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Effect of Protein Extraction Buffer and Blocking Buffer on Efficiency of Prepared Nitrocellulose Membrane for Assembly of Lateral Flow Immunoassay Strip

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ABSTRACT

Lateral flow immunoassay strip is a simple, reliable and rapid qualitative tool for primary screening of transgenic proteins. The extraction of proteins from sample products for performing lateral flow immunoassay (LFIA) strip test is an important step. In this study, the protein extraction efficiency of various buffers prepared in-lab and some extraction buffers from commercial kits were tested. In addition, the effects of blocking buffers along with hybridization timings, for blocking of unoccupied sites, on prepared nitrocellulose membranes were examined during assembly of LFIA strips. It was estimated that 1XPBS of pH 7 is best protein extraction buffer according to our sample, Bt-Cry 1Ac from Bt-Cotton seeds. Furthermore, during manual assembly of LFIA strips, we found that the sealing tapes used as covering for one-unit assay has no effect on efficiency of LFIA strip tests. In our experiments, we optimized hybridization timing for blocking of unoccupied sites on nitrocellulose membranes is 30 minutes. We also optimized a simple composition for blocking buffer.

Key words: *Transgenic proteins, Lateral flow immunoassay, Nitrocellulose membranes, Bt-Cotton, Protein extraction buffer*

INTRODUCTION

In agriculture biotechnology the basic use of immunoassays is to authenticate the presence or absence of transgenic material in a product or to measure the amount of transgenic material present in a product[1]. Two commonly used immunoassays are lateral flow immunoassay (LFIA) strips and enzyme-linked immunosorbent assays (ELISA). LFIA strips are used for qualitative or partial quantitative identification of antigens[2]. The scientific basis of the lateral flow immunoassay was consequent from the latex agglutination assay, which was developed in 1956 by Plotz and Singer. In the same decade, micro titter plate-based immunoassays were developed[3]. The primary principle of the lateral flow technology be advanced through the early 1980s. Since then, at least other 500 patents have been filed on various aspects of the technology[4].

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LFI strips for detection of transgenic proteins use same antibodies as in ELISA, except that the secondary antibody (goat anti rabbit antibody IgG) is conjugated with colloidal gold fairly than an enzyme for generating a visible signal. LFIA strip consists of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad assembled together on a thin plastic backing. The strip, in the conjugate pad, contains one mobile, labeled specific antibody and two immobilized capture antibodies present upstream in the nitrocellulose membrane[5]. The first immobilized capture antibody is specific for the transgenic protein and designated as test line. The second immobilized capture antibody is polyclonal secondary antibody (IgG) for the unbound detection and is designated as control line[6]. After application of test sample, if many gold-labeled antibodies attached with antigen accumulates at the test line results in a visible line, it is indication of transgenic protein's presence. Sample liquid continues to flow upstream in the strip by capillary action, and many unbound gold-labeled antibodies are captured by the control line capture antibody[7,8]. A colored line shows that the liquid flowed all the way through the test zone and the device is working properly; if control line is missing it indicates an invalid result. If transgenic protein is not present in the sample solution, only control line appears and the result is negative. If both lines appear, the result is positive[9]. The objective of the present study was to develop LFI strip for the primary screening of transgenic Cry1Ac protein expressed in Bt cotton. For this purpose, the technology was optimized, which included the selection of best nitrocellulose membranes for this assay, optimum pH of protein extraction buffer and blocking timing for blocking of unoccupied sites on nitrocellulose membrane.

MATERIAL AND METHODS

For nanocolloidal gold "Gold-in–a-Box™ kit (Cat. No. NGIB01-B044) was procured from BioAssay Works, LLc., USA It contained Naked Gold®-(44ml)-20nm-15 OD particles, buffer solution A, buffer solution B, buffer solution C, buffer solution D, BSA blocking solution and gold drying buffer. Highly purified polyclonal antibodies, which were Rabbit IgG (Cat. No. 41-GR30) and Bt Cry1Ac antibody (Cat. No. 70r-BR005) were purchased from Fitzgerald International, country. Bt Cry1Ac was in lyophilized form, while Rabbit IgG was supplied in phosphate buffered saline (PBS), pH 7.4 with 0.02% Na Azide. 1X PBS (10 mM), 5% sucrose solution, BSA blocking buffer (3% BSA in 10 mM PBS) and PBS-Tween 20 (3%) with 5% BSA and 2 ml polyvinyle alcohol (blocking buffer) were prepared separately.

2.1 Preparation of Nitrocellulose Membrane for Assembly of Lateral Flow Immunoassay Strip by Using Different Nitrocellulose Membranes without Treatment of Blocking Buffer

Conjugate pad contains dried antibody-gold conjugated solution, which was prepared as per supplier methode. For each 1 ml of gold-antibody conjugate solution 0.1 ml of gold drying buffer was mixed. In this experiment pH of gold-antibody solution was 8.8. 10 µl of prepared solution was dispensed on a conjugate pad of size 8 mm × 5mm. This pad was dried in an incubator at 37ºC for overnight. For this experiment three different nitrocellulose membranes (Millipore HF135, Millipore HF120 and Fermentas) were used, these membranes were varying from each on basis of pore sizes. The membranes were cut into same sized strips of 2.5 × 0 .5 cm. Four strips from Millipore HF135 NC membrane, four strips from Millipore HF120 NC membrane and two strips from Fermentas NC membrane were taken for assembly of 10 Lateral Flow Immunoassay strips. These NC membranes were labeled accordingly. Each membrane was treated with same protocol. Test and control lines of antibodies were placed in the middle of the NC membranes. The lines were 1 cm apart from each other. The control lines on each strip were placed by goat anti rabbit antibody (1 µl/line; 1 mg/ml) and test lines were consisting of immobilized anti-Bt Cry1Ac antibody (1µl/line; 1 mg/ml) mixed with 3% methanol. Membranes were placed in an incubator at 37ºC to dry for 1 hour.All required components for the assembly

of lateral flow immunoassay strip were assembled into coordinating manner as a one-unit assay. For support of one unit assay adhesive plastic backing was used. Conjugate pad was pasted on the card, near test line side, overlapping 2 mm on NC membrane. Sample pad (2.5× 1.5 cm) for each strip was placed as overlapping 2 mm with conjugate pad from lower side, Absorbent pad was placed (2.5 × 1.5 cm for each strip) above the NC membrane. This assembly was covered with transparent tape. These assembled Lateral Flow Immunoassay strips were cut manually. When 10 Lateral Flow Immunoassay strips were assembled, assay was performed by using Bt cotton seeds and non-Bt cotton seeds. Commercially available (Agdia ImmunoStrip®) strip was used as a control. 1XPBS (10mM) of pH 7.4 was used as protein extraction buffer.

2.2 Preparation of lateral flow Immunoassay strips for different protein extraction buffers

MilliporeHF135 NC membranes were prepared for assembly of four Lateral Flow Immunoassay strips. The purpose of this experiment was to observe the effect of different protein extraction buffers on antibody-antigen interaction on blocked and unblocked NC membranes used in Lateral Flow Immunoassay strips. Each membrane was prepared as above mentioned protocol (2.1). For blocking of unoccupied sites of nitrocellulose membranes after immobilization of test and control lines and drying of antibodies, 3 membranes were incubated with blocking buffer (1XPBS and 3% BSA) for 4 hrs at RT on orbital shaker. When blocking process was performed, these membranes were soaked in 5% sucrose solution (w/v) and dried for one hour at 37ºC. The 4th membrane was remained unblocked. Preparation of conjugate pad and Lateral Flow Immunoassay strips assembley were performed as above described method (2.1) Protein was extracted from source (Bt-Cotton seeds) with different protein extraction buffers 1XPBS, 0.5XPBS and protein extraction buffer (100 mM Sodium Phosphate buffer pH 7.0, β-mercaptoethanol, 5 mM EDTA, 0.1% Triton X-100, 2% PVP-40).

Table 2.1 Lateral Flow Immunoassay strips with different treatments and protein extraction buffers

Strip number	Treatment of NC membrane	Protein Extraction buffer
1	Blocked unoccupied sites	1XPBS
2	Blocked unoccupied sites	Protein Extraction Buffer
3	Blocked unoccupied sites	0.5XPBS
4	Un-blocked	1XPBS

2.3 Preparation of nitrocellulose membranes with different blocking timings for development of Lateral Flow Immunoassay strips

This experiment was performed to determine effect of blocking buffer (1XPBS, 5% BSA) hybridization timing on test and control lines of prepared nitrocellulose membranes. Millipore HF135 was used for this experiment. Five strips were prepared according to mentioned protocol (2.1). For blocking of unoccupied sites of nitrocellulose membranes after immobilization of test and control lines and drying of antibodies, 3 membranes were incubated with blocking buffer (1XPBS and 3% BSA) with different incubation timings. The detailed information about NC membranes hybridization is given in table 2.2.

Table 2.2 Different hybridization times for unoccupied sites on nitrocellulose membranes with blocking buffer

No of strip	Blocking Duration
1	30 minutes
2	1 hour
3	2 hours
4	Unblocked (negative)
5	Unblocked (positive)

Strip number 4 and 5 were remained unblocked for negative and positive control respectively. Blocked membranes were washed with 1XPBS after hybridization, then dried for 30 minutes at 37ºC after washing. Blocked membranes were soaked in 5% sucrose solution and dried again at 37ºC for 30 minutes. Preparation of conjugate pad and Lateral Flow Immunoassay strips assembly were performed as above described method (2.1) After assembly of 5 Lateral flow immunoassay strips, assay was performed with Bt cotton seeds and non-Bt cotton seeds. For a control the commercially available (Agdia ImmunoStrip®) strip was also used along these strips. Protein extraction buffer 1XPBS (10mM) of pH 7.4 was used.

RESULTS

3.1 Preparation of Lateral Flow Immunoassay Strips by using Different NC Membranes Without Blocking and Washing Treatments

The purpose behind this experiment was to determine either blocking and washing treatments on prepared nitrocellulose membranes do any effect performance of lateral flow immunoassay strips. So nitrocellulose membranes used in this experiment were not blocked or washed with any buffer or any reagent. Second purpose of this experiment was to ensure that sealing tape (lamination) used for the covering of strip has any effect on the membrane or not; therefore, two strips (Millipore HF135) were tested without lamination. Four strips with Millipore HF135 were dipped in extract from Bt-cotton sample, two strips of Millipore HF120 were tested (one in Bt positive sample and second in Bt negative sample) and two strips with Fermentas NC membrane were also used. It was observed that, 4 strips of Millipore HF135 gave a sharp control line and a light test line. The 1 strip of Millipore HF120 with positive sample gave both control and test line and 2nd gave only control line with negative sample. Strips with Fermentas NC membranes gave no signals i.e. any line with positive samples (fig 3.1 and Table 3.1).

Fig. 3.1 Lateral flow immunoassay strips prepared by using three types of membranes without any treatment. Agdia® Bt strip was also tested as a control

Table 3.1 Results of Lateral flow immunoassay strips prepared by using three types of membranes without any treatment. Agdia® Bt strip was also tested as a control

Strip No.	NC membrane Used	Blocking	Gold particles size	pH of conjugate Solution	Extraction Buffer	Results
1	Millipore HF135	No	20 nm	8.8	1XPBS	light test and control lines
2	Millipore HF135	No	20 nm	8.8	1XPBS	light test and control lines
3	Millipore HF135	No	20 nm	8.8	1XPBS	light test and control lines
4	Millipore HF135	No	20 nm	8.8	1XPBS	light test and control lines
5	Millipore HF135	No	20 nm	8.8	1XPBS	Not tested
6	Millipore HF135	No	20 nm	8.8	1XPBS	Not tested
7	Millipore HF120	No	20 nm	8.8	1XPBS	visual test line
8	Millipore HF120	No	20 nm	8.8	1XPBS	visual test line
9	Fermentas	No	20 nm	8.8	1XPBS	Not any line
10	Fermentas	No	20 nm	8.8	1XPBS	Not any line
11	Agdia® Bt strip				1XPBS	Both test and control lines

3.2 Effect of Protein Extraction Buffers on Efficiency of Lateral Flow Immunoassay Strips

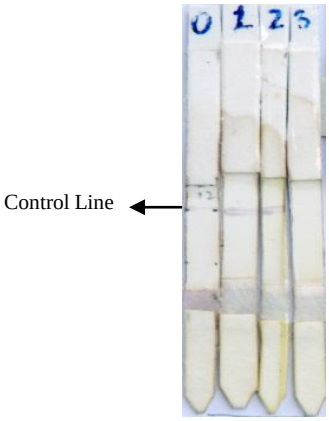

Fig 3.2 Lateral flow immunoassay strips tested using different extraction buffers. 1: membrane blocked and strip dipped in 1X PBS (pH 7.4), 2: membrane blocked and dipped in extraction buffer (pH 7), 3: membrane blocked and dipped in 0.5X PBS (pH 7.4), 4: membrane unblocked and dipped in 1X PBS (pH 7.4).

Table 3.2 Lateral flow immunoassay strips tested using different extraction buffers

Strips	Treatment of NC membrane	Protein extraction buffer	Line appearance
1	Blocked unoccupied sites	1X PBS (pH 7.4)	control Line
2	Blocked unoccupied sites	Extraction Buffer in Lab (pH 7)	control Line
3	Blocked unoccupied sites	0.5X PBS (pH 7.4)	Not any line
4	Un-blocked	1X PBS (pH 7.4)	Both test and control lines but light in colour

3.3 Effect of Hybridization Timings of blocking Buffer

This experiment was to explain the effects of different hybridization timings of blocking buffer (1X PBS, 5% BSA) on stability of antibodies immobilized on nitrocellulose membranes. For this purpose, un-blocked membrane strips were also tested at same time as positive and negative control. Results are given below in Table 3.5 and shown in Fig. 3.9.

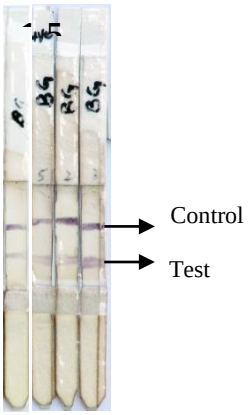

Fig 3.3 Lateral flow immunoassay strips with NC membranes blocked for different blocking duration. 1: 30 minutes, 2: 1 hour, 3: 2 hours, 5: unblocked membrane

Table 3.3 NC Membranes blocked for different blocking times

Strip No.	Blocking duration	Line appearance
1	30 minutes	A sharp purple color control and test line
2	1 hour	A sharp control line but less diffused test line
3	2 hours	A sharp control line but more diffused test line
4	Unblocked (negative)	A sharp control line
5	Unblocked (positive)	Both test and control lines very well appeared

DISCUSSION

In Lateral flow immunoassay, the primary function of a immobilized monoclonal antibody to a nitrocellulose membrane is to act as capture agent for the target antigen in a flowing sample[10]. In the present research work, Cry1Ac antibody was immobilized on nitrocellulose membrane to capture target antigen Bt Cry1Ac protein extracted from Bt cotton seeds or leaves. Since the test result is dependent on a good binding of the capture reagent to the membrane, therefore, a high and consistent level of protein binding is very important[11]. If one used a buffer that greatly reduces either hydrophobic or electrostatic interactions, the level of protein binding could be reduced[12]. Similarly, it is widely recognized that adequate drying of the membrane after protein application is an important measure for ensuring the long-term stability of the protein membrane bond[7]. In strip preparation antibody was dissolved in 1X PBS as 1 mg/ml and applied on NC membrane as 1 µl/line mixed with 5% methanol. The sufficient time was also given to the NC membrane for drying that is 30 minutes at 37ºC. Blocking the unoccupied sites of the NC membrane after application of antibodies, with a proper blocking buffer is also an important measure in a successful LFIA strip development[13]. Composition of blocking buffer and incubation times are key points in blocking process. As incubation time increases the binding of capture antibody on the NC membrane will be lose[14].

The components of blocking buffer must be chosen according to the properties of capture antibody. Materials can affect the binding of antibodies to nitrocellulose membranes[15]. Materials that interfere with protein binding can be divided into three general types: nonspecific proteins, materials that interfere with electrostatic interactions and materials that interfere with hydrophobic interactions[16]. Commonly used materials that reduce protein attachment include those that compete for binding sites, such as the classic bulking proteins (BSA, animal sera), as well as those that interfere with hydrogen bonding (formamide, urea) and those that interfere with hydrophobic bonding (Tween, Triton)[17]. Man-made polymers such as polyvinyl alcohol

(PVA), polyethylene glycol (PEG), and polyvinyl pyrrolidone (PVP) can also interfere with protein binding. Their mode of action may be a combination of effects that inhibit one or more of the forces essential to protein membrane binding[18] So, the optimized composition of blocking buffer was the 5% BSA in 1X PBS. All the detergents like triton X-100 and PVP were removed from the ingredients of the blocking buffer. The incubation time for blocking process was optimized as 30 minutes at room temperature (RT).Several experiments were made for appropriate protein extraction buffer to avoid false positive and false negative results. Because protein extracting reagents vary, therefore, maximizing the binding of an isolated protein may also require optimum buffer conditions[17]. There are two important factors that need to be ensured during modifications to the application buffer. These are the solubility e stability of the transgenic protein molecules[19]. To ensure that sufficient protein is available in the applied capture line, it is essential that the capture protein be soluble in the application buffer[20]. To enable the protein to be dissolved, it is necessary to have some ions present in the application buffer[2]. So, the ionic strength of the buffer can help to control the pH of the capture reagent[21,22]. The pH level of a protein extraction buffer can also have a significant effect on immunoassay properties. The solubility of a typical protein is minimum at its isoelectric point[11]. Since scientists are aiming to minimize the molecular stability of the capture protein in solution, the ideal pH of the extraction buffer should be around the pI of the capture protein being used[2,10]. With these experiments it was found that 1X PBS at pH 7.4 should be used as protein extraction buffer in initial experiments and during optimization process.

CONCLUSION

In conclusion, the developed LFIA strip can detect specific transgenic Cry1Ac protein. The results can be visualized by naked eyes without any instrumentation, which provides the ease for assay on-site. In addition, the test is performed within 10 minutes. It, therefore, could be used directly in the cotton fields for the qualitative screening of samples. Additionally, the method is economic, simple, and easy-to-use.

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