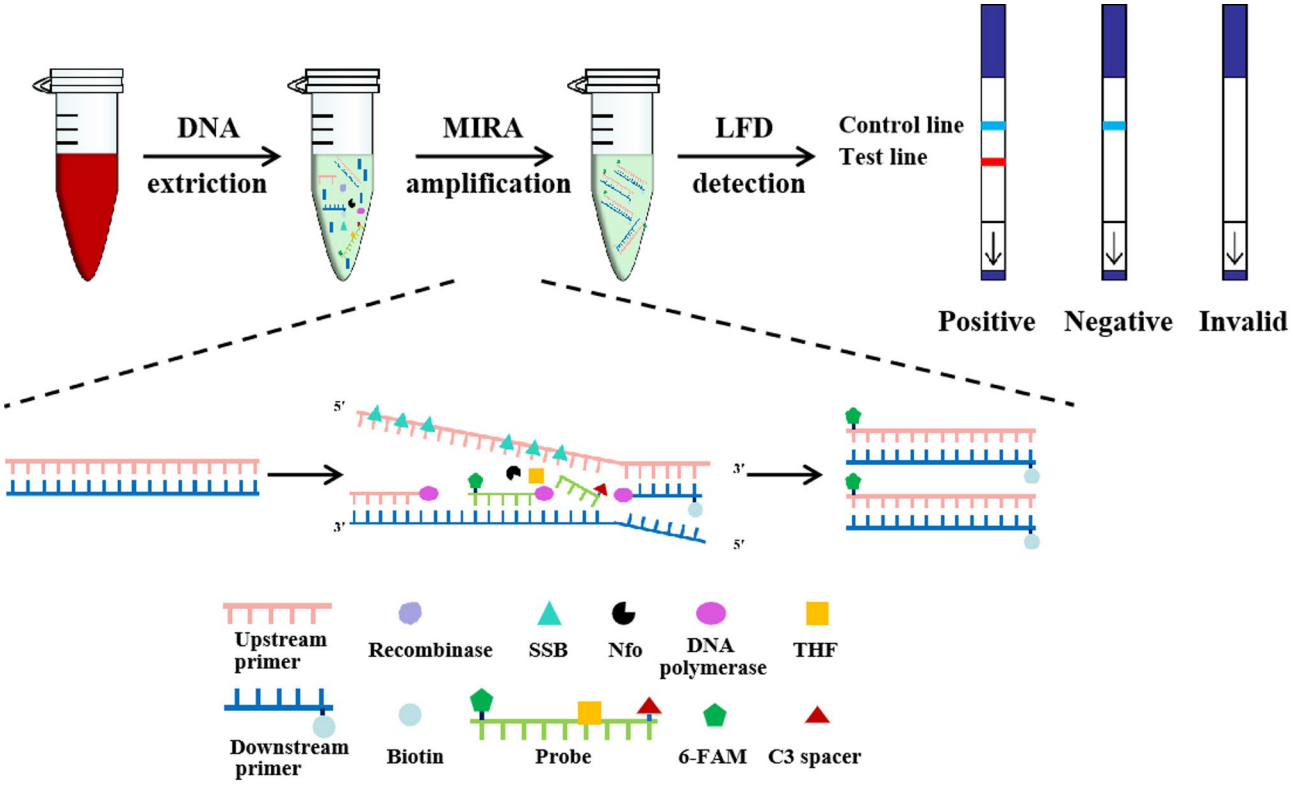

Fig. 3 The workflow of the MIRA-LFD assay

HTLV-1-2, HTLV-1-3, and HTLV-1-4) were firstly used to detect HTLV-1 plasmids of 10^4 copies/μL. As shown in Fig. 4A, the results showed that the three pairs of primers/probes(HTLV-1-1, HTLV-1-3, and HTLV-1-4) can identify HTLV-1, while HTLV-1-2 primers/probe could not detect the virus. Three pairs of primers/probes(HTLV-1-1, HTLV-1-3, and HTLV-1-4) were confirmed for second screening, and then ddH_2O was detected using these three pairs of primers/probe. The results showed that only the test line of the HTLV-1-1 primers/probe did not show color, indicating no non-specific reaction, while HTLV-1-3 and HTLV-1-4 showed non-specific bands (Fig. 4B). Therefore, HTLV-1-1 primers/probe were selected for subsequent detection.

Optimum reaction temperature and time

The MIRA-LFD assay was conducted at three different reaction temperatures(30 °C, 37 °C, and 42 °C). As shown in Fig. 5A, the LFD strip using plasmids with concentrations of 10^4 copies/μL as the amplification template only showed a quality control line at 30 °C, while both control and test lines appeared on the LFD strip at 37 °C and 42 °C. Therefore, the optimal reaction temperature for the MIRA-LFD assay was determined to be 37 °C.

Further, the MIRA reaction system was set up with five reaction times(5, 10, 15, 20, and 25 min). As shown in Fig. 5B, the LFD strip using plasmid with a concentration of 10^4 copies/μL as the amplification template began to show the quality control and test lines after 10 min of MIRA amplification, and the color intensity of the test line remained basically unchanged after 15 min. Thus, the optimal reaction time for the MIRA-LFD assay was determined to be 15 min.

The LOD of the MIRA-LFD assay

To determine the LOD of the MIRA-LFD method, standard plasmids of different concentrations(10^7, 10^6, 10^5, 10^4, 10^3, 500, 200, and 100 copies/μL) were detected at 37 °C for 15 min. As shown in Fig. 6, the LOD of this assay was 200 copies/μL.

The specificity of the MIRA-LFD assay

DNA/RNA of HTLV-1, HBV, HCV, HEV, CMV, HSV-1/2, B19V, and EBV, as well as HTLV-2 plasmid DNA were

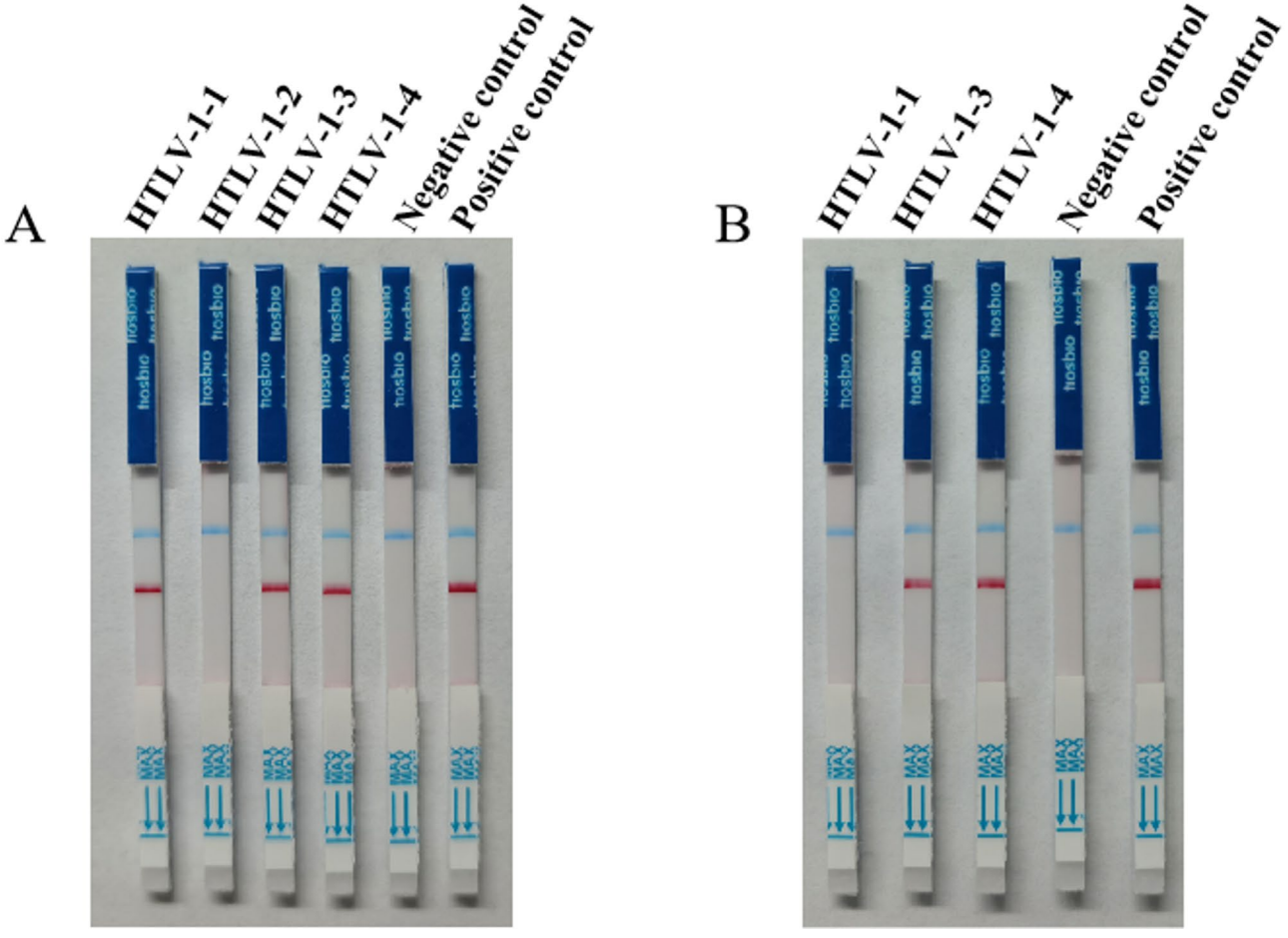

Fig. 4 Screening tests for four pairs of primers/probes. LFD strip change for MIRA-LFD amplification in four pairs of primers/probes

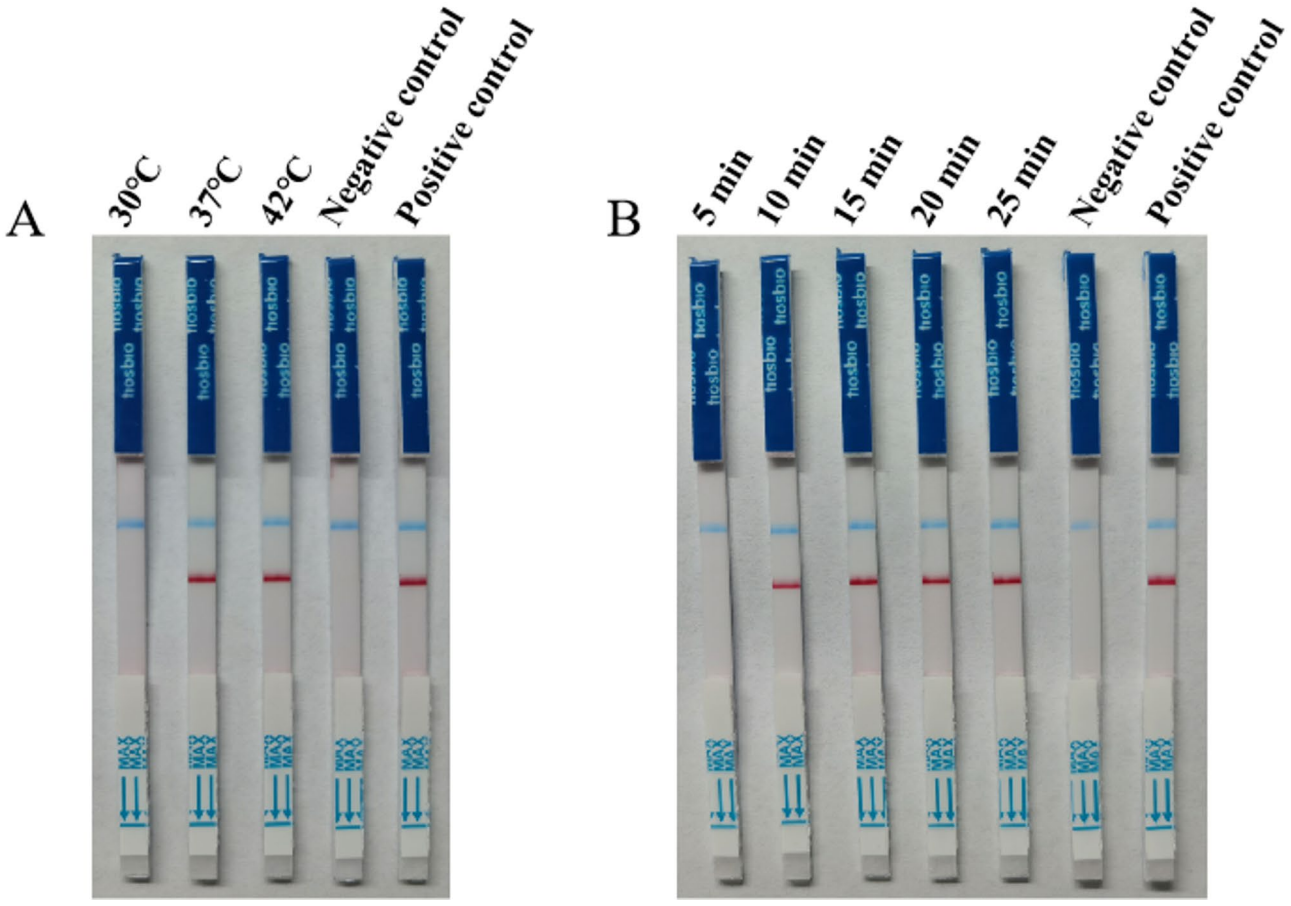

Fig. 5 Optimization of MIRA-LFD reaction temperature and time. (**A**) LFD strip change for amplification products at different temperatures (30 °C, 37 °C, and 42 °C). (**B**) LFD strip change for amplification products at different times (5, 10, 15, 20, and 25 min)

detected to determine the specificity of this method. As shown in Fig. 7, the LFD strip with HTLV-1 as the amplification template showed both quality control and test lines, while the negative control and other pathogens only showed quality control lines, indicating that the MIRA-LFD assay has good specificity.

The detection of clinical samples

HTLV-1 was detected in 500 clinical samples using qPCR and MIRA-LFD assays (Table 2). The MIRA-LFD assay accurately identified 7 positive samples and 493 negative samples, which was the same as the results of the qPCR assay. Clinical sample detection results based on qPCR assay as the standard, the diagnostic sensitivity and specificity of the MIRA-LFD assay both were 100.00%. The results indicated good consistency(100.00%) between qPCR and MIRA-LFD assays for detecting clinical samples, with a kappa value of 1.00. But MIRA-LFD was faster, indicating the potential of MIRA-LFD for clinical diagnosis.

Discussion

At present, there is no vaccine or specific drug for preventing and treating HTLV-1, and once infected, people will carry it for life [33]. The only effective way currently is to promptly detect HTLV-1 infected individuals and take measures to cut off the transmission route [34]. Blood screening is an effective way to block the transmission of HTLV-1 through blood transfusion [35]. Compared with antibody testing, nucleic acid testing can significantly shorten the window period for pathogen detection, thereby reducing the risk of pathogen transmission through blood [36, 37]. Developing a simple, rapid, good sensitivity, strong specificity, and cost-effective method is essential for HTLV-1 nucleic acid detection. Therefore, we developed a novel method for detecting HTLV-1 by combining MIRA and LFD techniques. This is the first report on the MIRA-LFD assay for detecting HTLV-1, which will inspire the establishment of this method for the detection of other human viruses.

This study developed a method based on detecting the HTLV-1 proviral DNA in the human leukocyte genome, rather than detecting RNA. When HTLV-1 invades cells,

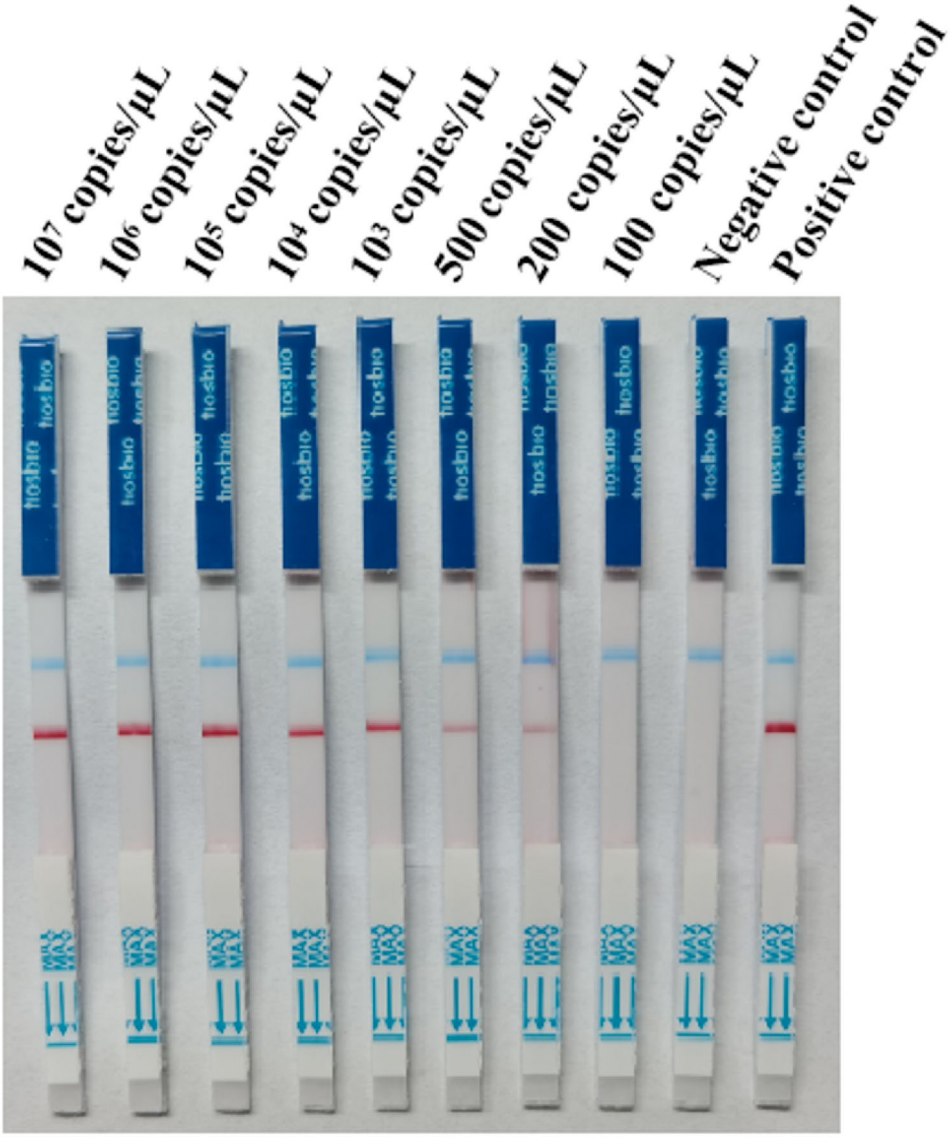

Fig. 6 The LOD of the MIRA-LFD amplification. LFD strip change for MIRA-LFD amplification in different concentrations of HTLV-1 plasmids

the viral RNA is reverse-transcribed into DNA and integrated into the genome of the host cell. The DNA integrated into the host cell genome is called a provirus [38, 39]. Due to the rare presence of HTLV-1 virus particles in plasma, although HTLV-1 genomic RNA can be detected in the plasma of a few patients, its level or frequency is not sufficient for clinical or diagnostic use [40]. In addition, DNA samples are more stable than RNA, they can be stored for a longer time at low temperatures, have a wider range of sources(which can be various tissues, organs, or blood, not limited to plasma), do not require reverse transcription, and are easier to handle [41].

Primers/probes in this study were designed to target the Tax region of HTLV-1, as it is one of the most conserved genes in the HTLV-1 genome and a commonly used target for PCR techniques previously reported [42–45]. This study established a rapid detection method for HTLV-1 provirus DNA based on the combined technology of MIRA and LFD. The MIRA-LFD assay has the characteristic of rapid reaction, and the reaction begins as soon as the sample comes into contact with the reagent. The entire detection time of the MIRA-LFD method is 20 min, including gene amplification(15 min) and the LFD strip detection(observing the color results within 5 min), which is significantly less than the LAMP and PCR assays(approximately 30–60 min) [46–49]. In addition, comparing with other isothermal amplification-based assays, such as RPA, MIRA-LFD assay has a more complete and efficient core functional enzyme system, and its cost is lower [24]. The RPA assay may cost 9 pounds/reaction, while the MIRA-LFD test has a lower cost than the RPA assay, with a cost of approximately £6

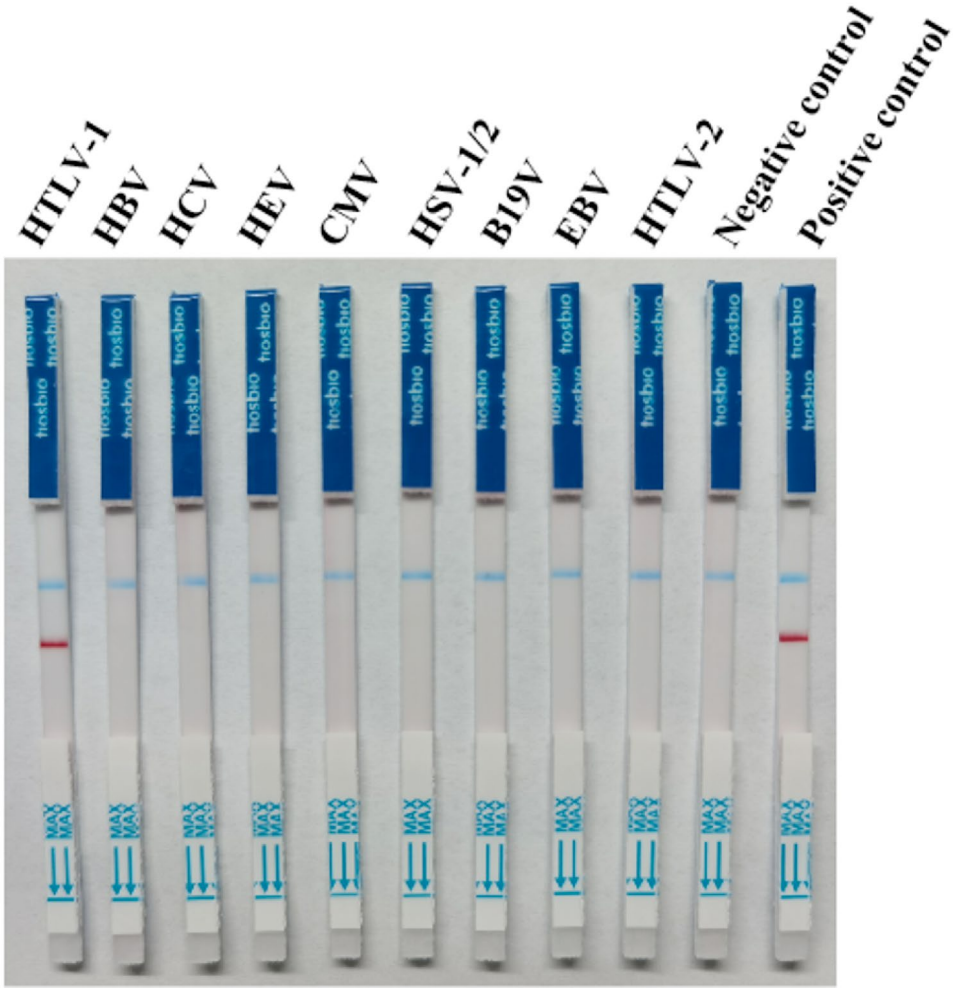

Fig. 7 Specificity validation of the MIRA-LFD assay. LFD strip change for MIRA-LFD for detection of HTLV-1, HBV, HCV, HEV, CMV, HSV-1/2, B19V, EBV and HTLV-2

Table 2 Detection of HTLV-1 in clinical samples using qPCR and MIRA-LFD assays

		qPCR			Sensitivity (%)	Specificity (%)	Concordance rate(%)	Kappa value
		Positive	Negative	Total				
MIRA-LFD	Positive	7	0	7				
	Negative	0	493	493				
	Total	7	493	500	100.00%	100.00%	100.00%	1.00

per sample. Compared with LAMP and MCDA, primers/probes in the MIRA-LFD assays are easier to design and the experiments are easier to operate [50, 51]. The amplification efficiency of long targets in SDA is relatively lower than that of MIRA-LFD [52]. The experimental conditions for PSR are 65 °C for approximately 70 min, both of which are higher than those for MIRA-LFD [53]. The entire gene amplification process of the MIRA-LFD method was completed in a water bath at 37℃. Compared with qPCR technology, the MIRA-LFD assay does not require high-temperature denaturation, annealing, and other steps, so it does not rely on expensive precision instruments. The MIRA-LFD method was used to detect HTLV-1 and eight other pathogens. Except for HTLV-1, all other pathogens were consistent with the negative control results, with only the quality control line showing color, indicating the high specificity of this method. 500 clinical samples were tested to compare the clinical performance of the MIRA-LFD and qPCR methods. Clinical sample detection results based on qPCR assay as the standard, the diagnostic sensitivity and specificity of the MIRA-LFD assay both were 100.00%, indicating good consistency(100.00%) between qPCR and MIRA-LFD assays.

In this study, our developed MIRA-LFD method has the advantages of low cost and short time compared to

existing HTLV-1 infection validation methods such as LIA and WB. It can be used to confirm HTLV-1 positive serological results and provide final conclusions for uncertain results obtained by LIA or WB.

This study has certain limitations. Due to the high sensitivity of fluorescent probes, aerosols are easily generated during the experimental process. Therefore, the experimental process requires strict zoning: reagent preparation area, gene amplification area, and LFD strip detection area. It is best to conduct color testing on LFD strip in a well-ventilated area to avoid false positives.

Conclusions

In summary, we have developed and optimized a rapid visual detection method for HTLV-1 provirus DNA based on MIRA-LFD. This method has good sensitivity, strong specificity, simple operation, time-saving, and high efficiency. Furthermore, the MIRA-LFD assay can complete all detection steps within 20 min and can be used for rapid diagnosis and confirmation of HTLV-1 infection, especially suitable for grassroots medical institutions and blood stations with limited laboratory resources.

Abbreviations

HTLV-1	Human T-cell lymphotropic virus-1
HTLV-2	Human T-cell lymphotropic virus-2
ELISA	Enzyme-linked immunosorbent assay
WB	Western blot
LIA	Line immunoassay
qPCR	real-time PCR
RPA	Recombinase polymerase amplification
LAMP	Loop mediated isothermal amplification
MCDA	Multiple cross displacement amplification
SDA	Strand displacement amplification
PSR	Polymerase spiral response
MIRA	Multienzyme isothermal rapid amplification
SSB	Single-stranded binding protein
LFD	Lateral flow dipstick
nfo	nucleic acid exonuclease IV
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HEV	Hepatitis E virus
CMV	Cytomegalovirus
HSV-1/2	Herpes simplex virus-1/2
EBV	Epstein-Barr virus
B19V	Parvovirus B19
THF	Tetrahydrofuran
ddH_2O	double distilled water
LOD	Limit of detection
NMPA	National Medical Products Administration
6-FAM	6-carboxyfluorescein

Author contributions

MML conceived the idea, carried out the experiments, analyzed the data, and wrote the manuscript. MJL and YSX collected samples, conducted some experiments, and analyzed the data. DWC analyzed the data and polished the manuscript. JX contributed to the conception and design of the experiments, obtained funding, and revised the manuscript. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed during this study are included in this article. The data associated with this manuscript will be made available by reasonable request with the corresponding author.

Declarations

Ethics approval and consent to participate

This study was conducted with the approval of the Ethics Committee of The First Affiliated Hospital, Zhejiang University School of Medicine (Reference Number2020-856). All participants signed the written informed agreement. This study was performed in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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