

Quantum dots nanoparticle-based lateral flow assay for rapid detection of *Mycobacterium* species using anti-FprA antibodies

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Abstract

A lateral flow (LF) device combined with quantum dots (QDs) technology was developed for rapid detection of a specific mycobacterial flavoprotein reductase (*fprA*). In order to develop the LF assay based on a double-antibody sandwich format, two monoclonal antibodies recognizing different epitopes located in separated *fprA* domains were identified. The first monoclonal antibody was immobilized onto the detection zone of a porous nitrocellulose membrane, whereas another monoclonal antibody was conjugated to QDs nanoparticles as a detection system. Using these monoclonal antibodies we recorded a good fluorescence signal, the intensity of which was directly proportional to the concentration of *fprA* protein. The use of antibodies conjugated with fluorescent semiconductor QDs via biotin-streptavidin bridge, allowed the detection of *fprA* protein at concentrations as low as 12.5 pg/μL in less than 10 min. The reported technology could be useful in the diagnostic investigation of *Mycobacterium tuberculosis* and other human pathogens in clinical specimens.

Introduction

In the past few years, the LF immunoassay-based technology has become a powerful tool in the rapid detection of pathogens in liquid samples.¹

Originally this technique was used as a pregnancy test but nowadays several kits based on LF technology have been developed for a large number of pathogens, toxins or drugs.²⁻⁸ LF technology provides qualitative information on the presence/absence of an analyte in a liquid

sample with several advantages, including reduced time, low cost and possibility of performing on field assays, thus avoiding any sample transport.¹ All these features make LF technology an interesting tool for diagnostic investigation of *Mycobacterium tuberculosis*. The last decade has seen a dramatic resurgence in the incidence of tuberculosis and related mycobacterial diseases, especially in poorer areas but also in developed world.⁹ Indeed, diagnosis of mycobacterial infections requires rapid assays for immediate therapy or treatments for disease prevention.¹⁰ Diagnostic investigations of mycobacterial infection are currently mostly based on microscopic analysis via the Ziehl-Neelsen or Auramine-rodhamine staining and culture isolation.¹¹ Even though microscopic investigations are relatively rapid, they have several disadvantages in term of sensitivity and specificity. In fact, the microscopic analyses show a detection limit as low as 10⁵ bacilli/mL and only provide information on the presence of acid-alcohol resistant bacilli.¹⁰⁻¹² Culture-based methods remain the gold standard even though the growth is slow and it may take 6-8 weeks.¹³ Substantial shortening of detection time was realized by use of automated systems based on liquid culture, such as the BATEC radiometric method, Septicheck AFB and MIGIT fluorimetric method.^{14,15} These methods are able to reduce the time of diagnosis to 1-2 weeks but require dedicated extremely expensive equipment. DNA amplification techniques and high-performance liquid chromatography provide rapid analyses but require specialized personnel and are difficult to be implemented in clinical settings.¹⁶⁻²⁰ Many studies have focused on *M. tuberculosis* diagnosis based on antigen detection by immune-based tests, such as dot immunobinding assay,²¹ capture enzyme-linked immunosorbent assay (ELISA),²² and by latex agglutination assay.²³

Several mycobacterial antigens, such as the 38 kDa antigen,²⁴ 30 kDa antigen,²⁵ 16 kDa antigen,²⁶ lipoarabinomannan (LAM),²⁷ A60,²⁸ Mtb81,²⁹ 55 kDa antigen,²² 14 kDa antigen, 19 kDa antigen,³⁰ and ESAT-6,³¹ have been characterized, purified and, in some cases, reported for a potential use in the detection of tuberculosis.^{21,22,32,33}

Using a dot immunobinding assay, Sumi *et al.*²¹ was able to detect a circulating mycobacterial antigen (14 kDa antigen) in cerebrospinal fluid at the lowest concentration of 100 ng/mL, within 6 h. An ELISA based method was suggested to detect lipoarabinomannan antigen (LAM) in human sputum samples.³² The author reported a detection limit of 1 ng/mL using a purified antigen and a concentration of 10⁴ *M. tuberculosis* whole cells per mL. Recently, using a Fast Dot-Enzyme-Linked Immunosorbent Assay (FD-ELISA), El-Masry *et al.*³³ reported a fast assay which can be applied

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Contributions: FC, principal investigator, design of lateral flow assay; MC, technical work and Biotecgen RTD supervisor; PP, quantum dot conjugation; EDeL, technician, lateral flow tests; AA, competence in mycobacterial infections. FprA mutant protein study; AS, protein biochemistry, lateral flow assay set up, critical review of the manuscript; LAS, competence in mycobacterial infections. Studies on recombinant FprA protein and critical review of the manuscript.

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in the field for the detection of a 20 kDa mycobacterium antigen in serum from patients with pulmonary tuberculosis. This method was able to detect 1.8 μg/mL of purified antigen as the lowest detectable concentration. Some of these rapid methods display high sensitivity but require expensive equipment, training of personnel for their completion and have not been sufficiently explored in clinical practice. Moreover this current markers are not able to differentiate latent and active tuberculosis disease.³⁴ Among possible strategies to effectively identify the active pathogen metabolism is the enzyme involved in Fe²⁺ availability in *Mycobacteria*, such as the mycobacterial ferredoxin reductase A (*fprA*), which seems a promising target to pursue.³⁵ *fprA* is an NAD(P)H- and FAD-binding reductase, structurally and evolutionarily related to adrenodoxin reductase.³⁶ Two genes, structurally related to the ferredoxin-NADP+ reductase (FNR) protein family, the flavoprotein reductases *fprA* and *fprB*, were identified in *M. tuberculosis* with the former cloned,

expressed and purified.³⁷ *fprA* is involved in the pathogen metabolism and is thought to support the activity of several mycobacterial enzymes.³⁸ Thus, *fprA* represents a potential target for antimycobacterial drugs and bacterial viability. In addition, the aminoacidic sequence of *fprA* is highly conserved in *M. paratuberculosis*, whereas, it differs in other mycobacteria species (*M. leprae*, *M. avium*). Due to these similarities, the detection of this protein could provide useful information about the presence and vitality of bacteria in clinical and environmental samples.

In the present work, we used a recombinant *fprA* protein to set up a rapid and easy method for diagnosis of *Mycobacterium tuberculosis*, based on the lateral flow immunochromatography. The rapidity of lateral flow assay was combined to the sensitivity provided by Cadmium Selenide Quantum Dots (QDs) technology. QDs are fluorescent semiconductor metal oxides with high fluorescence intensity and long photostability.³⁹ QDs, unlike organic dyes, display different colors corresponding to their sizes with a single excitation wavelength.⁴⁰

Materials and Methods

Expression and purification of *FprA*

fprA recombinant protein was expressed and purified according to Fischer *et al.*³⁷ by the University of Sassari, Department of Biomedical Science. Total genomic DNA was isolated from *Mycobacterium tuberculosis* (strain H37Rv) using the QIAamp DNA Kit (Quiagen cat. n. 51304). *M. tuberculosis fprA* sequence (Gene ID: 888839) was amplified from genomic DNA by PCR using the following oligonucleotide sequences: 5'-GCCATGATGCGTCCCTATTACA-3' and 5'-GTCATATGTCAGCCGAGCCCAAT-3', which contained the *Nde I* restriction sites (underlined), for subsequent cloning of *FprA* open reading frame into the pET-11a vector (Novagen cat. n. 69436-3), according to the procedure described by the supplier. *E. coli* BL21(DE3) cells were transformed with pET-*FprA* plasmid and the recombinant *FprA* protein was expressed and purified as described by Fischer *et al.*³⁷

Production of monoclonal antibodies against *FprA*

In order to produce monoclonal antibodies against the recombinant *fprA*, mice were injected intraperitoneally with 200 μ l of antigen (Ag). The immunization scheme was as follows:

30 μ g of antigen were injected at day 0, 30, 60 and 90, followed by a rest period of approximately 8-14 weeks for maturation to obtain antibodies with high affinity. Boosting was

performed with 30 μ g of antigen in phosphate-buffered saline (PBS: 10 mM phosphate, 150 mM NaCl, pH 7.5). Hybridoma cell clones were developed in 7-18 days as described by Leid *et al.*⁴¹ and were screened by ELISA test against *fprA*. Eight clones were selected: 5B8.1; 5G10; 3A10; 2B12; 1A7.1; 2G4; 4H9, and 4E8. The monoclonal antibodies were produced by Quattromed Ltd. (Tartu, Estonia).

Antibody QD conjugation

The monoclonal antibodies were desalted by gel filtration with Zeba™ Desalt Spin Columns (Pierce, cat. n. 89883). The antibodies were biotinylated using the biotin-XX microscale protein Kit (Molecular Probes™ cat. no. B30010) according to the procedure described by the supplier. The concentration of the biotinylated antibody was determined at 280 nm by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). Cadmium selenide (CdSe) QDs (15-20 nm in size) with a maximum emission wavelength of 565 nm, shelled with ZnS and coupled to streptavidin, were purchased by Invitrogen (Invitrogen cat. n. Q10131MP). The amount of 1 μ l of QDs 565 streptavidin conjugate was used to label 200 ng of biotinylated antibody in PBS.

Direct LF assay

Hi Flow plus HF135 membrane sheet (Millipore cat. n. HF13502XSS) was cut into 0.6-0.7 cm wide and 6-7 cm long strips. The proteins were first desalted by gel filtration with Zeba™ Desalt Spin Columns and diluted in PBS before immobilization onto a strip. The LF test device was prepared as follows (Figure 1A): 1.5 μ l of *fprA* recombinant protein (0.5 μ g/ μ l) was spotted on the membrane as test line and 1.5 μ l of Protein A (1 mg/mL, Sigma) was spotted as control line. A distance of 1 cm was chosen between the test and control lines. After protein spotting, the membrane was dried at 37° C for 2 h and then assembled on a glass adhesive backing to provide rigidity. Cellulose sheet (Millipore cat. n. CFSP173000) was cut into 0.6-0.7 cm wide and 4-5 cm long sections and used as sample and adsorbent pad, respectively applied at the origin of the sample flow and at the end of the membrane, overlapping with a 0.4-0.7 cm membrane. One μ l of monoclonal antibody conjugated with QDs (conjugated clone 4H9 at the concentration of 0.2 mg/mL) was loaded onto the sample pad of the lateral flow and then one end of the strip was immediately dipped in a tube containing the running buffer, allowing the migration, by capillary forces, along the dipstick into the adsorbent pad. A positive result was visualized in less than 10 min by the appearance of two fluorescent lines, both in the test and in the control area, on a UV lamp (Gel Doc 1000, Biorad).

Screening the better performing antibodies

Several different monoclonal antibodies (clones: 5B8.1; 5G10; 3A10; 2B12; 1A7.1; 2G4; 4H9 and 4E8) were used as capture probe and immobilized onto the test lines of the nitrocellulose strip (two antibodies per strip). The LF test assay was performed as follows: 1 μ l of the QD conjugated antibody (conjugated clone 4H9 at the concentration of 0.2 mg/mL) was incubated with 500 ng of *fprA* recombinant protein and the mixture was assayed on LF strips.

LF sandwich assay

The lateral flow Assay based on double-antibody sandwich format was prepared immobilizing 1.5 μ l of the monoclonal antibody (1mg/mL) raised against the *fprA* recombinant protein on the test lines. The LF test assay was performed as follows (Figure 1B): 200 ng of the QD conjugated antibody (clone 4H9) was incubated with *fprA* recombinant protein in a final volume of 40 μ l (0.2% Tween-20, 0.2% BSA in PBS pH 7.4). After 15 min incubation at room temperature, the mixture was applied onto the sample pad of the LF and then the bottom tip of the strip was immediately dipped in the running buffer, allowing the migration along the dipstick into the adsorbent pad.

The result of the test was evaluated visually after 10 min by the UV lamp and the intensity of the test zone was estimated densitometrically using the software UN-SCAN-IT™ (Silk Scientific Inc.).

Results and Discussion

Direct LF assays were performed in order to check the best experimental conditions based on general protocols (Figure 1A).

Several different running buffers were tested for the optimum separation of protein complexes along the membrane, the ability to avoid non-specific adsorption of QDs onto the nitrocellulose strip, and for maintaining an appropriate pH for physiological stability of QD conjugated antibodies.

We also tested different combinations of three detergents, namely Triton X-100, Tween 20 and SDS, BSA and glycine, for reduce non-specific adsorption and improved protein stability and solubility. The best results in terms of optimal flow rate and sensitivity were found using a running buffer consisting of PBS containing 1.5% BSA and 3% Tween-20 (Figure 2D). Lower or higher concentration of Tween-20 gave high background (Figure 2A) and fainter fluorescent signals (Figure 2B, C and E).

In order to perform the LF assay based on double-antibody sandwich format (Figure 1B),

the binding ability of antibodies directed to different *fprA* domains was screened.

For this purpose, some different monoclonal antibodies (clones: 5B8.1; 5G10; 3A10; 2B12; 1A7.1; 2G4; 4H9 and 4E8) were used as capture probe and immobilized onto the test lines of the nitrocellulose strip (two antibodies per

strip). 4H9 was QD conjugated and chosen as detection antibody. The 2G4 antibody showed the highest fluorescence intensity in comparison with other antibodies used as capture probe. Good but weaker signals were also recovered from 5G10 and 2B12 antibodies (Figure 3).

The sensitivity of the LF assay was later determined. Two hundred ng of QD conjugated 4H9 antibody was incubated with 0.05, 0.5, 5, 50 and 500 ng of *fprA* recombinant protein in a final volume of 40 μ l (0.2% Tween-20, 0.2% BSA in PBS) to obtain a final concentration of 1.25 pg/ μ L, 12.5 pg/ μ L, 125 pg/ μ L, 1.25 ng/ μ L and 12.5 ng/ μ L, respectively. After incubation at room temperature for 15 min, the mixtures were subjected to chromatographic separation onto LF strips, containing the antibody 2G4 in the test line and Protein A in the control line.

The minimum concentration of *fprA* protein, visually detectable on UV lamp, was 125 pg/ μ L (Figure 4A); however, the densitometric analysis of the test zones allowed the detection limit to be lowered to 12.5 pg/ μ L (Figure 4B).

Conclusions

We describe a method based on lateral flow immunochromatographic assay and quantum dot labeled antibodies as fluorescent tracers. The device was developed to detect *M. tuberculosis fprA* proteins in liquid samples. In this experimental condition it was possible to detect the *fprA* protein at the lowest dilution of 12.5 pg/ μ L.

The use of quantum dot labeled antibodies improved the sensitivity of lateral flow immunochromatographic assay compared to other immune-based tests such as ELISA^{32,33} and dot immunobinding assay.²¹ However, the major advantages of the LF are its speed (results may be available within 15 min) and simplicity.

The lateral flow set up in this work showed the potential to be used as an assay method for detecting the presence of mycobacterial proteins. The LF device is versatile and could be implemented with additional antibodies to detect several mycobacterial antigens important in clinics, such as the 38 kDa antigen,²⁴ 30 kDa antigen,²⁵ 16 kDa antigen,²⁶ lipoarabinomannan (LAM),²⁷ A60,²⁸ Mtb81,²⁹ 55 kDa antigen,²² 14 kDa antigen, 19 kDa antigen,³⁰ and ESAT-6.³¹

In fact, the possibility of using antibodies conjugated with various QDs makes this technology suitable for multiplexing, allowing the detection of different antigens in one LF assay.⁴² Further work is required in order to validate the antibodies cross-reactivity towards different *fprA* proteins from *Mycobacterium spp.* If the diagnostic value of *fprA* protein is confirmed, efforts should be focused on the clinical sample processing, in order to obtain the best exposure of *fprA* protein in the lateral flow assay.

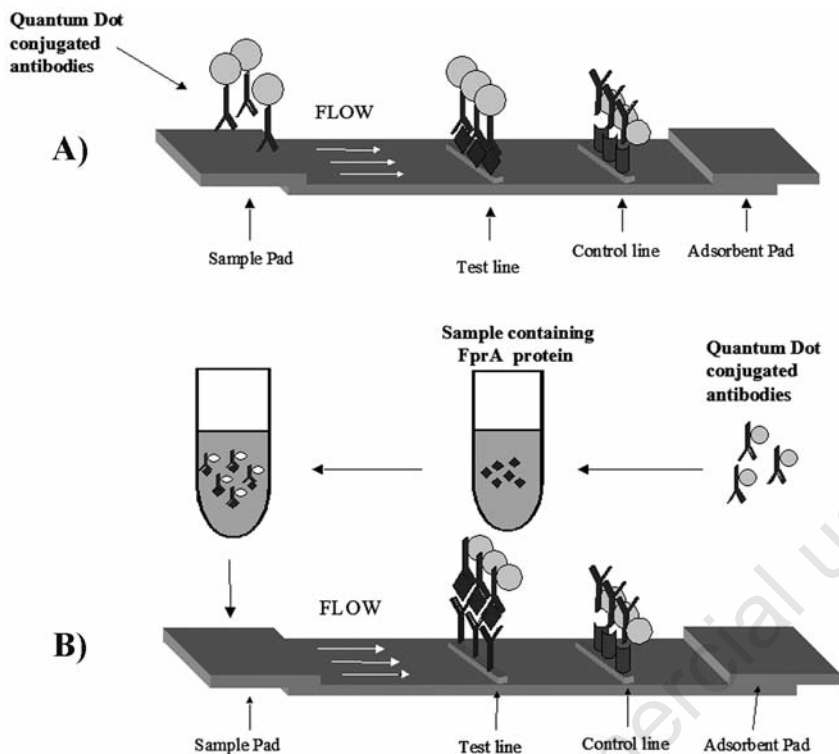


Figure 1. Schematic description of immunochromatographic test. (A) Direct lateral flow assay: QD conjugated antibody solution is applied onto the sample pad of the LF strip, containing on the test line the *fprA* recombinant protein and on the control line the Protein A. (B) Lateral flow assay based on double-antibody sandwich format: QD conjugated antibodies are incubated with the sample and then applied onto the sample pad of the LF strip. The complex QD conjugated antibody-protein is captured on the test line, by the immobilized antibodies and the excess of QD conjugated antibodies are captured on the control line, by the immobilized Protein A.

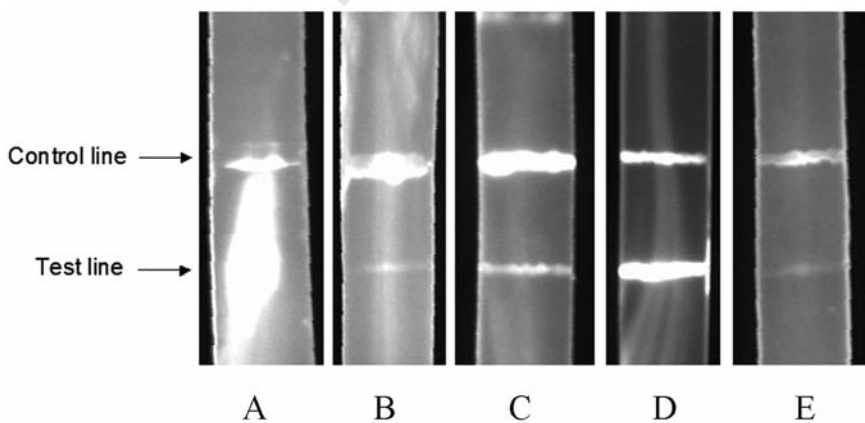


Figure 2. Lateral flow device setup was carried out using the following running buffers: PBS+BSA + (A) 0.5% Tween-20; (B) 1% Tween-20; (C) 2% Tween-20; (D) 3% Tween-20; (E) 4% Tween-20.

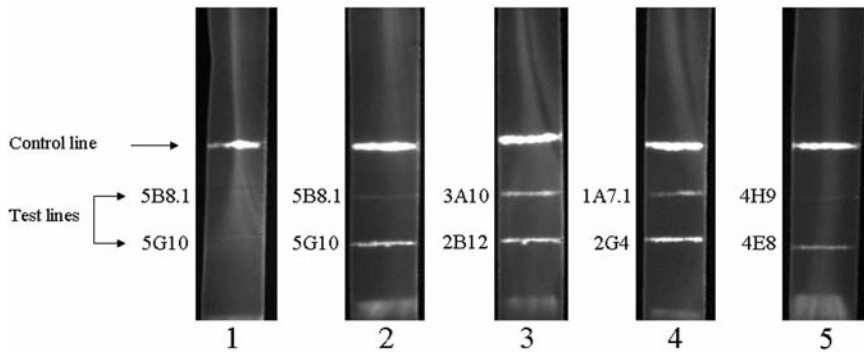


Figure 3. Lateral flow assay based on double-antibody sandwich format. Different monoclonal antibodies (clones: 5B8.1; 5G10; 3A10; 2B12; 1A7.1; 2G4; 4H9 and 4E8) were immobilized onto the test lines of the nitrocellulose strip (two antibodies per strip). Protein A was immobilized on the control line. The sample pad was loaded with QD conjugated antibody (clone 4H9) previously incubated with *fprA* recombinant protein (Lanes 2-5). As a negative control, QD conjugated antibody was incubated with BSA (Lane 1).

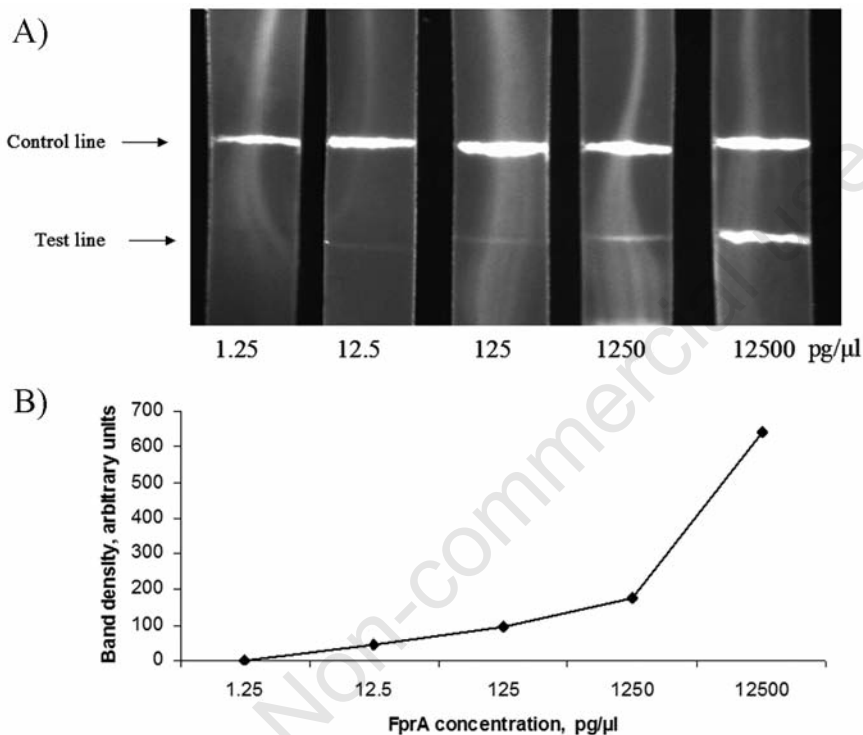


Figure 4. LF strips were tested for sensitivity using decreasing concentrations of *fprA* antigens. (A) QD conjugated 4H9 antibody was incubated with increasing concentration of *fprA* recombinant protein, ranging from 1.25 to 12500 pg/μL, and assayed onto a LF strip device containing on the test line the capture antibody 2G4 and on the control line the Protein A. (B) Densitometric analysis of the test zones of the strip. The density of the test zone was plotted against the concentration of *fprA* protein loaded on the strip.

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