34th Annual International Conference of the IEEE EMBS
San Diego, California USA, 28 August - 1 September, 2012

A mathematical model to predict the optimal test line location and sample volume for lateral flow immunoassays

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Abstract— **Lateral flow immunoassay (LFIA) platform is one of the most relevant technologies for screening and diagnosing clinical conditions [1]. However due to low sensitivity and poor repeatability of the platform it has been used only for limited and non-critical tests [2] [5]. Mathematical models have been used to understand the principles of capillary flow and antibody antigen based immunoreactions in nitrocellulose membrane typically seen in LFIA [4]. The model presented in this paper predicts the optimized location of test line on LFIA strip, sample volume and total reaction time that is needed to achieve the required sensitivity for different analytes on a case to case basis. The membrane properties like capillary flow time (s/cm), concentration and affinity constants of antibodies can be varied and the corresponding effect on strip design can be found. Hence this model can be used as a design tool to optimize the LFIA strip construction and reagent development processes.**

I. Introduction

Lateral flow immunoassay (LFIA) test strip is a platform for performing rapid and easy to use diagnostic tests. The LFIA platform technology has matured over the years, from its main application of pregnancy tests to other applications in the clinical diagnostics field like HbA1c, cardiac troponin I and molecular diagnostics for infectious disease detection. It's been reported that over 200 companies worldwide are manufacturing tests in LFIA platform, with a total value of approximately $2.1 billion dollars (USD), as of 2006 [1].

The LFIA platform has several advantages over the conventional ELISA (Enzyme-linked immunosorbent assay) and other methods of immunoassay such as high shelf life without the need for refrigeration, involvement of only a single process step, low cost and lesser turnaround time [2]. Hence, LFIA is very relevant and appropriate technology for point of care technology applications [11]. Particularly, it is most relevant in the context of the public healthcare management in India and other developing countries where infrastructure and skilled personnel are limited.

However, this technology has not been widely applied where sensitive, highly reproducible and quantitative results are required [2]. For example, immunoassays for thyroid stimulating hormone, prostate specific antigen and cardiac troponin I require a very high sensitive platform for useful clinical information to be derived [5]. Furthermore, LFIA development itself requires knowledge of several diverse principles from biology, chemistry, physics and engineering like nitrocellulose membrane manufacturing, antibody generation, chemical manipulation methods, fluid dispensing etc [3]. Although several companies are providing LFIA platform solutions for diagnosis of such high sensitive analytes, the cost of these systems are very high due to use of complicated reader technologies [6]. Hence there is a need for focused innovations on LFIA strips to improve its performance and reduce development cost. [9, 11]

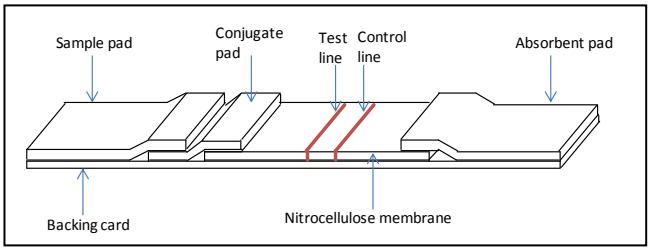

Figure 1. Typical configuration of a LFIA strip; Test line is where the results are read; Presence of control line is to confirm the validity of the assay results.

A typical LFIA strip is shown in Fig.1. The LFIA strip works on the principle of immunoassay which is commonly used in ELISA. Here a target analyte is captured by an antibody fixed onto a solid support like ELISA plate well or the test line in LFIA strip and a second antibody binds to the analyte to a different epitope thereby forming a sandwich. The second antibody usually has a detection species like dye or gold nano particles (GNP) attached to it. This detection species is usually measured to determine concentration of the target analyte [7, 1, 10].

Optimizations on the LFIA platform can be fourfold namely strip construction, assay technology, reader technology and manufacturing processes. Strip construction involves material selection for components like sample pad, conjugate pad, membrane, absorbent pad etc, their dimensions and arrangements, use of actuation mechanisms like hydrophobic gate [8], multiplexing methods, housing and backing card [1]. Assay technology involves selection of the right antibody pair against the analyte of interest with the required affinity, specificity and target epitopes, chemical modification of antibodies with dyes, optimizing final concentrations of the reagents and buffers and stabilizers [1]. The choice of reader technology further determines the sensitivity, linearity, signal to noise and dynamic range of the reader. Colorimetry and fluorescence are the most widely used detection methodology which results in good sensitivities. New detection methodologies like capacitance measurements based on change in dielectric properties due to immunoreactions are interesting and exciting technologies to look into [6]. Finally, the ease of manufacturing processes, innovations in the construction and material robustness play an important role in eliminating the lot to lot variations in the assay results and in reducing the cost per test [3, 1].

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All these four modules are equally important and merits focussed research. The aim of this work is to optimize the construction of the LFIA strip and reagent development processes for any analyte of interest with a target sensitivity. Qian et al have published an elaborate mathematical model of the capillary flow and the immuno reactions happening on the lateral flow membrane. As an extension to this, our work involves understanding the various components on the LFIA strip, their functions and host of other variables that need to be optimized. Since the immunoreaction happens while the sample flows through the membrane, we have considered the effects of flow rate, antibody affinity, concentration of antigen and antibody, distance of test line from sample pad, volume of sample and reaction time, on the kinetics of the reaction. These parameters are then varied to achieve the optimized kinetics of reaction which could improve the sensitivity of the test.

II. MATHEMATICAL MODEL OF THE SYSTEM

A typical LFIA membrane made of a flat porous membrane is considered. A sandwich antibody antigen reaction is considered for the model. The two main principles based on which LFIA strip works are antibody-antigen reaction and capillary flow.

$$Ag + Ab \xleftrightarrow{K} Ab - Ag ; K = \frac{[Ag - Ab]}{[Ag][Ab]} \quad (1)$$

The bond between antibody (Ab) and antigen (Ag) is a non covalent bond and reaction is assumed to be a first order equilibrium reaction where the rate of the reaction is proportional to the concentration of the reactants [4]. In equation (1), K is the equilibrium constant or the affinity constant that determines the rate of the reaction. [Ag-Ab] represents the concentration of the complex formed between antibody and antigen [4].

LFIA system is a capillary driven system, where the capillary flow of the antibodies (reagent) and target analyte (sample) happen though the reaction membrane. When sample containing the target analyte is added, it wets the detection antibody at the conjugate pad (where detection antibody is present in dried form) into solution, and the mixture flows through the nitrocellulose membrane, by capillary action. While analyte and antibody flows through the membrane, an immunoreaction takes place to form analyte-detection antibody complex. The analyte-detection antibody complex as well as free analytes react via the available site of analyte and bind with the immobilized capture antibodies at the test line to form capture antibody-analyte-detection antibody and capture antibody-analyte complex respectively. The signal from the test line is the measure of amount of capture antibody-analyte-detection antibody complex which is related to concentration of analyte present in the sample [7, 1, 10].

Square brackets are used to denote the concentrations of various species. [A], [P], [PA], [R], [RA] and [RPA] are analyte (whose concentration is to be detected), detection antibody (that binds with analyte and has a detectable signal attached with it), analyte-detection antibody complex, capture antibody (that is present on test line), capture antibody-analyte complex (that is formed by free A on the test line) and capture antibody-analyte-detection antibody complex (that is measured as a signal for concentration of analyte) respectively [4].

A_0, P_0, R_0, K_{ai}, K_{di}, x, t, T and D are concentration of analyte in sample, concentration of conjugate, initial concentration of capture antibody, association rate constant of the reaction (where i depicts the reaction number) dissociation rate constant of the reaction (where i depicts the reaction number), starting location of test line as measured from sample pad end, time to reach x, time to flow through the test line and diffusion coefficient respectively. Total time between adding sample on sample pad and observing signal on test line = t + T.

The model has two stages of optimization that is carried out. One is before the test line and other is on the test line optimization. Also there are few assumptions that were made to simplify the model. No reaction between P and A takes place before entering membrane and no hydrophobic / time actuation gate is present. P is released uniformly from conjugate pad throughout the duration sample flows through it. Membrane velocity provided by manufacturer is constant throughout the length of membrane. Matlab® Partial differential equation toolbox™ was used to solve the equations in the model.

A. Optimization model before test line

The binding of the analyte and the detection antibody has to be maximized because concentration of analyte is measured in terms of complex it forms with the detection antibody. The objective of the optimization is to predict the location of test line on the membrane where the concentration of analyte-detection antibody complex is at its maximum. The farther is the test line from the conjugate pad, more is the time for reaction between analyte and detection antibody to form complex. The membrane property that controls this optimization is capillary flow time (s/cm). More the flow time, slower would be the flow and the test line would be located much closer to conjugate pad.

$$A + P \xleftrightarrow{K1} PA \quad (2)$$

Equation (2) represents the reaction between A and P. Here K1 is the equilibrium reaction constant which is K_{a1}/ K_{d1}. Before test line only the above reversible reaction takes place as the mixture flows through the membrane. The test line needs to be placed such that when the mixture enters it has maximum concentration of PA. Since, initially no PA was present, thus maximum [PA] would be same as [PA] at equilibrium of (2). The convection-diffusion mass balance equation is given in equation (3) [4].

$$\partial[PA]/\partial t + u\,\partial[PA]/\partial x = -D\,\partial^2[PA]/\partial x^2 + ka_1[A][P] - kd_1[PA] \quad (3)$$

Here $[A] = A_0 - [PA]$, $[P] = P_0 - [PA]$. The equation reduces to

$$\partial[PA]/\partial t + u\,\partial[PA]/\partial x = -D\,\partial^2[PA]/\partial x^2 + ka_1[PA]^2 + \{Ka_1[A][P] - Kd_1(A_0 + P_0)\}[PA] + Ka_1A_0P_0 \quad (4)$$

The initial condition is $[PA](0,t) = 0$ and the boundary conditions are $[PA](x,0) = 0$ & $\partial[PA]/\partial x = 0; for\_x = ut$. At equilibrium no change in [PA] occurs and upon solving gives [4],

$$Ka_1[PA]^2 - \{Kd_1 + Ka_1(A_0 + P_0)\}[PA] + Ka_1 A_0 P_0 = 0 \quad (5)$$

$$[PA]_{opt} \sim 0.9[PA]_{eq} = 0.9\left\{A_0 + P_0 + Kd_1/Ka_1 - \left((A_0 + P_0 + Kd_1/Ka_1)^2 - 4A_0P_0\right)^{1/2}\right\}/2 \quad (6)$$

$[PA]_{opt}$ is needed because equilibrium takes long time for achieving that may result in very long length of reaction membrane before test line, which may not be practically feasible. Thus, positioning the test line at a location where $[PA]_{opt}$ is reached would maximize the amount of analyte-detection antibody complex and thus provides an ample chance for better reaction kinetics.

B. Optimization model on the test line

It is on the test line that the signal is measured, and thus the amount of complex that the capture antibody on test line forms with the complex of analyte and detection antibody determines the ultimate sensitivity of the assay. Since the capture antibody immobilized on the membrane is fixed on the test line, more the volume of solution that passes through the capture, more is amount of complex of capture, analyte and detection (sandwich) formed. Thus, this optimization predicts the volume of sample and the total time for which the test should be carried to achieve certain target sensitivity. Again, the membrane property that plays most important role is capillary flow time (s/cm). More the flow time, slower is the flow more is the interaction time for the reaction to happen and lesser the amount of solution required.

Following reactions takes place on the test line [4]:

$$A + P \xleftrightarrow{K1} PA \quad (7)$$

$$A + R \xleftrightarrow{K2} RA \quad (8)$$

$$PA + R \xleftrightarrow{K3} RPA \quad (9)$$

$$RA + P \xleftrightarrow{K4} RPA \quad (10)$$

On test line the variation of [RPA] with respect to t should be determined, and the time when it tends to become nearly constant is to be found. Corresponding to this time the volume of sample required for the test can be found. Volume of sample required = T x u x A. where, T is time for flow through test line required, u is membrane velocity and A is cross sectional area of membrane. Thus, T = (time since starting of test – time to reach start of test line); Also let X = distance from start location of test line;

$$\partial[A]/\partial T + u\,\partial[A]/\partial X = -D\,\partial^2[A]/\partial x^2 - (Ka_1[A][P] - Kd_1[PA] + Ka_2[A][R] - Kd_2[RA]) \quad (11)$$

$$\partial[P]/\partial T + u\,\partial[P]/\partial X = -D\,\partial^2[P]/\partial x^2 - (Ka_1[A][P] - Kd_1[PA] + Ka_4[P][RA] - Kd_4[RPA]) \quad (12)$$

$$\partial[PA]/\partial T + u\,\partial[PA]/\partial X = -D\,\partial^2[PA]/\partial x^2 - (Ka_1[A][P] - Kd_1[PA] + Ka_3[PA][R] - Kd_3[RPA]) \quad (13)$$

$$\partial[RA]/\partial T = (Ka_2[A][R] - Kd_2[RA] - Ka_4[P][RA] + Kd_4[RPA]) \quad (14)$$

$$\partial[RPA]/\partial T = (Ka_3[PA][R] - Kd_3[RPA] + Ka_4[P][RA] - Kd_4[RPA]) \quad (15)$$

$$[R] = R_0 - [RA] - [RPA] \quad (16)$$

Equations (11) to (16) represent the mass balance equation of species in reactions happening on the test line and are simultaneously solved to determine their effect with respect to time and distance.

Initial conditions:

$$[A](X,0) = 0; [P](X,0) = 0; [PA](X,0) = 0; [RA](X,0) = 0; [RPA](X,0) = 0$$

Boundary conditions:

$$[A](0,T) = [A]_{opt} = A_0 - [PA]_{opt}; [P](0,T) = [P]_{opt} = P_0 - [PA]_{opt};$$
$$[PA](0,T) = [PA]_{opt}; \partial[RA]/\partial x = 0 \;\&\; \partial[RPA]/\partial x = 0, for, x = 0;$$

At the beginning of the test line, i.e. at x=0, [RA] and [RPA] is at its minimum and at the end of the test line (whose width is say 3mm) i.e. beyond x=3mm, no further changes in concentration takes place. So,

$$\partial[A]/\partial x = 0; \partial[P]/\partial x = 0; \partial[PA]/\partial x = 0;$$

$$\partial[RA]/\partial x = 0; \partial[RPA]/\partial x = 0; for, x = 0.003$$

III. RESULTS & DISCUSSIONS

A. Before test line

Following values for constants were used for calculations; $A_0 = 10^{-8}$; $P_0 = 10^{-8}$; $k_a = 10^6$; $k_d = 10^{-3}$; $D = 10^{-12}$. Fig. 2 shows the variation of [PA] with distance along the membrane for a capillary flow time of 400s/4cm. The plot is a grouped representation of four results for t as 150s, 300s, 450s and 600s since at a particular distance x the concentration is different at different times. That division of time whose end point is closest to point of intersection with $[PA]_{opt}$ is be considered. The results are summarized in Table 1. As the membrane velocity was increased the location also moved farther from the conjugate pad, by the same factor. However the time for reaching the optimum concentration is same for all membrane velocities. If the location is too far or impractical for strip construction, say greater than 4 cm from conjugate pad, then use of actuation time gate [8] at the conjugate pad is recommended to achieve the required sensitivity.

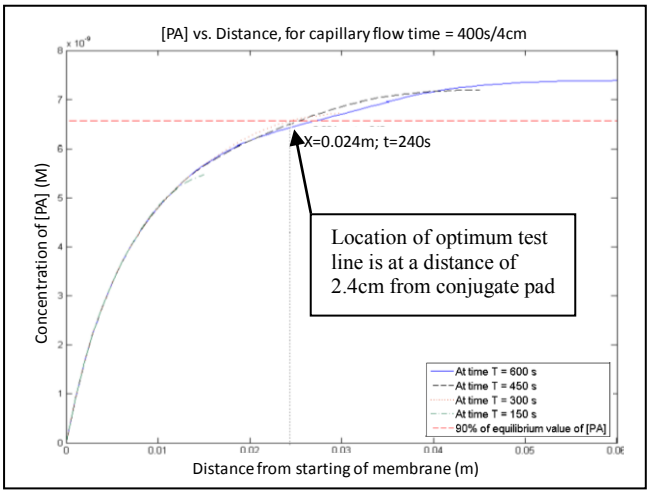

Figure 2. Figure shows variation of [PA] with distance moved along the membrane for capillary flow time = 400s/4cm

TABLE I. RESULTS OF BEFORE TEST LINE OPTIMIZATION

Membrane velocity (m/s)	*Capillary flow time (s/4cm)*	*Location of test line (cm)*	*Time to reach test line (s)*
0.0001	400	2.4	240
0.0002	200	4.8	240
0.0003	133.33	7.2	240
0.0004	100	9.6	240
0.0005	80	12.1	240

TABLE II. RESULTS OF ON THE TEST LINE OPTIMIZATION

Membrane velocity (m/s)	*Capillary flow time (s/4cm)*	*Volume of sample (uL)*	*Passage time through test line (s)*
0.0001	400	14.5	363
0.0002	200	28	350
0.0003	133.3	39.6	330
0.0004	100	52	325
0.0005	80	64	320

B. On the test line

Fig. 3 shows the variation of all the reactant and product concentrations along the width of the test line. Concentration f all species remains constant throughout the test line but slight dip in concentrations is observed in slower membranes. Fig. 4 shows the variation of average [RPA] over time on the test line. The volume of sample required for test increases as the membrane velocity is increased. There is also a small decrease in time required for the process as the membrane velocity is increased. The results are summarized in Table 2.

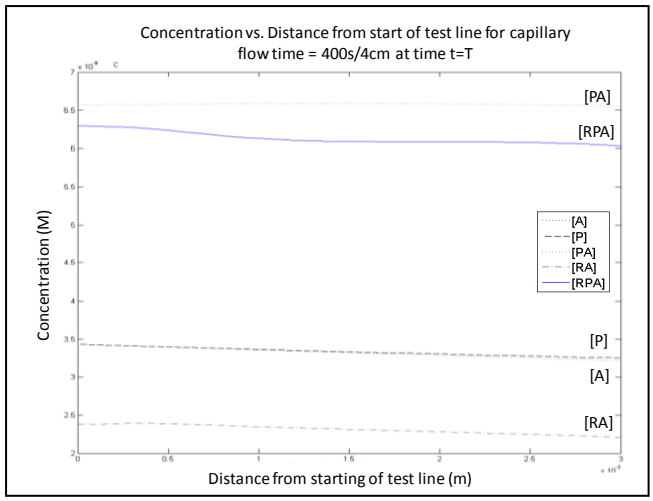

Figure 3. Figure shows variation of all concentrations with distance from start of test line for capillary flow time = 400s/4cm.

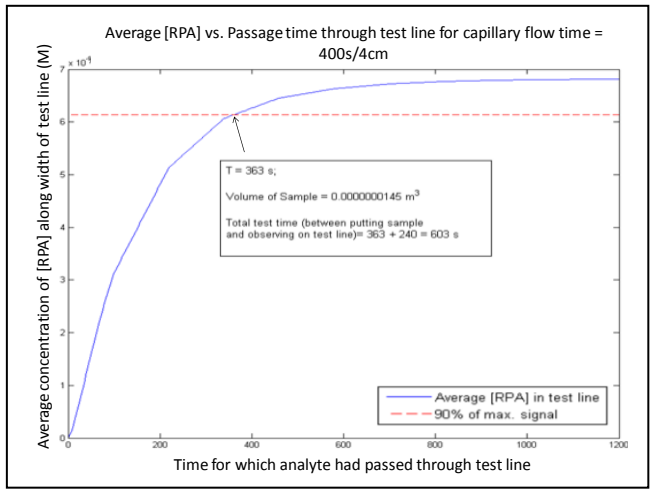

Figure 4. Figure shows variation of Average [RPA] along x vs. time of flow through test line for capillary flow time = 400s/4cm.

IV. Conclusions

The work models the LFIA strip and helps in optimizing the construction of the strip based on the analyte for which the test is being developed and assesses the performance requirement for the same. The 'before test line' optimization results determine the location of the test line where maximum analyte-detection antibody is formed. The 'on the test line' optimization results determine the minimum volume of the sample and duration of the test that is needed to achieve the required sensitivities. Further, the membrane type, flow time, antibody characteristics, affinity and cross reactivity can be varied in the model to get the right construction for any required analyte. Hence this is a very useful tool which can be used as a first step towards the development of the LIFA strip, which will help decrease the timeline and cost.

ACKNOWLEDGMENT

We would like to thank Dr. Venkat Kalambur, Dr. Sindhulakshmi Kurup and Dr. Zubin Varghese for their guidance and support to the work.

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