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Sensitivity and colour intensity enhancement in lateral flow immunoassay tests by adjustment of test line position

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ABSTRACT

In this article, we have enhanced the sensitivity and limit of detection of lateral flow immunoassay tests, exemplified for the detection of human chorionic gonadotropin (pregnancy) through determination of proper test line position. Test line position affects the flow velocity and concentration profile of analytes at the test line, which itself impacts the reaction rate and thus the colour intensity. The flow velocity was precisely modelled, both analytically and numerically, and the predicted analytes' moving front (lateral) velocity was experimentally verified. Evolution of the velocity magnitude was determined to locate points with proper capillary velocity and accumulated bed-volume. This provides insights into the essence of LFIAs performance for simple and easy to practice improvements. The colour intensity was measured by quantifying the RGB of the resulting test line colours. The response colour intensity was enhanced for at least 20%, up to 40% by proper displacement of the test line position, and the limit of detection was cut by half to 10 IU/L.

1. Introduction

Lateral flow immunoassay (LFIA) is one of the most useful techniques in diagnostic areas such as medical, veterinary, agricultural, environmental, industrial, sports diagnostics and food safety. This method has rapidly developed due to its benefits such as accuracy, rapid readout and smooth operation [1–4] and low detection limit [5]. The result of the LFIA test is a visual signal [1,6] that appears in a few minutes [4,7]. Rapid tests are qualitative tests, but they can be used in the quantitative or semi-quantitative analysis using techniques such as computer image analysis [8], strip scanners/signal readers, cell-phone accessories [9,10], magnetic or radioactive signal readers [7,11].

The principle of the LFIA is based on the movement of the sample liquid and labelled-conjugate through a porous pad [12]. The test strip consists of porous pads of sample and conjugate, a test membrane, absorbent pad and a backing card as a backbone of the strip (Fig. 1). The sample pad is often made of cellulose and might have some surfactants that facilitate the liquid flow. Conjugate pad contains a conjugate of label particles (markers) and antibody or antigen, depending on the assay type. The label particles are the source of test/control line signal, and their choice depends on the desired type of output signal, its intensity, and the assay type [8,13], among them, gold nanoparticles are the most common [14]. On the test membrane or detection zone, a test line and a control line is printed. The reaction between the sample liquid and antibody/antigen of the test line determines the result of the test. Depending on the test format (competitive or sandwich), the formation of a coloured test line will indicate the negative or positive result of the test. The colour of the control line must change in both competitive and sandwich assays; otherwise, the results are not reliable. Finally, the absorbent pad acts as a reservoir and absorbs the sample liquid [15,16].

Sensitivity and performance of LFIA devices is a function of flow velocity and concentration profile of analytes at the test line, which the two latter parameters influene the reaction rate, and thus, the colour intensity. Therefore, the parameters that change the velocity profile at the test line, and bed volume after test line, can affect the sensitivity and performance of the test. Other influential parameters include pads and test membrane materials [12,15], size and material of the marker particles [6,12,15,17], recognition elements, membrane thickness, structural properties of porous pads [18,19] and 2D geometry of the test pads [6,20].

A proper velocity magnitude also enhances the reaction progress rate, and thus, the performance of the LFIA, which manifests as an increase in the response colour intensity. The immunoreaction never

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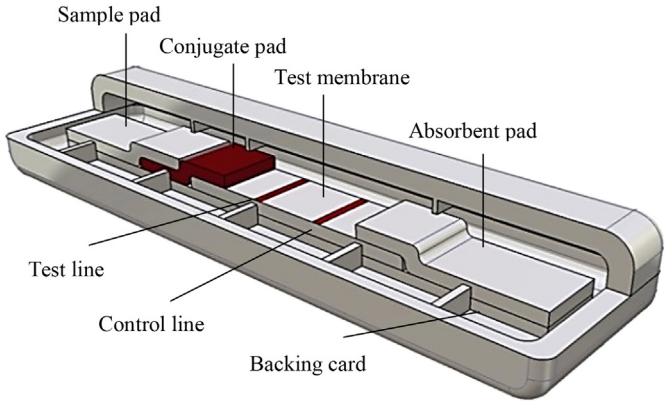

Fig. 1. LFIA test strip components.

completes in the short 10-minute read-out interval. Experimental reports clear that the antibody/antigen reaction reaches equilibrium at 37 °C after 4 h, and only 40% of the reaction completes in the first 15 min [21]. Therefore, arriving at the best possible velocity profile of analytes in the test line is an essential parameter for boosting the antibody/antigen reaction. As will be demonstrated later, upon moving forward in porous pads, the average sectional flow velocity reduces. Thus, the test line position in the length of the pad can affect the sensitivity of the LFIA pad. Moreover, the contrast between capillary velocity magnitude and the accumulated bed-volume before the test-line, and accordingly their impact on the performance of the LFIA has not been comprehensively studied.

In this article, the flow velocity at different test line locations was quantified. Through proper prediction and adjustment of the test line position, the sensitivity and response colour intensity of LFIA test strips were measured for pregnancy biomarkers. Nevertheless, approach, methodology and research findings are general and offer insights into simple, and easy to practice methods that may extend the performance of all LFIA test strips. The flow velocity was anticipated through numerical and analytical modelling, and the average analytes lateral velocity was experimentally verified. Test lines were printed in four different distances of the test membrane. The sensitivity and test line colour intensity were evaluated by developing an RGB quantification subroutine. In order to study the effect of test line position on the sensitivity and limit of detection of the strip, all other influential parameters, such as the concentration of the test line antibody, was considered to be the same for all of the strips.

2. Materials and methods

The experimental procedure for sensitivity and performance enhancement of LFIAs is summarized in the following.

2.1. Chemicals and equipment

Sample pads (GFB-R7L), absorbent pads (AP110), conjugate release matrix (PT-R7) backing cards (L Type) and nitrocellulose test membranes (CNPCSS-12, 12 μm, 30 mm wide) were purchased from Advanced Microdevices PVT LTD (Ambala, India). Anti-human chorionic gonadotropin beta monoclonal antibody (C102), produced in mouse (Leinco Technologies Inc., St Louis, USA) was used as the test line antibody. Rabbit anti-beta hCG secondary antibody produced in goat (Rouzhan Azma, Tehran, Iran) was used as a control line antibody. Anti-human chorionic gonadotrophin polyclonal antibody (produced in rabbit) and 40 nm particle diameter gold nanoparticles were purchased from Rouzhan Azma (Tehran, Iran). Micropette plus pipette (Dragon Lab, Beijing, China) was used for handling the sample liquid. Test line and control line antibodies were printed on the test membrane by Biodot XYZ 3060 Dispensing Platform (California, USA). For diluting the analytes, 40 mM PBS (phosphate buffer saline) and Tween20 were purchased from DNA Biotech (Tehran, Iran). Standard ELISA kit (HCG-96) as the sample analyte and other reagents, used for preparing gold-antibody conjugate were purchased from Pishtaz Teb (Tehran, Iran).

2.2. Test strip preparation

To prepare Gold-Antibody solution, rabbit anti-hCG polyclonal antibody solution (100 μg/mL) was added to the gold nanoparticle solution of pH 9, while the solution was on the stirrer. Then, the solution was centrifuged at 650 rpm for 20 min in an incubator, followed by centrifugation at 14000 rpm for 20 min. The conjugation of gold-antibody was then suspended in 2 mM borate buffer (pH of 7.4) containing 10% of sucrose, following the same protocol used by others [20]. The conjugate pad was soaked into the conjugation of the gold-antibody solution and then dried for 30 min in a vacuum drier.

Test line and control line antibody solutions (1 mg/mL of antibody in 10 mM PBS) were dispensed on the test strip using Biodot XYZ 3060 dispensing system at a rate of 1 $\mu L \cdot cm^{-1}$. Control line antibody was dispensed at a distance of 20 mm from the beginning of the test membrane. Then the nitrocellulose pads were dried at 60 °C for 30 min. After that, the pads were divided into four groups; G1, G2, G3, and G4. Test line antibody was dispensed in distance of 2.5 mm, 7.5 mm, 12.5 mm and 17.5 mm from the beginning of the test membrane for groups G1, G2, G3, and G4, respectively (Fig. 2). Then the pads were dried at 60 °C for 30 min. After preparation of nitrocellulose pads, all the pads were assembled on the backing cards with 0.5 mm overlap. Then, a slight pressure was applied to the membranes using a manual pressing machine, the cards were cut into 3.2 mm wide test strips by a semi-automatic strip cutter and the total length of strips was 60 mm. 5 strips of each group were compared to the pregnancy LFIA tests (purchased from Rouzhan Azma Co.) as control strips, using the same concentration. The test line and control line of all the commercial strips turned into red and positive results were observed, using the same sample analyte used for the modified test strips under evaluation.

2.3. Test procedures

To determine the detection limit by visual inspection (unaided eye), 30 μL of sample analyte with hCG concentration of 0, 5, 10, 20, 50, 100 and 250 IU/L was dispensed on the sample pad. The strips were priory washed by the Tween20/PBS solution to reduce the effect of non-specific binding of antibodies and analytes to the membrane surface [22,23]. After 10 min and developing the colour of the lines, the strips were read by strip reader (ten replicates for each concentration). The colour intensity was quantified by processing the RGB of images captured from the response line (the strips were scanned without drying), using a MATLAB code [24,25], and the stronger the colour intensity, the higher the percent value acquired. The minimum concentration that resulted in a detectable/reliable colour change in test line in all the strips was designated as the minimum limit of detection for colour intensity tests. A USB digital microscope (Coolingtech, Guangdong, China) recorded the process, to enable measuring the average flow velocity in four sections of the test membrane.

2.4. Flow velocity determination

One of the most critical factors in the sensitivity and performance of lateral flow is flow velocity, which was calculated here analytically and numerically, and then the average flow velocity in the test membrane was measured experimentally.

2.4.1. Analytic method

Upon release of the fluid analyte on the porous medium, the capillary pressure (force) draws the fluid on the medium, according to