

Lateral Flow Genochromatographic Strip for Naked-Eye Detection of *Mycobacterium Tuberculosis* PCR Products with Gold Nanoparticles as a Reporter

Nazari-Vanani R.¹, Tondro G. H.¹, Dehdari Vais R.¹, Haghkhah M.², Heli H.³, Sattarahmady N.^{3,4*}

ABSTRACT

Background: *Mycobacterium tuberculosis* (MTB) is a pathogen causing tuberculosis (TB) in human, and TB can cause enormous social and economic disruptions. Lateral flow test strips (LFTSs) are inexpensive, portable, disposable, rapid, and easy-to-use analytical tools.

Objective: LFTSs were prepared for the detection of MTB. LFTSs were fabricated using a new specific probe for MTB H37Rv, based on IS6110 sequence gene, and tailed with poly deoxyadenine (dA).

Material and Methods: In this experimental study, to create test and control zones, streptavidin (STP) and a 150-mer dA were dotted on a nitrocellulose membrane. Gold nanoparticles (GNPs) were conjugated with poly deoxythymidine sequence and placed on the conjugate pad. The composition of immersion buffers for sample pad and conjugate pad, running solution, solutions of GNPs-S-dT150 and STP were introduced. DNA genome of MTB and *Mycobacterium bovis* in clinical samples was amplified with PCR, and then detected by the LFTSs. During the assay, samples were firstly hybridized in two steps and then placed on a conjugate pad in a manner that positive and negative samples provided two and one red lines, respectively, on the detection pad.

Results: After PCR reaction with biotinylated primer, hybridization process with specific MTB probe-dA70-100 took 10 min, and running process on the strip was performed within 5 min.

Conclusion: We showed that LFTS can discriminate a particular bacteria strain from others. The LFTSs can be redesigned for detection of other pathogenic genomes.

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Keywords

Lateral Flow; Test Strip; Tuberculosis; Nucleic Acid Hybridization; Metal Nanoparticles; Gold

Introduction

Tuberculosis (TB) is one of the first bacterial infectious diseases [1], and an infection rate of one-third of the world's population by TB is estimated [2]. The World Health Organization (WHO) re-

¹MSc, Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
²PhD, School of Veterinary Medicine, Shiraz University, Shiraz, Iran
³PhD, Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
⁴PhD, Department of Medical Physics, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

*Corresponding author:
 N. Sattarahmady
 Department of Medical Physics, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
 E-mail: sattarahmady@yahoo.com

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ported in 2015 that there were nearly 10.4 million new TB cases [3] and 1.5 million annual deaths are related to TB [4]. *Mycobacterium tuberculosis* (MTB) complex consists of seven organisms [5]. MTB is an aerobic pathogenic bacillus which establishes infection in the lungs. TB symptoms include coughing, night sweats, moderate fever, fatigue, abnormal chest X-ray manifestation, blood in the sputum, loss of appetite, and weight loss [6, 7].

Currently, TB diagnosis methods are performed by the approaches of radiology, tuberculin skin test, blood test, and microbiological culture, microscopic examination of body fluids such as sputum, and molecular assays including immunoassays, DNA detection, interferon-gamma release assay, aptamer-based methods and PCR-based assays [8-11] Tuberculin skin test was used as the gold standard despite some recognized limitations [12]. The requirement for tuberculin skin test reading after 48-72h increases the loss to follow-up [13]. Tuberculin skin test may also present lower sensitivity, and false negative results may occur [12]. Microbiological culture-based methods are excessively time-consuming, requiring complicated media and need laboratory infrastructure. Despite several advantages and high potential, molecular diagnostic tests are usually expensive and time consuming, with poor specificity, and require specialized equipment and trained personnel. These diagnostic tests are also unreliable when used in HIV-positive patients and suffers from several other pivotal drawbacks [9, 14]. To overcome these limitations, considerable efforts are now directed towards the development of methods that can rapidly detect pathogens in clinical samples [15]. Most of the published papers have not proceeded beyond proof of concept, and few assays have been applied on real clinical samples, since artificially spiked samples or complex DNA detection routes have been often used.

Nanostructured materials have been used in many medical applications, for instances,

drug delivery, diagnostic and theranostic assays, and treatments [16-19]. Paper-based microfluidic technologies such as lateral flow test strips (LFTSs) have attracted much attention for rapid diagnostic test applications based on nanomaterials [20]. They are inexpensive, portable, disposable, rapid, and easy-to-use analytical tools for point-of-care diagnosis [21]. LFTSs are composed of i) a sample pad in which the sample is added into the strip; ii) a conjugate pad, where the analyte is conjugated with nanoparticles; iii) a detection pad that includes two different lines where the main reactions take place, i.e. control and test lines. The test line is typically printed with a biomolecule targeting the analyte, while the control line is formed by a biomolecule that binds to the probe (or the labeled recognition element) that is tagged with nanoparticles and comes from the conjugate pad; iv) an absorbent pad, which absorbs the materials that reach the end of pads; and v) a plastic backing that maintains the other parts [22]. Thus, after loading a sample, the labeled biomolecules in the conjugate pad flow throughout the detection pad until they reach the test line, where a sandwich complex is performed, give a signal depending of the analyte concentration in the sample [23]. LFTSs can work with the blood, plasma, serum, urine, or saliva samples for testing [24]. It is also applied for protein detection, viral antigens and small molecules, and enables rapid point-of-care diagnosis of infectious diseases such as influenza [25], hepatitis B [26], leishmaniasis [27], malaria [28], and HIV [29] as well as biomarkers [30, 31].

Up to now, LFTSs have been reported for TB detection. Ma *et al.* combined a LFTS with recombinase polymerase amplification for detection of MTB complex [32]. Using the IS6110 sequence of tuberculous meningitis as a target, fragments were amplified by nested PCR and two sets of primers, and then detected by an antibody-labeled probe on a LFTS [33]. A PCR-nucleic acid lateral flow immunoassay was also reported for the indirect de-

tection of labeled PCR amplification products of multidrug-resistant TB [34]. Multidrug-resistant TB was also detected after rolling circle amplification of padlock probes using a LFTS [35]. Loop-mediated isothermal amplification assay targeting *sdaA* gene combined with a lateral flow dipstick [36]. In the present study, LFTSs were fabricated, optimized and used for rapid and lower cost diagnosis of MTB in simple PCR products using one set of primers and without needing antibodies. It takes about one hour for the identification of mycobacterium in clinical samples. Accuracy of the results was confirmed by PCR and gel electrophoresis.

Material and Methods

Reagents and biologicals

In this experimental study, nitrocellulose (NC) membrane (Hi-flow plus), absorbent pad (cellulose fiber sample pad roll), sample pad (cellulose fiber sample pad roll) and conjugate pad (glass fiber conjugate pad rolls) were purchased from Merck Millipore (Germany). Plastic adhesive rigid back were prepared from a local store. Chemicals were acquired from Scharlau (Spain) or Sigma (USA). Taq DNA polymerase was obtained from CinnaGen Co. (Iran). Terminal deoxynucleotidyl transferase (TdT) kit was prepared from Thermo Scientific (USA). Deoxyadenosine triphosphate (dATP) was from Jena Bioscience (Germany).

All solutions were prepared with RNAase free water.

Preparation of the gold nanoparticles (GNPs)

GNPs were synthesized by the citrate reduction method as reported elsewhere [17]. Briefly, 250 mL of chloroauric acid (1.0 mmol L^{-1}) was heated, and then 25 mL of sodium citrate (38.8 mmol L^{-1}) was added. The mixture was boiled for 15 min, then cooled down to room temperature and finally characterized by visible absorption using a UV-vis spectrophotometer of Rayleigh 2100 (China).

Primers and probes

All the oligonucleotides were received from Bioneer Co. (Korea), and their sequences are presented in Table 1. A 20-mer single stranded DNA sequence was used as the specific probe of MTB H37Rv (MTB probe) and checked using NCBI BLAST nucleotide search tool, mfold [37], Primer3 and oligo software. MTB probe was then tailed with a dA70-100 sequence (denoted as MTB probe-dA70-100, vide infra). A thiolated 150-mer deoxythymidine (S-dT150) and 150-mer deoxyadenine (dA150) oligonucleotides were employed.

Preparation of MTB probe-dA70-100

For preparation of MTB probe-dA70-100, 7 μL MTB probe, 1.5 μL TdT, 1.5 μL buffer, and 10 μL dATP ($10 \text{ }\mu\text{mol L}^{-1}$) were mixed and in-

Table 1: Oligonucleotide sequences used in this study.

Name	Sequence (5' → 3')
PCR forward primer	TAAACCGGACTATTTCTTCAACC
PCR reverse primer	Biotin-GGTGATGACCTACTTAGCACGAT
MTB probe	ATGAACGGCTCGTTGAAGAC
S-dT150	SH(CH ₂) ₆ (T) ₁₅₀
dA150	(A) ₁₅₀
MTB probe-dA70-100	MTB probe-(A) ₇₀₋₁₀₀

cubated at 37 °C for 60 min. Then, the mixture was incubated at 70 °C for 10 min to inactivate the enzyme.

Sample collection and PCR amplification

Ten bovine (from lymph nodes) and human (from phlegm) specimens infected by *Mycobacterium bovis* and MTB, respectively, were collected and confirmed in Razi Vaccine and Serum Research Institute (Iran) by culture on Lowenstein-Jensen medium following by both microscopic analysis and PCR detection.

DNA extraction was done based on a procedure reported elsewhere [38], and the obtained samples were dissolved in deionized water and stored at -20 °C. After recording the optical densities at 260/280 nm of the samples, they were amplified using a 5'-biotinylated reverse primer. For the PCR reaction, the reaction mixture was prepared containing PCR buffer, MgCl₂, dNTP, primers, sample, Taq DNA polymerase and nuclease-free water. The PCR amplification conditions were respectively as follows: initial denaturation step at 95 °C for 5 min, 35 cycles of post-denaturation at 95 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s. Confirmation of the assay was carried out by agarose gel electrophoresis.

Gel electrophoresis

The electrophoresis analysis was carried out on 2% agarose gels and run in 1X Tris-EDTA-acetate (TEA) buffer, pH 8.0. The gel was polymerized for 40 min at room temperature. 7 µL of the PCR product of each sample was mixed with a loading buffer and subjected to gel electrophoresis. The resulting gel was imaged with UV light in a gel documentation system from ATP Technology Vietnam Co., Ltd.

Preparation of GNPs-conjugated S-dT150 (GNPs-S-dT150)

S-dT150 (1 µmol L⁻¹) was suspended in di-

thiothreitol (DTT) in a 1:100 ratio, wrapped in an aluminum foil and shaken at room temperature for 2 h. Then, GNPs (of 14.1 nmol L⁻¹) were added to reach a final volume 1000 µL and shaken for 16 h at room temperature. Then, sodium dodecyl sulfate (SDS, 1%, 5 µL) and phosphate buffer saline (PBS), 10 mmol L⁻¹, pH=7.0 were added and shaken for 30 min. After that, a solution of 2.0 mmol L⁻¹ NaCl in 10 mmol L⁻¹ PBS was added during 48 h. The solution was centrifuged at 13000 rpm for 20 min and the precipitate was washed with 500 µL of 10 mmol L⁻¹ PBS, containing 0.15 mol L⁻¹ NaCl and 1% SDS. It was allowed to stand in the dark at 4 °C until use.

Preparation and optimization of detection conditions

To achieve high detection sensitivity, several key parameters should be optimized, such as the composition of running solution, the composition of matrices for dissolution of GNPs-S-dT150 to place on the conjugate pad, and the composition of STP solution to form the test line. These parameters could influence the final color of the test and control lines.

Two different running solutions were identified and investigated by selecting the suitable components [39, 40]. The best running solution was selected based on the flow rate and time of detection. The examined running solutions were contained (I): 4% glycerol and 1% SDS in PBS, and (II): 4% glycerol, 1% SDS and 0.15 mmol L⁻¹ NaCl in PBS.

LFTSs were made by manual cutting of the strip components with a ~5 mm width. The sample pad was pretreated by immersion in PBS containing 2.5% triton X-100 and 0.15% NaCl followed by drying in the air. Prior to the deposition of GNPs-S-dT150, the conjugate pad was pretreated by immersion in PBS containing 1.0% Triton X-100, 3.0% bovine serum albumin (BSA), and 0.05% polyvinyl alcohol (MW of 8,000), and dried overnight. Three matrices were selected for dissolution of GNPs-S-dT150 to immobilize on the con-

jugate pad [39, 40]: (I) 0.25% SDS, 0.25% tween 80, 30% sucrose, and 45 mmol L⁻¹ NaCl; (II) 5.0% BSA, 0.05% tween 80, and 5% sucrose, and (III) 5.0% BSA, 0.1% tween 80, 15% glycerol, and 4.5% NaCl. Each of these mixtures was prepared with a concentration of GNPs-S-dT150 of 7.2 fmol mL⁻¹. About 2 μL of the GNPs-S-dT150 solution was applied onto the conjugate pad and allowed to dry at room temperature.

STP solutions of two concentrations were prepared in PBS and dotted on the test zone. These solutions comprised [39, 40]: (I) 4.0 mg STP, 6.0% methanol and 2.0% sucrose; (II) 4.0 mg STP, 3.0% methanol and 2.0% sucrose in PBS; (III) 4.0 mg STP, 3.0% methanol and 4.0% sucrose; (IV) 4.0 mg STP, 1.5% methanol and 2.0% sucrose; (V) 2.0 mg STP, 1.5% methanol and 2.0% sucrose; (VI) 2.0 mg STP and 5.0% sucrose; (VII) 4.0 mg STP, 3.0% methanol and 8.0% sucrose.

A dA150 solution of 4.0 μmol L⁻¹ was prepared in PBS containing 0.41 mol L⁻¹ NaCl,

0.45 mol L⁻¹ KCl, 5% methanol, and 2% sucrose. 2 μL of this solution was dotted on the control zone. To immobilize dA150 on the control zone, UV light was illuminated onto the control zone for 3 h.

The absorbent pad was used as a capillary force driver as well as the reservoir for the used assay reagents.

Fabrication of LFTSs

Different parts of each strip were assembled on a plastic backing as presented schematically in Figure 1. The width of the test strips was about 5 mm.

Detection of the TB

2 μL of MTB probe-dA70-100 with a typical concentration of 3.0 pmol μL⁻¹ was added to 10 μL of each biotinylated PCR product. After that, 1.0 μL of a NaCl aqueous solution of 2 mol L⁻¹ was added to the solution, heated to denature at 95 °C for 5 min, cooled down, and incubated at 37 °C for 15 min. 1.0 μL of

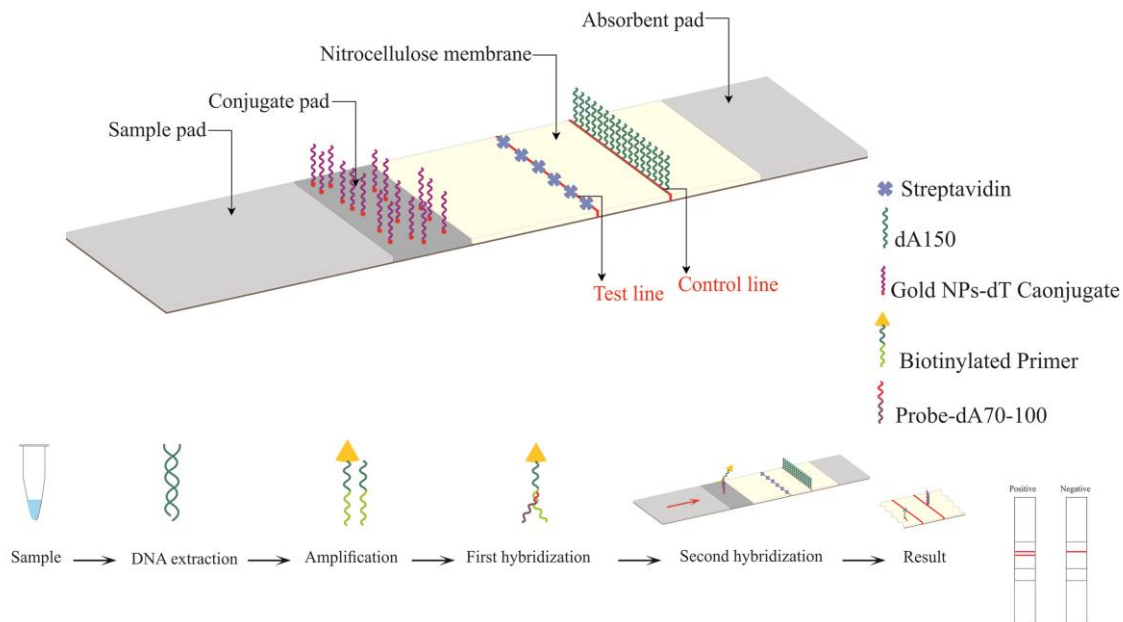


Figure 1: Schematic representation of different parts of LFTS assembled on a plastic backing and its working principle.

the above mixture was loaded on the conjugate pad. The detection of genome was carried out by applying running solution to the bottom of the strip, allowing the analytes flow along the test strip within 5 min. During this time, the product of first hybridized MTB probe-dA70-100 and PCR product (of positive or negative samples) was hybridized for the second time with GNPs-S-dT150 on the conjugate pad and they were subsequently captured at the test-line, while the excess GNPs-S-dT150 conjugate was captured at the control line. The results were observed by the naked eye. Positive results produced two bands corresponding to the test and the control lines. Negative results produced a single band corresponding to the control line.

Results

A visible spectrum of GNPs solution in Figure 2 showing a strong absorbing peak at 530 nm. This confirmed the successful synthesis of ~ 15-nm GNPs. A 20-mer single stranded DNA sequence as a specific MTB H37Rv probe was

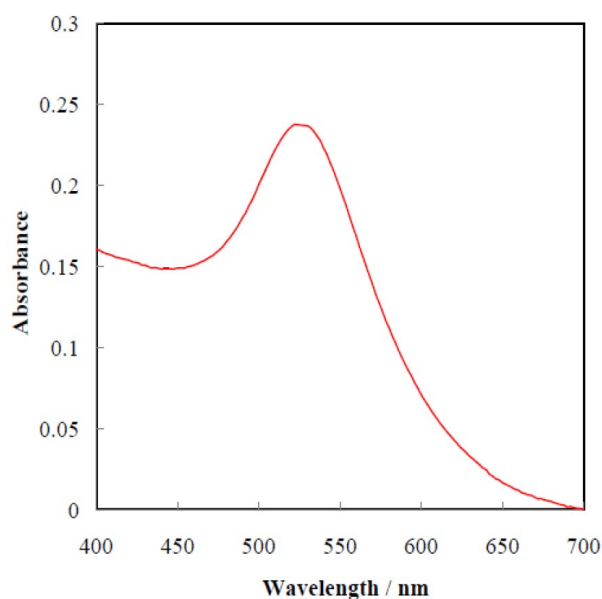


Figure 2: A visible spectrum of GNPs solution.

selected from IS6110 sequence gene.

Here, we aimed to detect the MTB genome as a target analyte (positive sample), and *Mycobacterium bovis* was employed as negative sample. DNA of positive and negative samples was extracted from confirmed specimens and amplified by PCR. Following amplification of positive samples by PCR, denaturation was applied by the sample incubation at 95 °C for 5 min. Then, hybridization of the amplified fragments with MTB probe-dA70-100 was done at 37 °C for 5 min. It should be noted that MTB probe-dA70-100 has a tail for a second hybridization step. After the first hybridization step, the product was placed on the conjugate pad. The second hybridization step occurred between the adenine nucleotides of MTB probe-dA70-100 and thymidine residues of GNPs-S-dT150 previously immobilized on the conjugate pad. Then, the strips were soaked in the running solution. Running solution was developed upward by capillary force and rehydrated GNPs-S-dT150. Upon penetration of the running solution on the strip surface, a second hybridization complex was captured with the immobilized STP in the test line, and a red signal formed in this zone. In the case of negative samples, the flowing complex on the strip was only GNPs-S-dT150 without hybridization with PCR products to occur; therefore, interaction between biotin and STP did not occur. Hybridization of GNPs-S-dT150 with dA150 on the control line occurred for these samples, and a red signal was observed at the control line. As for the positive samples, excess GNPs-S-dT150 accumulated at the control line.

It has been reported that the choice of a suitable running solution can have a significant effect so that the highest sensitivity is achieved. Based on the results presented in Figure 3A, the running solution (I) had a sufficient flow rate for efficient reaction between the recognition probe and the target on the strip. The other tested running solution (II) induced certain drawbacks including the incomplete test line

Test Strip for Tuberculosis PCR Products

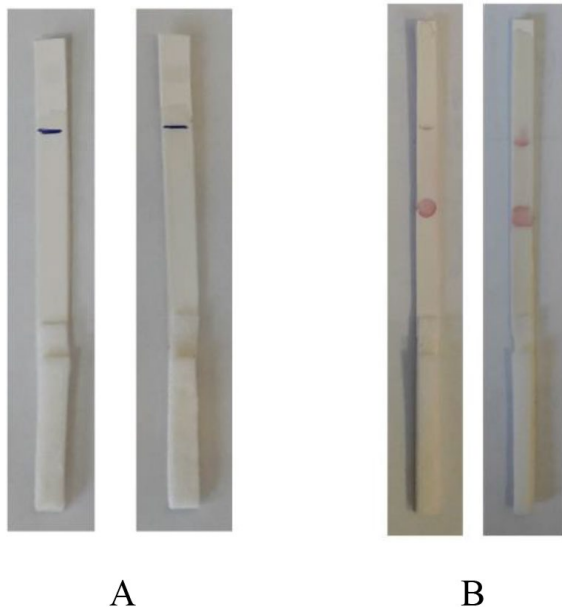


Figure 3: (A) Images form blank LFTSs (without loading the test and control lines) when they immersed in running buffer I (left) and II (right). The time to reach the running buffers to the blue dashes was 3.2 (left) and 5.7 (right) min. (B) Images form LFTSs upon loading with a positive sample when they immersed in running buffer I (left) and II (right) (Absorbent pad was omitted).

or control line, unclear test or control lines and high background signals, as depicted in Figure 3B.

The matrices for GNPs-S-dT150 immobilization were tested and the results are shown in Figure 4A. By visualization, the best and most bold colors at both test and control zones were attained using the matrix (I).

Some different media for STP were optimized to find the best immobilization conditions. From the matrices, matrix (I) provided the best immobilization conditions for STP immobilization on the detection pad, while providing the best color located at the place of STP immobilization, as shown in Figure 4B.

Stability of the LFTSs was evaluated by their storage at 4 °C with a simple cover for about 3 months, and every three days, one of them was assayed. No obvious change in the formed colors was observed during this time, and 3 months was considered as the stability time of LFTSs.

As for applicability of LFTSs, the biotinylated PCR product of the samples was added to MTB probe-dA70-100 solution and placed at the hybridization conditions. Then, 1 μ L of the above mixture was loaded on the conju-

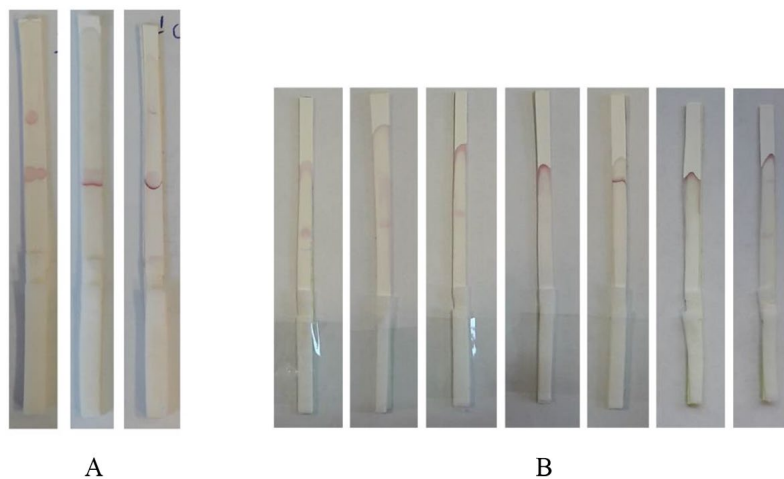


Figure 4: (A) Images form LFTSs upon loading with a positive sample when they prepared with different matrices for GNPs-S-dT150 immobilization of I (left), II (middle), and III (right) (Absorbent pad was omitted). (B) Images form LFTSs upon loading with a positive sample when they prepared with different matrices for STP immobilization. From the left to the right: matrix (I) to matrix (VII) (Absorbent pad was omitted).

gate pad and after applying running solution to the bottom of the strip, allowing flow of the analytes along the test strip within 5 min. Product of the first hybridized MTB probe-dA70-100 and the PCR product was hybridized for the second time with GNPs-S-dT150 on the conjugate pad and they were subsequently captured at the test-line. The excess GNPs-S-dT150 conjugate was captured at the control line. The results were observed in Figure 5 by the naked eye. Positive samples (7 samples) produced two bands corresponding to the test and the control lines, while negative samples (2 samples) produced a single band corresponding to the control line.

Discussion

NCBI BLAST nucleotide results, the E-value and percent of identity between the all nucleotides indicated the selected 20-mer single stranded DNA sequence is a specific MTB H37Rv probe from IS6110 sequence gene. The 20-mer DNA sequence is just specific for MTB *spp*.

Point of care assays built primarily around

the lateral flow assay usually employ antibodies and detection of the ultimate immune complex formation is visually performed, using a nanoparticles label. However, one another class of nucleic acids hybridization assays to fabricate LFTSs is based on complementary DNA sequence hybridization. Hybridization is the key element for success of this assay, and an advantage of nucleic acids hybridization strategy is that it can function under conditions that often cause antibodies to become inactivated.

Here, the MTB genome and *Mycobacterium bovis* were selected as positive and negative samples, respectively. DNA of positive and negative samples amplified by PCR. It should be mentioned that after PCR reaction, just positive samples were amplified and biotinylated. Amplified target was acquired with a biotin modification, allowing for conjugation to streptavidin. Therefore, PCR products of positive samples had a biotin functional group in 5' terminal and also had complementary sequence of the specific MTB probe, which can hybridize with this sequence. First and second



Figure 5: Images form LFTSs upon loading with different samples when they prepared at optimized conditions. 7 left samples are positive, and 2 right samples are negative.

hybridization of the PCR products of positive samples were occurred with MTB probe-dA70-100 and thymidine residues of GNPs-S-dT150 on the conjugate pad, respectively. Then, the strips were soaked in the running solution. Products of second hybridization complex was captured with the STP in the test line and formed a red signal in this zone due to accumulation of gold nanoparticles in the hybridization complex. In the case of negative samples, the flowing complex on the strip was only GNPs-S-dT150 without hybridization with PCR products to occur; therefore, interaction between biotin and STP did not occur. Hybridization of GNPs-S-dT150 with dA150 on the control line occurred for these samples, and a red signal was observed at the control line. As for the positive samples, excess GNPs-S-dT150 accumulated at the control line.

For high detection sensitivity of LFTS, the composition of running solution, matrices for dissolution of GNPs-S-dT150, and the composition of STP solution were optimized. The key considerations for choosing a running solution for strip detection are controlling the flow rate of the running solution and providing suitable conditions for the reaction between the recognition probe and the target analyte or capture probes on the strip. Here, the running solution including 4% glycerol and 1% SDS in PBS had a sufficient flow rate for efficient reaction between the recognition probe and the target on the strip.

The matrices for GNPs-S-dT150 immobilization were selected based on the best and most bold colors at both test and control zones including 0.25% SDS, 0.25% tween 80, 30% sucrose, and 45 mmol L⁻¹ NaCl; (II) 5.0% BSA, 0.05% tween 80, and 5% sucrose. It seems that BSA had a negative effect, and increment in the ionic strength of the matrix positively affected the appearance of colors.

Some different media for STP were optimized to find

The best immobilization conditions for STP was the matrix (I) included 4.0 mg STP, 6.0%

methanol and 2.0% sucrose on the detection pad.

The applicability of LFTSs was confirmed with testing clinical positive samples (7 samples) and negative samples (2 samples). The results indicated that LFTSs could successfully recognize the positive and negative samples.

Conclusion

In summary, under the above optimized conditions, one LFTS for rapid and on-site detection of MTB genome has been developed. To display strain-specific detection in the present system, LFTS was built from paring a probe-poly A/target having a biotin at its 5'-end with a poly T conjugated with 15-nm GNPs. This LFTS detected the TB genome without any observable cross-reactivity with other strains (*Mycobacterium bovis* as negative samples). Furthermore, optimization of the factors which may affect the detection sensitivity and method assessment was also described. Under the optimized conditions, this LFTS achieved genome detection qualitatively.

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Conflict of Interest

None

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