

Establishment of Dipstick Development Technology for Detection of Cry1Ac in Transgenic Plants

Muhammad Irfan^{1*}, Muhammad Asif¹, Aftab Bashir¹⁺, Kauser Abdullah Malik¹⁺⁺

^{1*}Gene isolation lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad
Email: attari_binm@yahoo.com

¹Gene isolation lab, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad.
Email: asif@nibge.org

¹⁺, ¹⁺⁺ Department of Biological Sciences, Forman Christian College, Lahore.
Email: aftabb.pk@gmail.com

Abstract— The insecticidal Bt Cry1Ac protein is, currently used for transgene expression in numerous crops or deliberating resistance against lepidopteron pests. Since the introduction of Bt cotton in Pakistan. It has been demonstrated that the technology has achieved the goal of providing an effective tool for lepidopteron control.

In this study, single step, sensitive and specific dipstick strip test for the revealing of recombinant Cry1Ac protein in the transgenic plants was established. Anti-Bt-Cry1Ac antibodies and goat anti-rabbit IgG antibodies were used in test and control lines, respectively. The distance between these lines were optimized as 0.5 cm. Polyclonal rabbit anti Bt-Cry1Ac antibody conjugated to nanocolloidal gold (20 nm of OD 15 and 40 nm; OD 1 in separate experiments) at pH 9.2 was used to serve as a probe for detecting Cry1Ac protein in transgenic Bt cotton samples. Both conjugate solutions were coated on separate polyester conjugate pads (0.7 cm × 0.5 cm). The total size of strip was 7.5 cm × 0.5 cm. For 20 nm gold conjugated strip, purple color test line and for 40 nm gold red color test line indicated the binding of gold labeled antibodies to antigen. The assay was corroborated with transgenic cotton samples with protein extraction buffer 1X PBS of pH 7.

This on-site test offers fast screening for any genetically modified crop devouring Cry1Ac transgenic protein.

Keywords— Nitrocellulose membrane, Cry1Ac, Dipstick, Nano-colloidal gold particles.

quantitative traits. Many genetically modified (GM) crops have been developed with different important traits by introducing various transgenes like insecticidal genes (*Cry1Ab*, *Cry1Ac*, *Cry1F*, *Cry2Ab*, *Cry3A*, *Vip3*) [1], herbicide tolerant genes (*epsps*, *bar*, *pat*, *als*) [2], virus resistant (*cp*, *prsv-cp*, *rep*, *hel*), delayed ripening genes (*sam-k*, *acc*, *pg*) [3] genes for color modification (*dfr*, *hfl*, *bp40*) in a numeral crops species like canola, cotton, corn, potato, tomato, brinjal, papaya, rice, tobacco, soybean, wheat, sunflower, alfalfa, etc. The two most important traits that have been successfully introduced in different commercially available GM crops are insect resistance and herbicide tolerance [4].

Transgenic plants expressing insecticidal genes that were initially derived from common soil bacterium, *Bacillus thuringiensis* (Bt), have been found to give an environmentally safe and efficient control of many insect pests Bt-cotton containing Cry1Ac gene provides protection against the lepidopteron insect pests commonly known as cotton bollworms. Bt cotton has been introduced in many other countries like Australia (1996), China (1997), Argentina (1997), South Africa (1998), Mexico (1998), Colombia (2002), India (2002) and Pakistan (2010).

(<http://www.agbioworld.org/biotech-info/articles/biotech-art/safety-bt-cotton.html>).

Since GM crops have been entered the food chain, public and scientific domain discussions related to their safety and manipulability have been continued. Before the commercial release of any GM crop, their biosafety evaluation is required to assess the environmental influence and effect on health of the consumers. It was demonstrated that unauthorized and possibly unsafe GM products may

I. INTRODUCTION

Transgenic technology has provided a very powerful tool to develop crop varieties, which are tolerant to various biotic and abiotic stresses, and improved qualitative or

sometimes be found in the market [3]. Unauthorized GM crops altogether present a significant socioeconomic risk through their possible undesired effects on human and animal health, and the environment. Therefore, several countries have implemented thresholds for unintended mixing of GM crops; fixed at 5% in Taiwan and Japan, 3% in Korea, 1% Australia, New Zealand, Brazil, and 0.9% in the European Union. Hence, for regulatory compliance of GM labeling, there is a dire need for easy and steadfast detection methods of such GM crops. Protein based GM crops detection is particularly useful for monitoring transgene expression both at qualitative and quantitative levels [5].

Previously methodologies are being used to detect the manifestation of GM materials in food stuff which emphasis on target either transgenic DNA or transgenic protein expression in GM crops[6]. Recently, Dong et al. have developed a database “GMO Detection method Database (GMDD)” which placid almost all the earlier developed and conveyed GMOs detection methods. The frequently used DNA based methods include polymerase chain reaction (PCR) and real-time PCR, while protein based methods include immuno PCR, near infrared (NIR) spectroscopy, micro fabricated devices, chromatography mass spectrophotometry and DNA chip technology which offer solutions to current technical issues in GM crop analysis [7], but these methods are costly, time taking and advantages along with the disadvantages[8]. Other protein based methods include enzyme linked immunosorbent assay (ELISA) and western blot. These are more accurate, cost effective but required trained and well equipped lab. Almost all of the above mentioned methods are not suitable for onsite testing of transgenic crops. Another protein based method named ‘dipstick test’ has been found quicker, simpler, less expensive, and suitable for onsite testing and does not require specific skills [9]. The current study is fixated on immunological based detection of transgenic plants expressing Bt (Cry1Ac) gene through developing a dipstick.

The scientific basis of the lateral flow immunoassay was consequent from the latex agglutination assay, which was established in 1956 by Plotz and Singer. During this period, plate-based immunoassays were being settled[10]. The basic principles of the lateral flow technology continual to be advanced through the early 1980s and were further recognized during the latter years with the filing of several major patents on this technology format by companies such as Becton Dickinson & Co. and Unilever and Carter Wallace. Since then, at least other 500 patents have been filed on various aspects of the technology [11]. The

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technology has been successfully applied for diagnostic purposes in the areas of agriculture, veterinary, environmental health, food and safety, industrial testing, as well as new areas such as molecular diagnostics and theranostics[12].

The dipstick (Lateral Flow Immunoassay) assay uses a membrane based detection system. Previous studies shown a number of names found for the strip based immunoassay tests, such as lateral flow devices (LFD), immunochromatographic (IC) tests, one-step tests, lateral flow technology (LFT) and dipstick tests[1]. The dipstick technology is a variant of enzyme-linked immunosorbent assay (ELISA), using nitrocellulose membrane strips rather than micro-titer wells and offers a qualitative or semi-quantitative test.

The objective of this study was to develop a quick, unpretentious, qualitative, and subnanocolloidal gold based sandwich IC strip assay for one step detection of transgenic Cry1Ac protein expressed in Bt cotton. The developed dipstick strip can successfully be used in diagnostic labs and by the cotton growers and farmers to perform the purity test of seed lots[13].

II. MATERIAL AND METHODS

2.1 Chemicals and reagents

The Material Starter Kit (Cat. No. 010) was purchased from Diagnostic Consulting Network UK, For nanocolloidal gold “Gold-in-a-Box™ kit (Cat. No. NGIB01-B044) was procured from BioAssay Works, LLC., USA 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.

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 polyvinyl alcohol (blocking buffer) were prepared separately.

2.2 Preparation of Dipstick Strip by using Seven Different Types of NC Membranes

Dilutions of anti Cry1Ac Antibody (2 mg/ml in 1XPBS buffer) were prepared. The IEF point for Cry1Ac was estimated to be 8.8 (CLC bio workbench). Conjugated solution were prepared having pH 8.8 and 9.2 after optimized conditions.

Seven membranes of different pore sizes were short listed. Each membrane was cut into strip of 3×1.5 cm size. The each membrane was treated with same protocol. Two lines were assigned in the middle of the membrane; which were 1 cm apart from each other, upper line was named as control line the goat anti rabbit antibody (1 μ l/line; 1 mg/ml) was

dispensed on control line and lower was named as test line. Anti-Bt Cry1Ac antibody (1 µl/line; 1 mg/ml) mixed with 3% methanol was immobilized on test line. Each strip of NC membrane was placed in an incubator at 37°C to dry for 1 hour. After drying of antibodies the unoccupied sites were blocked with blocking buffer (10 mM PBS containing 3% tween-20, 5% BSA and 2% polyvinyl alcohol). These membranes were incubated with blocking buffer for 4 hrs. at RT on orbital shaker. When blocking process was completed then these membranes were soaked in 5% sucrose solution (w/v) and dried for overnight at 37°C

finally parts of strip were assembled into coordinating parts as a one unit assay on adhesive plastic backing. After assembly of dipstick strips, assay was performed using Bt positive (Cry1Ac) cotton seeds and commercially available Immuno Strip (Agdia) were also used as a control.

2.3 Blot test for the determination of antibody and antigen reaction

A blot test was performed for the confirmation of antibody and antigen reaction according to protein extraction buffers. For this purpose following four different experiments were conducted using Fermentas NC membrane.

1. Nanocolloidal gold conjugated Cry1Ac antibody (3 µl) was dropped on NC membrane and labeled this spot number
2. Seeds of Bt cotton were crushed in 1X SEB4 buffer of pH 10.5 and used its 2 µl supernatant as antigen (Cry1Ac protein) and mixed it with 3 µl nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped on NC membrane and labeled it as spot number 2.
3. A secondary antibody IgG (goat anti rabbit antibody) was dropped 1 µl on NC membrane and dried at 37°C for 30 minutes. After 30 minutes Bt positive seeds were crushed in 1X SEB4 buffer of pH 10.5 and used its 2 µl supernatant as antigen (Cry1Ac protein) and mixed it with 3 µl nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped on NC membrane exactly at the same place where IgG was dropped and labeled it as spot number 3.
4. 1 µl of Anti-Cry1Ac Antibody was dropped on NC membrane and dried it at 37°C for 30 minutes. After 30 minutes Bt positive cotton seeds were crushed in 1X SEB4 buffer of pH 10.5 and used its 2 µl supernatant as antigen (Cry1Ac protein) and mixed it with 3 µl nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped on NC membrane exactly at the same place where Anti-Cry1Ac Antibody was dropped and labeled it as spot number 4.

2.3 Blot test for the determination of antibody and antigen reaction by using 1X PBS as protein extraction buffer

Again a blot test was performed for the determination of antibody and antigen reaction. For this purpose following four different experiments were conducted by using Fermentas Nitro Cellulose Membrane as an experimental base. Secondly, simple 1X PBS (10mM) buffer was used as protein extraction buffer.

1. Non-Bt cotton seeds were crushed in 1X PBS buffer of pH 7.4 and used its supernatant 2 µl and mixed it with 3 µl Cry1Ac antibody conjugated nanocolloidal gold. Total volume of 5 µl was dropped on NC membrane and labeled it as spot No. 1.
2. Bt cotton seeds were crushed in 1X PBS buffer of pH 7.4 and took 2 µl of its supernatant as antigen (Cry1Ac protein) and mixed with 3 µl Cry1Ac antibody conjugated nanocolloidal gold. Total volume of 5 µl was dropped on NC membrane and labeled it as spot No. 2.
3. A secondary antibody IgG (goat anti rabbit antibody) was dropped (1 µl) on NC membrane and dried at 37°C for 30 minutes. After 30 minutes Bt cotton seeds were crushed in 1X PBS buffer of pH 7.4 and used its (2 µl) supernatant as an antigen (Cry1Ac protein) and mixed with 3 µl nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped exactly at the same place on NC membrane where IgG was dropped and labeled it as spot No. 3.
4. Anti-Cry1Ac Antibody was dropped (1 µl) on NC membrane and dried at 37°C for 30 minutes. After 30 minutes Bt cotton seeds were crushed in 1X PBS buffer (pH 7.4) and used its 2 µl supernatant as an antigen (Cry1Ac protein) and mixed with 3 µl of nanocolloidal gold conjugated Cry1Ac antibody. Total volume of 5 µl was dropped exactly at the same place on NC membrane where Anti-Cry1Ac Antibody was dropped and labeled it as spot No. 4.

NOTE. The gold antibody conjugate used in all these experiments for determination of antibody and antigen reactions was of pH 8.8.

2.4 Preparation of dipstick strips by using different NC membranes without any treatments

In this experiment three NC membranes of different pore sizes from different manufacturers were selected. These were Millipore HF135, Millipore HF120 and Fermentas NC membranes. Each membrane was cut into strip of 2.5 × 0.5 cm size. Each membrane was treated with same protocol as mentioned above. Unoccupied sites on membranes were not blocked by any treatment.

After assembly of 10 dipstick strips, assay was performed by using Bt positive cotton seeds and Bt negative cotton seeds. Commercially available (AgdiaImmunoStrip®) strip was also used for a control. For protein extraction 1XPBS (10mM) of pH 7.4 was used.

2.5 Prepared dipstick strips and test for different protein extraction buffers

Four dipstick strips were prepared by using MilliporeHF135 NC membrane. The purpose of this experiment was to observe the effect of different extraction buffers in attachment of antibody-antigen on blocked and unblocked NC membranes used in dipstick strips.

Four strips of HF135 NC membrane were prepared for construction of dipsticks with 2.5 × 0.5 cm size as reaction area. The each membrane was treated with same protocol as mentioned above. After drying of antibodies the unoccupied sites of 3 membranes were blocked with blocking buffer (1XPBS and 3% BSA). These membranes were incubated with blocking buffer for 4 hrs. at RT on orbital shaker. When blocking process was completed then these membranes were soaked in 5% sucrose solution (w/v) and dried for one hour at 37°C. The 4th membrane was remained unblocked and untreated and labeled as 4. Conjugate pad was prepared and strip was assembled.

Bt positive Cotton seeds were taken in 3 pestle mortars and crushed them separately with different 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) and labeled the each pestle and mortar accordingly. 200µl of each supernatant was taken in different eppendorf tube and dipped the strip (table 2.4).

Table.2.4: Different treatments of strips and different types of 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

2.6 Preparation of unblocked NC membranes for strips with different antibody concentrations

The hypothesis behind this experiment was to observe that concentration of test antibody on NC membrane and its distance from conjugate releasing pad has any effect on color intensity of test line. For this purpose NC membrane (Millipore HF120) of high pore size and with a back protection sheet was used.

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Two strips of MilliporeHF120 NC membrane (was in 2.5cm × 0.5 cm) were activated by 1XPBS. Anti-Bt Cry1Ac antibody (1mg/ml) mixed with 5% methanol was immobilize on 3 test lines; 0.5 mm apart, from bottom to top arrangement of lines were ; line 1(1µl/line; 1mg/ml) ,line 2(2µl/line; 1mg/ml) ,Line 3(3µl/line; 1mg/ml).

These strips were dried at 37°C for 1 hr. One membrane was washed with 1XPBS buffer after drying process and other was not washed so labeled them accordingly. Washed membrane was dried for 30 minutes at 37°C.

After assembly of 2 dipstick strips, assay was performed with Bt (Cry1Ac) positive cotton seeds along with AgdiaImmunoStrip®. For protein extraction 1XPBS (10mM) of pH 7.4 was used.

2.7 Preparation of unblocked NC membranes for strips with different antibody concentrations (washed, unwashed and reverse orientation of lines)

In this experiment strips were prepared according to above mentioned protocol but test lines were in reverse order.

2.8 Preparation of NC membranes with different blocking timings for development of strips

This experiment was designed to check the effect of different hybridization (blocking of unoccupied sides) timings of blocking buffer (1XPBS, 5% BSA) on NC membranes for the attachment of antibodies.

Five different NC membranes were prepared according to optimized parameters. the unoccupied sites were blocked with blocking buffer (10 mM PBS containing 5% BSA) for different times.

Table.2.5: Blocking times for unoccupied sites on NC membranes with blocking buffer

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

After assembly of 5 dipstick strips, assay was performed with Bt (Cry1Ac) positive cotton seeds and Bt negative cotton seeds by using protein extraction 1XPBS (10mM) of pH 7.4. For a control the commercially available (AgdiaImmunoStrip®) strip was also tested along with these strips.

2.9 Final preparation of dipstick strip with optimized conditions

The Gold conjugated antibodies were prepared according to optimized conditions. For preparation of conjugate pad The pH of gold-antibody solution which was used in this

experiment was 9.2. About 10 μ l of this prepared solution was finally dispensed on a fiber glass conjugate pad of 7mm \times 5 mm. This conjugate pad was placed in an incubator at 37°C for overnight incubation. NC membranes were prepared according to above optimized conditions and assembled them as dipstick strip and test was performed.

2.10 Final preparation of dipstick strip under optimized conditions by using 40 nm colloidal gold particles

In above experiments 20 nm colloidal gold was used of 15 OD. In this experiment 40 nm colloidal gold was used of OD 1 under optimized conditions. The Gold conjugated antibodies were prepared per supplier instruction given in the Gold in a Box kit. For preparation of conjugate pad instructions provided by supplier in kit was followed. Each 1 ml of gold-antibody conjugate solution was mixed with 0.1 ml of gold drying buffer. The pH of gold-antibody solution which was used in this experiment was 9.2. About 15 μ l of this prepared solution (40nm of OD 1) was finally dispensed on a fiber glass conjugate pad of 7mm \times 5 mm. This conjugate pad was placed in an incubator at 37°C for overnight incubation. This incubation made gold-antibody conjugate dried on fiber glass pad and this was called as dried conjugate pad. Nc membranes were prepared according to above optimized conditions and assembled them as dipstick strip and test was performed.

III. RESULTS

3.1 Selection of Suitable NC membrane

Variation was found when different membranes were tested using Agdia SEB4 extraction buffer at pH ranging from 7 to 10.5 (Table 3.1; Fig 3.1)

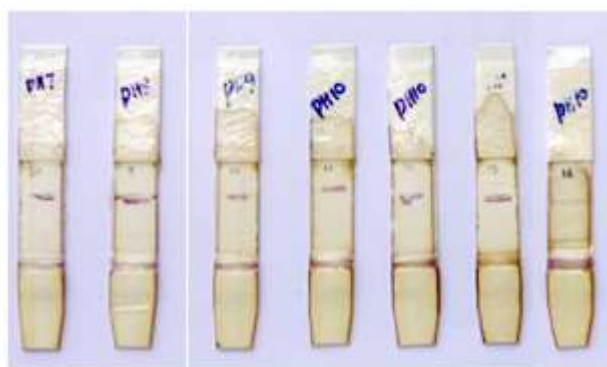


Fig.3.1: Variations in dipstick strip performance due to different NC membranes. 1: Immunopore FP, 2: Millipore HF075, 3: Millipore HF090, 4: Millipore HF120, 5: Millipore HF135, 6: Millipore HF240, 7: Whatman Fusion5

Table.3.1: Results of dipstick strips for detection of CryIAC protein using different membranes at varying pH of extraction buffer.

Strip No	pH*	Membranes	Line appearance
1	7	Immunopore FP	Control line
2	8	Millipore HF075	Control line
3	9	Millipore HF090	Control line
4	10	Whatman Fusion5	No line
5	10.5	Millipore HF120	Control line
6	10.5	Millipore HF135	Control line
7	10.5	Millipore HF240	Control line

* pH of agdia SEB4 extraction buffer.

3.2 Blot Test for the Determination of Antibody and Antigen Reaction

1. First spot gave reddish purple colour such as antibody-gold conjugate colour was (Fig. 3.2).
2. Second spot gave a circular appearance of dual lining with light green colour on inner side and light reddish purple colour on external margins (Fig. 3.2).
3. On 3rd spot, where a secondary antibody goat-anti-rabbit antibody (IgG) was immobilized and a mixture of conjugate solution (pH 8.8) and Bt positive extract was applied. Spot of IgG repelled that mixture and gave two overlapping circles of dual margins. The external margin of one circle was overlapping with external and smaller with internal margin of second (Fig. 3.2).
4. The 4th spot gave two semi circles of dual margins; internal margin was smaller interacting with antibody spot (Fig. 3.2).

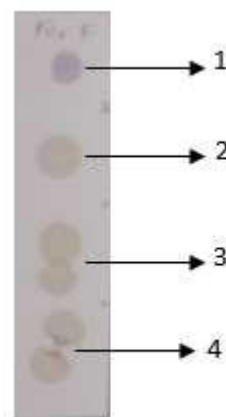


Fig.3.2: Determination of antibody-antigen interaction. 1: antibody gold conjugate solution, 2: conjugate solution and antigen mixture, 3: IgG, conjugate solution and antigen mixture, 4: CryIAC antibody, conjugate solution and antigen mixture.

3.3 Blot Test for the Determination of Antibody and Antigen Reaction by using 1X PBS as Extraction Buffer

The purpose behind this experiment was to check the role of protein extraction media in antibody–antigen interaction. So to determine antibody and antigen reaction, 1X PBS was used as protein extraction buffer (Fig 3.3) and results were the followings:

1. A light green colored spot appeared when Bt negative sample and antibody-gold conjugate was used.
2. A light reddish circle of double margin appeared by using Bt positive sample and antibody-gold conjugate.
3. On 3rd spot goat-anti-rabbit antibody (IgG) was spotted, dried on 37° for half an hour. A mixture of antibody-gold conjugate and Bt positive extract in 1X PBS was dropped. The mixture interacted with the antibody spot and gave dark reddish purple colored circle.
4. On 4th spot Anti-Bt Cry1Ac antibody was spotted, dried on 37° for half an hour. A mixture of antibody-gold conjugate and Bt positive extract in 1X PBS was dropped, the mixture interacted with the antibody spot and gave dark reddish purple colored circle like shape.

Both the spots of antibodies (Anti-Bt Cry1Ac antibody and goat-anti-rabbit antibody) gave almost similar results, which showed the antibody-antigen interaction.

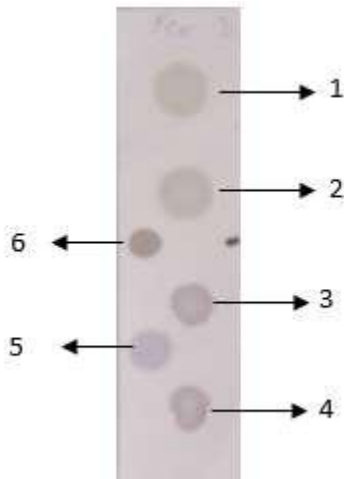


Fig.3.3: Blot test for the determination of antibody-antigen reaction by using 1X PBS as protein extraction buffer. 1: Bt negative sample and antibody-gold conjugate, 2: Bt positive sample and antibody-gold conjugate, 3: Goat-anti-rabbit antibody (IgG), Bt positive sample and antibody-gold conjugate, 4: Cry1Ac-antibody, Bt positive sample and antibody-gold conjugate, 5: only antibody labeled gold, 6: nano-colloidal gold without antibodies.

3.4 Preparation of Dipstick Strips by using Different NC Membranes without blocking and washing treatments

This experiment was conducted to check that do the blocking and washing treatments have any effect on NC membranes performance. So NC membranes used in experiment were neither blocked nor washed with any buffer or any reagent. Second purpose of this experiment was that either sealing tape (lamination) used for the covering of strip has any effect on the membrane; therefore, two strips (Millipore HF135) were tested without lamination. Four strips with Millipore HF135 were dipped in extract from Bt positive sample, two strips of Millipore HF120 were dipped (one in Bt positive sample and second in Bt negative sample) and two strips with Fermentas NC membrane were also tested.

It was observed that, 4 strips of Millipore HF135 gave a sharp control line and a light line on 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 3.5 and Table 3.2).



Fig.3.5: Dipstick strips prepared by using three types of membranes without any treatment. Agdia® Bt strip was also tested as a control.

Table.3.2: Results of dipstick 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

3.5 Preparation of Dipstick Strips and Test for Different Protein Extraction Buffers

Table.3.3: Dipstick 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

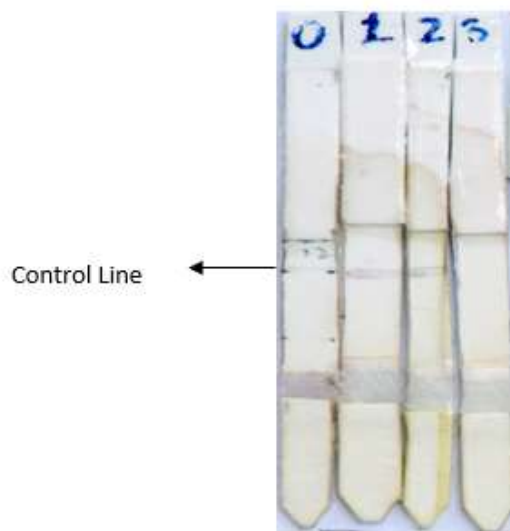


Fig.3.6: Dipstick strips tested using different extraction buffers. 1: membrane blocked and strip dipped in 1X PBS (pH 7.4), 2: membrane blocked and dipped in extraction buffer (pH 7), 3: membrane blocked and dipped in 0.5X PBS (pH 7.4), 4: membrane unblocked and dipped in 1X PBS (pH 7.4).

3.6 Preparation of Strips with unblocked NC Membranes and different Antibody Concentrations

In this experiment Millipore HF120 NC membrane was used and it was pre-soaked with 1X PBS buffer to check either it has any effect on antibody binding efficiency with NC membrane with the hypothesis that it might be possible that pre-soaking may help for proper application of antibodies on membrane. Anti-Bt Cry1Ac (1 mg/ml) antibody mixed with 5% methanol was applied on this membrane in different concentrations at different distances from the conjugate pad. Anti-Bt Cry1Ac antibody (1 mg/ml) was immobilized on 3 test lines; 0.5 mm apart each (Fig. 3.7). Results are tabulated in Table 3.4.

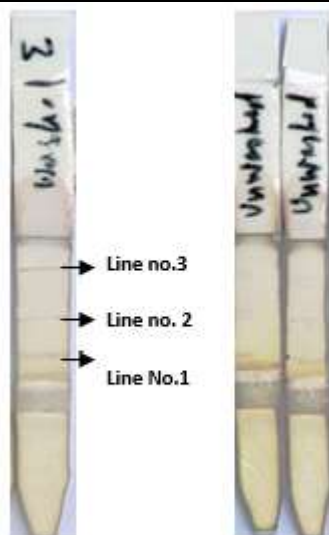


Fig.3.7: Unblocked NC membranes for strips with different volumes of antibody (1 mg/ml). 1: 1 μ l/line, 2: 2 μ l/line, 3: 3 μ l/line.

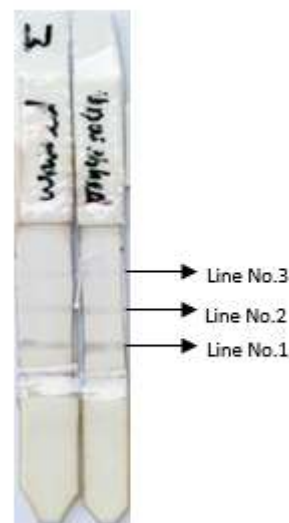


Fig.3.8: Results of unblocked NC membranes for strips with different antibody concentrations; washed: Line No.1; 3 μ l/line; 1mg/ml, Line No.2; 2 μ l/line; 1mg/ml, Line No.3; 1 μ l/line; 1mg/ml, unwashed; Line No.1; 3 μ l/line; 1mg/ml, Line No.2; 2 μ l/line; 1mg/ml, Line No.3; 1 μ l/line; 1mg/ml.

Table.3.4: Strips with unblocked NC Membranes and different antibody concentrations as test lines on same membrane.

Line No. (bottom to top)	Antibody (1 mg/ml)	Washed membrane	Un-washed membrane
1	1 μ l/line	light purple colour line	sharp light purple colour line
2	2 μ l/line	very light purple colour line	very light purple colour line
3	3 μ l/line	invisible line	invisible line

Table.3.5: Unblocked NC membranes for strips with different volumes of antibody (1 mg/ml) washed with 1X PBS, unwashed and reverse orientation of antibody lines.

Line No. (bottom to top)	Antibody (1 mg/ml)	Washed (treated) strip	Unwashed (un-treated) strip
1	3 μ l/line	A sharp purple colour line	A sharp purple colour line
2	2 μ l/line	Light sharp purple colour line	Light purple colour line
3	1 μ l/line	A light visible line (sharp)	light visible line

3.7 Preparation of strips with unblocked NC membranes and different antibody concentrations (washed, unwashed and reverse orientation of antibody lines)

This experiment was designed to evaluate that either colour the intensity of signal line (test line) depends upon antibody concentration in line at NC membrane or distance of antibody line from conjugate pad or any other factor. The major difference between this and previous experiments was that here antibody gold conjugated solution of pH 9.2 was used for the preparation of antibody gold conjugate pad. Anti-Bt Cry 1Ac antibody (1 mg/ml) mixed with 5% methanol was immobilized on 3 test lines; 0.5 mm apart each (Table 3.4; Fig. 3.8).

3.8 Preparation of NC Membranes with different Blocking Timings for development of Strips

This experiment was designed to see the effects of different time durations for blocking the membranes with blocking buffer (1X PBS, 5% BSA) on stability of antibody binding with membranes. For this purpose, un-blocked membrane strips were also tested at same time with positive and negative sample. Results are given below in Table 3.5 and shown in Fig. 3.9.

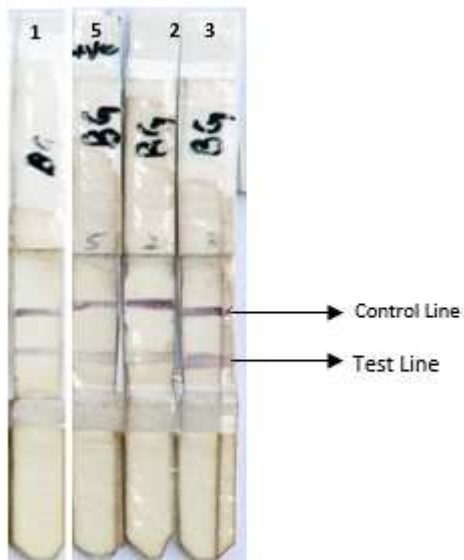


Fig.3.9: Dipstick strips with NC membranes blocked for different blocking duration. 1: 30 minutes, 2: 1 hour, 3: 2 hours, 5: unblocked membrane

Table.3.6: NC Membranes blocked for different blocking times.

Strip No.	Blocking duration	Line appearance
1	30 minutes	A sharp purple colour control and test line
2	1 hour	A sharp control line but smaller 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

3.9 Final Preparation of Dipstick Strip with Optimized Conditions using 20 nm Colloidal Gold Solution of OD 15

Under optimized conditions Anti-Bt Cry1Ac-Antibody gold conjugate solution was prepared. It gave no precipitation or change in colour; that showed the labeling of antibody or correct concentration of antibody for gold particles for conjugation.

After the experimentation, finally strips were prepared under optimized conditions. 1x PBS buffer was used to extract protein from both Bt positive and Bt negative cotton

seed samples. Three strips were tested with Bt positive extract 200 µl and one strip with Bt negative extract as negative control. The sample solution through wicking migrated onto the strip by capillary action. As the sample flowed successively through the detection antibody (conjugate pad) and the capture antibody, the Cry1Ac proteins got captured on test line and a dark purple coloured band was visible with positive samples (Fig. 3.10). A second dark purple coloured line was also observed on the control line on the membrane, generated by excessive gold conjugates, indicating the proper test performance. The detection was completed in less than 10 minutes.

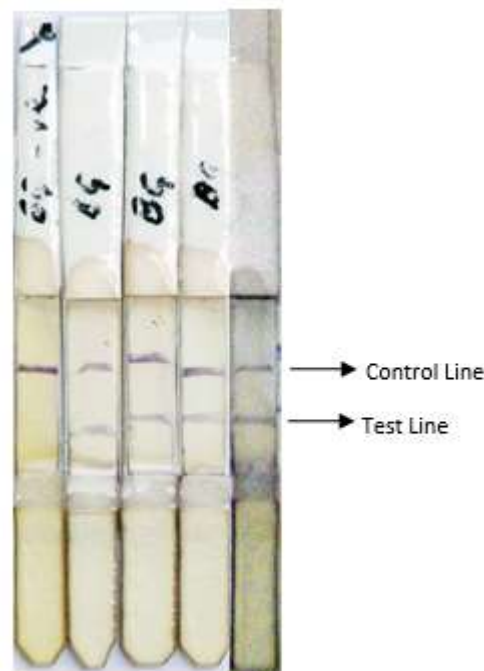


Fig.3.10: Dipstick strips prepared with optimized conditions and using 20 nm colloidal gold Solution of OD 15.

3.10 Final Preparation of Dipstick Strip with Optimized Conditions by using 40 nm Colloidal Gold Particles of OD 1

Antibody gold conjugate solution did not show any precipitation and change in colour; remained red. And strips were tested with positive and negative samples. The sample solution by means of wicking traveled onto the strip by capillary action. As the sample flowed continually through the conjugate pad and the capture antibody, the Cry1Ac proteins got captured on test line and a red colored line appeared. A second red coloured line was also observed on the control line of the NC membrane, produced by excessive gold conjugates, indicating the appropriate test

performance (Fig. 3.11). The detection was completed in less than 10 minutes.

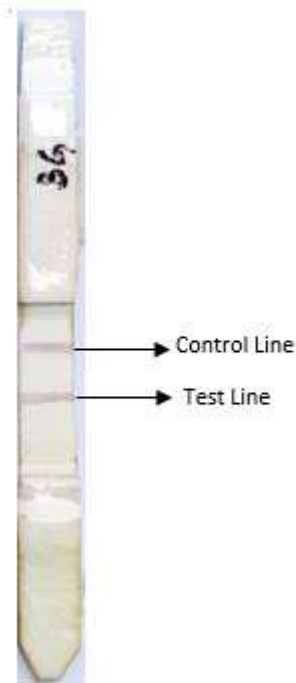


Fig.3.11: Dipstick strip prepared with optimized conditions and using 40 nm colloidal gold particles of OD 1, and tested with Bt positive cotton seed sample.

IV. DISCUSSION

During the past decade, a large number of genetically modified (GM) crops have been established. These GM crops shown inimitable agronomic traits such as insect resistance or herbicide tolerance, which offer noteworthy remunerations to the farmers. The development of GM crops is consummate by molecular biology methods, fundamentally by the incorporation of novel DNA sequences into the plant genome. The new DNA encodes in the novel protein expression in the targeted tissues, resulting in the distinctive agronomic trait. The DNA and novel protein are present in the plant, in harvested grain, and often in the food and feed prepared from them [14].

There are different detection methods of GM crops, which are based on phenotype or DNA and protein detections. The protein based detection of GM crops depend on solely on the immunoassay technology applications [15]. Commercial immunoassays are available for GM crops and have been used in a variety for large-scale applications.

Immunoassays are based on the reaction of an antigen (Ag), e.g., transgenic protein, with a specific antibody (Ab) to give a product (Ag-Ab complex) that can be measured.

Dipstick strip test is purely qualitative method for proteins detection in transgenic plants in which the results are interpreted by visual observation. The result provides yes or no answer for presence or absence of protein in the test samples. The appearance of two lines on dipstick strips after performing test shows the positive results and appearance of control line gives negative results as shown in fig 3.10.

The objective of the present study was to mature for dipstick strip for the Cry1Ac protein detection expressed in Bt cotton. For this purpose the technology was optimized, which included the selection of best NC membranes for this particular assay, optimum pH, optimum extraction buffer, optimum antibody concentration and size of the gold nano particles suitable for detection.

In order to achieve quality results and stable protein-nano gold particles, numerous parameters should be deliberated. These include: the optimum pH of the reaction, the appropriate concentration of protein loaded onto the colloids, determining the isoelectric point (pI) of the protein, and the stability of the colloids. Generally, most proteins can be adsorbed onto the metal surface in an optimal manner using buffer systems close to their pI value, away from this pH the adsorption decreases [16]. Gold nano particles were being used in current study as color producing probes because these are stated to be non-toxic, inert and have long retention of their optical properties, which creates them a better choice as a signal generator. The gold nanoparticles accumulation produces a characteristic red color on the surface plasmon resonance [17]. Macromolecular ligands adsorb onto colloidal gold through a combination of electrostatic and hydrophobic interactions. Cry1Ac antibodies (macromolecules) were conjugated with gold particles after adjusting the pH of colloidal gold solution. gold with a suitable amount of adsorbed macromolecules are not subject to flocculation with high salt, making this an excellent tool for determining whether the colloid has been sufficiently “protected” [6]. After preparation of antibody gold conjugate solution and to check stability of conjugation reaction; 10 µl of coated gold solution was mixed with 10 µl 1M NaCl. The results under the optimized condition indicated color stability, which indicated that antibody-gold particles are stable enough to be utilized in the strip development assay.

In colloidal gold conjugation process, it is significant to control the pH of the colloidal and gold ligand. Both preparations should be attuned to a pH slightly above the isoelectric point (pI) of the ligand before conjugation. Below the pI of the ligand, ligand-induced flocculation may ensue, whereas, above the pI of the ligand, there is limited

adsorption due to charge repulsion between the ligand and the colloid. In experiments, which were designed for optimization process of gold antibody conjugation, 20 nm gold was used with OD 15. Optimized pH values for conjugation process were 8.8 and 9.2, which were close and slightly above the calculated pI value of Cry1Ac antibody i.e. pH 8.8.

The conjugation of antibody with colloidal gold is controlled by pH of the solution and that concentration was used for full saturation of colloidal gold surface, which increased the chance of antigen-antibody interaction after collision with antigen and also increased the stability by protecting the surfaces of colloidal gold particles against coagulation[18]. The optimized concentration of Cry1Ac antibody for conjugation with colloidal gold was 14 μ l of 2 mg/ml antibody solution in 1X PBS.

After addition of optimized concentration of antibody to the colloidal gold solution, BSA blocking stabilizer buffer was added with the concentration of 50 μ l/0.5ml. A major disadvantage of the affinity of colloids for proteins is that the colloidal particles can also adsorb to other components of the system, causing non-specific labeling. Hence, colloidal metal conjugates must also be steadied with an inert macromolecule such as gelatin, bovine serum albumin (BSA) or polyethylene glycol. This can usually be done through washing the conjugates with buffer containing the macromolecule after the absorption of the wanted protein onto the nanoparticles. In addition to the non-covalent adsorption, proteins can also be devoted to the nanoparticles through covalent bonds via functional groups such as amines and thiols[19].

Both the antibody loading concentration and the pH of the reaction as well as the buffer conformation and incubation time, resolute the degree of adsorption of the protein onto the nanoparticle and the aggregation probability[20]. For the proper adsorption of antibody on the surface of gold particles, the gold conjugate solution should be left for 30 minutes of incubation after addition of antibody. It is necessary to give a proper time to the blocking buffer, so incubate the gold conjugate solution for overnight after addition of BSA blocking buffer. It is important to know that if antibody or nano particle is changed, then the optimal conjugation conditions will also differ. An optimised protocol for one system may not necessarily be readily applicable for another one, because once OD or size of colloidal gold changes the conditions for conjugation with antibody will also change [21].

In immune chromatographic assays, the primary function of a protein applied to a membrane is to act as a capture

reagent for the target analyte in a sample [8]. In the present research work, Cry1Ac antibody was applied on membrane as a capture reagent and target reagent was Bt Cry1Ac protein extracted from Bt cotton seeds or leaves. Since the test result is entirely dependent on attaining a good binding of the capture reagent to the membrane, therefore, triumphing a high and consistent level of protein binding is imperative.

If one selects a buffer that too greatly reduces either hydrophobic or electrostatic interactions, the protein binding level could be vividly reduced. Similarly, it is extensively recognized that adequate drying of the membrane after protein application is an essential for ensuring the long-term constancy of the protein membrane bond[22]. In strip preparation antibody was dissolved in 1X PBS as 1 mg/ml and applied on NC membrane as 1 μ l/line mixed with 5% methanol. The sufficient time was also given to the NC membrane for drying that is 30 minutes at 37°C.

Blocking the unoccupied sites of the NC membrane after application of antibodies, with a proper blocking buffer is also an important factor in a successful dipstick strip development. 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 (Fig. 3.9).

The components of blocking buffer should be selected according to the properties of capture protein (antibody). Materials and chemicals can have an effect on the binding of antigens and antibodies to nitrocellulose membrane. These materials can interrupt protein binding can be divided into three types: nonspecific antigenic proteins, materials that can interfere with electrostatic interactions and materials that interact with hydrophobic interactions [23]. Generally used materials that inhibit protein attachment are those that compete for binding sites, such as the bulking proteins (BSA, animal sera), as well as those that can interfere with hydrogen bonding (formamide, urea) and those that can disturb hydrophobic bonding (Tween, Triton). synthetic polymers such as polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP) and polyethylene glycol (PEG) can also effect protein binding. Their mode of action may be a combination of effects that reduce one or more of the forces essential to protein membrane binding.

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 efforts were made for appropriate protein extraction buffer and optimized to evade false positive and false negative results. Because protein capture reagents vary, therefore, maximizing the binding of a given protein may also require optimum buffer conditions. There are two important factors that need to be optimized through modifications to the application buffer. These are the solubility of the protein and the stability of the protein molecules.

To ensure that required protein is vacant in the dispensed capture line, it is essential that the capture protein be soluble in the appropriate application buffer. To allow the protein to be dissolved, it is mandatory to have some ions present in the application buffer. So, the ionic strength of the buffer can help to control the pH of the capture reagent. The pH level of an extraction buffer can also have a significant effect on immunoassay properties. The solubility of a typical protein is minimum at its isoelectric point. 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. With these experiments it was found that 1X PBS at pH 7.5 should be used as protein extraction buffer in initial experiments and during optimization process [21].

The discovery sensitivity of the test was also reliant on the pore size of the NC membrane. The assay was accomplished separately with NC membranes of different pore size. We perceived that the detection sensitivity was reduced with growing pore size of NC membrane (Fig 3.1). It is due to the faster wicking rate in case of NC membranes with large pore size (10 and 15 μm) which, in turn, provides no sufficient time for antibody and protein interaction. The better sensitivity was obtained with NC membrane of 5 μm pore size (MilliporeHF120); it was, therefore, selected for final assay development (Fig. 3.10).

Table.4.1: Comparison of optimized method with other studies of strip development

Ref*	Pre- Treatment of pads	Test and Control lines on NC membrane	Drying of antibodies on Test and Control lines	Blocking NC membrane
1		I) Pab-Dog IgA solution of 650 μl was mixed with 20wt% sucrose solution diluted with 50mM KH ₂ PO ₄ buffer (pH7.5) of 50 μl and 50 μl of 2-propanol. (IgA solution at 1mg ml ⁻¹ in PBS) II) 40 μl of polyclonal anti-mouse IgG mixed with both 60 μl of 2-propanol and 1100 μl of 50mM KH ₂ PO ₄ buffer (pH7.5).	drying for 1 h at room temperature	
2	Sample and the conjugate pads treated with PBS	DON-CBSA (1 mg /mL) conjugate and goat anti-mouse IgG antibody (1.5 mg/ mL) were sprayed onto the bottom and the top of NC membrane	vacuum-dried at 37 °C for 2 h.	By immersing in 50mM boric acid solution containing 0.5% casein (pH8.5) and incubating for 30min at RT
3		(NC membrane HF135MC100, Millipore) A solution of 1 mg/ml of recombinant Staphylococcus Protein-A (Sigma) and a solution of 1 mg/ml of mAb MM3, both in PBS	Dried overnight at 37°C.	
4	Sample pad was treated with 50 mM borate buffer, pH 7.4, containing 1% BSA, 0.5% Tween-20, and 0.05%	Clenbuterol-BSA (0.038mg/mL) and goat anti-mouse antibody (1.123 mg/mL)	Dried at 35°C	

	sodium azide, and dried at 60°C			
5		3µl anti-HBsAg antibody (0.1 µg / ml) test line. 3µl of goat anti-rabbit IgG (0.1 µg / ml)	air dry for 45 minutes at 4°C	Incubating in mixture 3% BSA and 2% gelatin in TBS for 30 minutes.
6		Anti-O1 LPS mAb (2 mg/ml), anti-O139 LPS mAb (2 mg/ml) and goat anti-mouse Ab (1 mg/ml) 4 µl/cm	Dried overnight in a desiccator at room temperature	With 50 mM PBS (pH 7.4) containing 1% western blocking reagent and 0.05% Tween-20.
7		MAB 4D1 (2mg/ml) and goat anti-mouse IgG (2mg/ml) were dispensed at the test or the control line	drying for 2 h at 37 °C	Incubating with PBS (pH 7.4) containing 2% (w/v) nonfat driedmilk for 30min Wash three times with PBS containing 0.1% (v/v) Tween-20 for 3min each time
8*		Anti-Bt Cry 1Ac antibody (1µl/line; 1mg/ml) mixed with 3% methanol, Goat anti rabbit antibody(1µl/line; 1mg/ml)	Dried for 1 hr at 37°C.	10 mM PBS with 3% BSA, 0.05 %,. Incubate NC membrane 30 minutes at RT. Soaked with 5 % sucrose solution.

1: [24] 2: [25] 3: [26] 4:[27] 5: [28] 6: [29] 7: [30] 8*: methode adopted in present study.

V. CONCLUSION

In conclusion, the developed technology for qualitative colloidal gold based dipstick strip using antibody sandwich immunoassay format can detect specific transgenic Cry1Ac protein. The results can be visualized by naked eyes without any complex instrumentation, which provides the convenience for assay on-site. In addition, the test is performed within 10 min without the need of using expensive equipment. It, therefore, could be used directly in the field for the rapid qualitative screening of GM samples. Additionally, the method is economic, simple, and easy-to-use.

Conflict of interest: All authors have no conflict of interest.

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