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COLLOIDAL GOLD BASED DIPSTICK STRIP FOR DETECTION OF GENETICALLY MODIFIED CROPS AND PRODUCE

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ABSTRACT

The production of genetically modified (GM) crops is rapidly increasing every single year worldwide. The safety and quality evaluation of these crops is an important issue regarding to consumers' health. This led the regulatory authorities to implement a set of very strict procedures for the approval to grow and utilize GM produce. Therefore, for fulfilling regulatory compliance on GM labeling and identification of genetic traits, it generated a demand for analytical methods capable of detecting either transgenic DNA introduced or the protein expressed in GM crops. The detection methods include either DNA based PCR assay or protein based ELISA and dipstick assays. This article illustrates the advances in colloidal gold based dipstick procedure for visual discrimination of GM crops/produce from non-GM.

KEYWORDS

Genetically modified crops, Dipstick, Colloidal gold.

INTRODUCTION

For generations, humans have searched and opted for crop plants that show resistance to pests, persist and yield sufficient produce. Many important traits incorporated through genetic engineering techniques include – insect resistance, herbicide tolerance, drought and salt tolerance, improved colors in fiber and flower crops, resistance to water logging, nutritionally enhanced produce, and longer shelf-life[1]. The genetically modified (GM) crops have contributed to the successful incorporation of different traits by introducing transgenes such as *Bacillus thuringiensis* (*Bt*) insecticidal genes (*cry1Ab, cry1Ac, cry1F, cry2Ab, cry3A, vip3*), herbicide tolerant genes (*cp4epsps, bar, pat, als*), virus resistant (*cp, prsv-cp, rep, hel*), delayed ripening genes (*sam-k, acc, pg*), genes for color modification (*dfr, hfl, bp40*) in a number of crop species such as cotton, canola, corn, potato, tomato, rice, tobacco, brinjal, papaya, soybean, sunflower, alfalfa, wheat, etc.[1].

Several approvals for commercial GM crops have been granted for cultivation in different countries[1]. However, several possible risks have been associated with the growing of GM crops and one of them is their potential adverse affect on non-target organisms[2]. Since GM crops have been entered the food chain, public and scientific debates concerning their safety and traceability have continued. The bio-safety assessment of genetically modified organisms (GMOs) is requires for their environmental impact and also for health of the consumers. It was demonstrated that unauthorized and potentially unsafe GM products may sometimes be found in the market[3,4]. Unauthorized GMOs altogether pose a significant socioeconomic risk through their potential effects on trade and trust in industry and authorities, as well as potentially to human and animal health, and the environment[5]. Therefore, several countries have implemented labeling thresholds for unintentional mixing of GM crops; defined as 5% in Taiwan and Japan, 3% in Korea, 1% Australia, New Zealand, Brazil, and 0.9% in the European Union[6]. Hence, for regulatory compliance on GM labeling and identification of genetic trait, the need for easy and reliable detection methods for these GM crops is necessary[7]. Reliable detection methods are important both for detection of unauthorized GMOs and as a first step in labeling control[8]. The detection of GM crops is useful for monitoring of crop production during the growing season based on transgenic DNA or its expressed proteins.

GLOBAL STATUS OF COMMERCIALIZED GM CROPS:

The farmers have continued to cultivate more GM crops year by year. In 2010, the global area of GM crops was reached to 148 million hectares, spread across 29 countries while it was 134 million hectares in 2009 and the apparent growth was about 10% or 14 million hectares between 2009 and 2010[9]. This increased cultivation indicates very high adoption rate of GM crops by farmers, which reflects its consistent performance. On the other hand, GM crops provide significant economic, environmental, health and social benefits to both small and large farmers in developing and industrial countries. In 2010, 29 countries comprised 19 developing countries, 10 industrial countries planted GM crops, and the USA was on the first place with 66.8 million hectares cultivated area including eight commercially approved GM crops (**Table 1**) and about 40 countries expected to adopt biotech crops by 2015. So far, a total of 964 approvals have been granted for 184 events for 24 GM crops in 59 countries to import for food and feed use and to release into the environment since 1996[9]. GM soybean continued to be the principal GM crop in 2010, occupying 73.3 million hectares followed by GM maize, GM cotton, and GM canola. From

the genesis of commercialization in 1996 to 2010, herbicide tolerance has consistently been the dominant trait[9].

Table 1
Global area of GM crops in different countries in 2010.

Rank	Country	Area (million hectares)	Biotech crops
1*	USA*	66.8	Maize, soybean, cotton, canola, sugarbeet, alfalfa, papaya, squash
2*	Brazil*	25.4	Soybean, maize, cotton
3*	Argentina*	22.9	Soybean, maize, cotton
4*	India*	9.4	Cotton
5*	Canada*	8.8	Canola, maize, soybean, sugarbeet
6*	China*	3.5	Cotton, tomato, poplar, papaya, sweet pepper
7*	Paraguay*	2.6	Soybean
8*	Pakistan*	2.4	Cotton
9*	South Africa*	2.2	Maize, soybean, cotton
10*	Uruguay*	1.1	Soybean, maize
11*	Bolivia*	0.9	Soybean
12*	Australia*	0.7	Cotton, canola
13*	Philippines*	0.5	Maize
14*	Myanmar*	0.3	Cotton
15*	Burkina Faso*	0.3	Cotton
16	Spain*	0.1	Maize
17	Mexico*	0.1	Cotton, soybean
18	Colombia	<0.1	Cotton
19	Chile	<0.1	Maize, soybean, canola
20	Honduras	<0.1	Maize
21	Portugal	<0.1	Maize
22	Czech Republic	<0.1	Maize, potato
23	Poland	<0.1	Maize
24	Egypt	<0.1	Maize
25	Slovakia	<0.1	Maize
26	Costa Rica	<0.1	Cotton, soybean
27	Romania	<0.1	Maize
28	Sweden	<0.1	Potato
29	Germany	<0.1	Potato
	Total	**148.0**	

**17 biotech mega-countries growing 50,000 hectares, or more, of biotech crops.*
Source: James[9]

In India, only *Bt* cotton containing the c*ry1Ac* gene, was for the first time introduced in 2002. During the past few years, Indian cotton scenario has changed dramatically, largely due to the adoption of *Bt* cotton[10]. The Indian farmers had grown 50,000 hectares of *Bt* cotton in 2002. *Bt* cotton area has increased to 9.4 million hectares in 2010 and the apparent growth was about 1 million hectares from 2009 to 2010; gaining fourth ranking place[9]. This is an

indication of extraordinary impact and acceptance of Bt technology in cotton by the Indian farmers. In India, the next GM crop, i.e. *Bt* brinjal, got the approval of India's Genetic Engineering Approval Committee (GEAC) in 2009, but it was faced huge outcry of Indian scientists and farmers; therefore, Environmental Ministry of India put a moratorium on release of the *Bt* brinjal after several public consultations across the country[11].

METHODS OF GM CROPS DETECTION:

Various methodologies is being employed to detect the presence of GM materials in food products which focus on target either transgenic DNA or expressed transgenic protein in GM crops. Recently, Dong et al.[12] have developed a database "GMO Detection method Database (GMDD)"which collected almost all the previously developed and reported GMOs detection methods. The commonly used DNA based methods include polymerase chain reaction (PCR) and real-time PCR; while protein based methods include immuno-PCR[13], chromatography[14] mass spectrophotometry[15], near infrared (NIR) spectroscopy[16,17], micro-fabricated devices and DNA chip technology[18,19] which offer solutions to current technical issues in GM crop analysis, but these methods are expensive and time taking. Other protein based methods include enzyme linked immunosorbent assay (ELISA) and western blot. These are sensitive, less expensive, but needs trained people. Besides these characteristics, above methods are not suitable for on-site testing. Another protein-based method named 'dipstick test' has been found quicker, simpler, less expensive, and found suitable for on-site testing by untrained people. This article is focused only on dipstick that is easier to perform than other techniques.

DIPSTICK STRIP ASSAY:

The dipstick assay format was initially developed in the 1970s. The dipstick assay uses a membrane based detection system. In the literature, a number of names are found for the strip based immunoassay tests, such as lateral flow technology (LFT), lateral flow devices (LFD), immunochromatographic (IC) tests, one-step tests and dipstick tests. The dipstick technology is a variant of ELISA, using nitrocellulose (NC) membrane strips rather than micro-titer wells and offers a qualitative or semi-quantitative test.

1. Components of dipstick strip:
   The dipstick comprised a NC membrane and three pads, i.e. sample, absorbent, and conjugate pads. The schematic diagram of typical dipstick is shown in Fig. 1.

1.1. Nitrocellulose membrane (NC):
The NC membrane used as a strip material in a majority of the dipstick assay systems and it is most important part of the test system, available from Whatman, Pierce, Advanced Microdevices (India) or Millipore. Besides NC membrane, several other attempts were tried using materials such as nylon[20] and polyethersulfone[21] but these were rarely used for the preparation of dipstick. The NC membrane has certain characteristics low-cost, true capillary flow, high protein binding capacity, relative ease of handling that makes it useful in this application in comparison to other membranes[22]. The membrane attached on plastic baking card, has two capture zones, one captures the bound antibody that is against transgenic protein, the other captures control antibody. A different protein binding capacity and pore size of NC membranes are available and used according to the suitability of the test. The 5 – 15 µm pore size of NC membranes is often used for the assay but the selection of membrane pore size depends on the test assay because it influences the wicking rate of the test. Increasing the membrane nominal pore size could also increase the speed of test dot development, but assay sensitivity suffered as a result[23].

1.2. Conjugate pad (Release pad):
It is usually made up of glass fiber, accepts the colloidal gold conjugated antibody and acts as reservoir for conjugate. It keeps the conjugate stable during the product's shelf life, and releases it efficiently and

reproducibly. The additives in this pad may be added according to requirements of users. Depending on the system, while it may be more important to achieve fast or slow release of the conjugate, the release must always be consistent.

1.3. Sample pad:

Sample pad is one of the most important elements after NC membrane in the dipstick assay, located at upstream end of the strip. It is made up of cellulosic fiber exhibits high absorption capacity and does not bind proteins. It is used to control the pH of the sample, movement of the gold conjugate. In dipstick assay, the sample pad accepts the sample and treats it to make compatible with the assay. This process must be done without destabilizing the analyte and by delivering it with high-efficiency and reproducibility to the rest of the assay. It helps to spread out the sample volume so that liquid enters the conjugate pad in an even and controlled manner.

1.4. Absorbent pad (Sink pad):

The absorbent pad is made up of cellulosic fiber. It is hydrophilic in nature and has excellent absorbent capacity. Absorbent pad, located at downstream end of the strip, has the ability to control sample flow through the device by pulling excess immuno-reagents from the test zone in assay.

1.5. Colloidal gold and conjugate:

In the conjugate system, the choices of labels and conjugation methods are critical. Several labeling agents have been used for development of strip such as colloidal gold, latex, silver, carbon, dye and enzyme. Because of their greater stability, sensitivity and precision over other labels, gold label was introduced into membrane-based rapid tests in the late 1980s and make gold suitable for use in IC tests[24]. As material of the label nowadays colloidal gold is used most often. The shape, size, and stability of the colloidal gold particles are the key factors of the success of the dipstick assay. The size of the colloidal gold particles can be controlled during manufacturing and the sodium citrate reduction method is most often followed for preparation as described by Frens[25]. A range from 10 – 40 nm colloidal gold particles has been employed in the dipstick assay to get sensitive results[26-30]. For conjugation, antibody is directly adsorbed on the colloidal gold particle surfaces, mediated by charge attraction, Vander Waals force, hydrophobic interaction and sulfur binding[24]. The pH of the colloidal gold solution is also important for conjugation and it necessary to maintain the pH close to isoelectric point of the antibody. The antibody firmly attached to the colloidal gold particle in the Fc region, leaving the Fab region protruding through the double ionic layer surrounding the gold particle and is able to bind protein[24]. More than one antibody may be adsorbed on per colloidal gold particle size greater than one nm[31]. The conjugate can be treated with sucrose solution according to assay need for consistent release of conjugate.

2. Dipstick test: assembly and principle:

There are two main format of dipstick commonly used for testing: sandwich type and competitive type.

2.1. Sandwich type dipstick assay:

Sandwich assays are mostly employed for larger analytes, such as transgenic proteins, bacterial pathogens and viruses, with multiple antigenic sites. The dipstick strip of 7.5 x 0.5 cm^2 size is generally selected which comprises NC membrane, sample pad, absorbent pad, and conjugate pad. On each strip, two lines (a test line and a control line) are assigned in the middle of the NC membrane separating 1 cm from each other. The specific antibody against the protein is immobilized on test line and the second antibody against species-specific antibody conjugated to colloidal gold particles is immobilized on control line. After drying strip, block the unoccupied site of NC membrane with suitable blocking buffer. In order to assist free mobility of the conjugated antibody, soak the membrane with sucrose solution. Concurrently, conjugate pad is treated with colloidal gold antibody. Then,

conjugate, sample and absorbent pads are pasted on their respective place as shown in **Fig. 1.** The strips can be stored in dry condition in a desiccator at 4 °C until use.

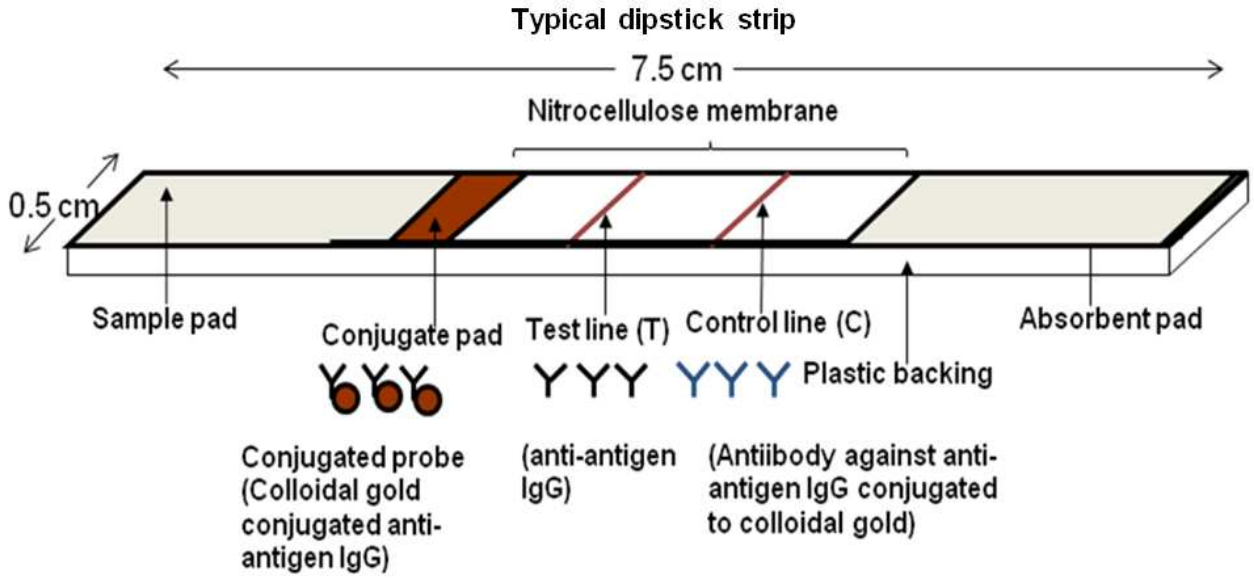

Figure 1

Schematic diagram of typical dipstick strip used for GMOs detection. The strip consists of a conjugate pad, a NC membrane, sample pad and absorbent pad. The conjugate pad contains colloidal gold conjugated antibody. Antibodies are immobilized on test line and control line on NC membrane.

The dipstick assay is essentially required the movement of a liquid sample, or its extract containing the protein of interest along the strip thereby passing various zones where antibodies have been attached that exert more or less specific interactions with the protein. The 50 – 200 µl assay volume of extract is generally used in the dipstick assay. When the strip is placed in a vial containing an extract from plant tissue or seed harbouring a transgenic protein, the sample migrates up the strip by capillary force of the strip material, but to maintain a flow an absorbent pad is attached at the distal side of the strip. This absorbent pad wick the liquid to the end of the strip, thus maintaining the flow. As the sample flows sequentially through the detection antibody (conjugate pad) and the capture antibody, the protein get captured/accumulated on test line and a red colored band was visible. A second red colored line is also observed on the control line of the membrane, generated by surplus gold conjugates, indicating the proper test performance while negative test sample yields no color on the test line indicating absence of protein in the test. The detection is completed in less than 10 min. The strong positive samples show marked bands, on test line, within 3-4 min. If no band is appeared on test line within 10 min, the results are interpreted as negative (**Fig. 2**). The colored band must be visualized on the control line, so the test could be considered invalid if there will be no color line present in the control region. The intensity of developed color on the test line is directly proportional to a certain concentration range of protein present in the test sample. The detection sensitivity decreases with increase in pore size of NC membrane due to the faster wicking rate in case of NC membranes with large pore size which, in turn, provides no sufficient time for antibody and protein interaction[23,32].

Sandwich type dipstick

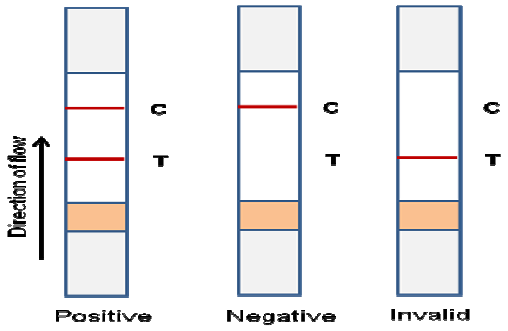

Figure 2

Sandwich type dipstick strip: two red lines shows positive result for protein and only one red line on control line shows absence of protein in the sample. If no red line was observed on the control line, the test should be treated as invalid.

2.2. Competitive type dipstick assay:

Another type of dipstick, i.e. competitive assay is used most often when testing for small molecules with a single antigenic determinant. It differs from the double antibody sandwich format in that the test line contains immobilized target antigen. In this format, free antigen in the sample solution competes with the immobilized antigen on the test line to bind colloidal gold conjugated antibodies at a defined concentration. The dense of the test line is dependent on the concentration of free antigen present in the test samples. The more amount of antigen present in the test sample, the weaker was the test line. A single control line on the membrane is a positive result whereas two visible lines in the capture and control zones is a negative result (**Fig. 3**).

Competitive type dipstick

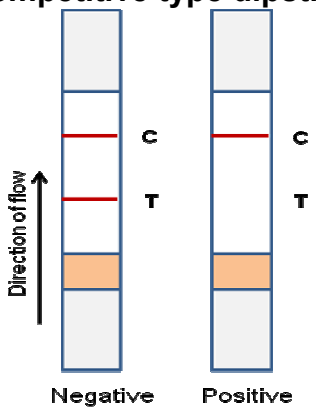

Figure 3

Competitive type dipstick strip: two red lines shows absence of antigen and only one red line on control line shows presence antigen in the sample.

Before the assay development, optimization is necessary to determine the NC membrane pore size, size of colloidal gold particles, optimal immobilization concentration of antibodies, optimal ratio of gold–antibody conjugate, blocking buffer and incubation time. Optimal immunoreagent concentration should be selected as a clear appearing in the

negative control with the shortest time, and the comparison of the intensity of color among test samples and negative control could be easily distinguished by eye.

The dipstick test generally provides qualitative or semi-quantitative results, which are interpreted by visual observation. The detection of the test samples takes usually 3–20 min. The result provides yes/no answer for presence or absence of protein in the test samples and results give no extra information concerning the presence of GM material at the ingredient level in food. However, quantification is technically possible by using special strip test readers. The qualitative performance parameters such as false positive and false negative rates, sensitivity and specificity rates are also important in developing qualitative test[33]. The sensitivity of test will also be enhanced by using silver enhancer[26,34] or gold enhancers[35]. It is also important the availability of certified reference material (CRM) for GMO testing that can be used as an external standard, to validate a method or determine sensitivity and specificity. The antibody-based immunoassay has been widely used to detect transgenic proteins in a variety of applications including testing in the breeding process, testing for unapproved events, and determining GM content, ensuring compliance with non-GM labeling requirements.

Compared to ELISAs, the dipstick tests have the same or even higher sensitivities. Dipstick can detect the antigen up to pg/ml range in the test samples[36,37] while for transgenic protein it can detect up to 5 ng/ml[38]. Dipsticks are simple to use by untrained operators and offers reproducible, reliable and sensitive results within few minutes. The results can be read directly by naked eyes, which ensure the convenience of assay on-site. These features make them ideal for use at the point-of-care and for testing of single or multiple analyte at a time in the field, as well as in the laboratory. It is economical and suitable as an initial screening method early in the food chain. The strips are very stable and robust, having a long shelf-life and usually not required refrigeration. There is no need for operations as incubation, washing and enzymatic reactions during signal generation, which distinctly lengthen the detection time. The dipstick test is currently using to detect transgenic proteins in leaves, seeds and grains.

FACTORS AFFECTING DIPSTICK ASSAY:

Because of numerous factors affecting the performance of dipstick[39], it is a little bit complex to develop. These crucial factors sometimes cause poor test performance that result in the generation of false positive and false negative signals. In an immunological assay, protein detection is relying on the specific binding between a protein and an antibody and therefore, any conformational changes in the tertiary structure of the protein render the test ineffective. Such conformational changes are induced frequently during food processing. Another component of an immunoassay is antibodies, which should be more specific for the target protein. The specificity of the antibodies must be carefully checked to elucidate any cross-reactivity with similar substances, which might cause false positive results. The antibodies may be polyclonal or monoclonal, but should be highly purified.

The ionic strength of the antibody coating buffer is also interfering with electrostatic interactions essential for protein binding. It is, therefore, important to determine the optimum buffer conditions that will result in a sufficient coating of capture antibody in the test line. Kaur et al.[35] demonstrated that by adding a small percentage of methanol (3%) in the buffer give considerable improvement in antibody coating on NC membrane, which in turn giving a sharp line to improve the performance of the immunoassay.

Selection of suitable blocking agent is necessary to reduce non-specific binding in the assay. A range of blocking reagents has been used such as polymers (PVP, PVA, and PEG), gelatin, casein, and BSA, but it should be optimized according to the assay requirement. Another factor is the stability of the colloidal gold conjugate. The stability of

colloidal gold should be checked in the conjugate. The shape of colloidal gold should be spherical and of appropriate size. Excess gold conjugate will create several problems in the test, which will increase the possibility of false positive signals.

The wicking rate is an important characteristic feature of NC membrane and primarily determines the reaction kinetics. The consistency and stability of NC membranes is necessary for assay performance. The sensitivity and specificity of the test are important parameters to determine before test kit development. Highest sensitivity is obtained with smaller pore size (slower wicking rate) and vice-versa[23]; and for high affinity antibody-protein reaction use of larger pore size (faster wicking rate) can result in faster test with adequate sensitivity.

COMMERCIAL DIPSTICKS FOR DETECTION OF GM CROPS:

Dipstick formats have been used instantaneous detection of transgenic proteins in leaves, seeds and grains of GM crops/foods. One can get on-site yes/no results in 10 min or less. It provides rapid, reliable and cost-effective screening in the production, verification of purity of GMO and non-GMO material. Dipstick test can be used to discriminate between authorized and unauthorized GM material or use of material, to identify safe or potentially unsafe material. However, the detection may be rendered difficult in the samples by low levels of expression of transgenic proteins and processed materials. In the dipstick strip for GMOs, both monoclonal and polyclonal antibodies against transgenic proteins were used. Currently, dipsticks are commercially available in the market to detect transgenic proteins such as Cry1Ac, Cry1Ab, Cry2Ab, Cry1F, CP4-EPSPS, etc. in different GM crops/produce[38, 40-44] listed in **Table 2**. Few literatures and patents are also available related to the dipstick based detection of GM proteins[45-47].

Table 2

Comprehensive overview of rapid GMO dipstick test kits which are commercially available.

GMO Test Kits	Transgenic protein	GM Crops	Limit of detection
Biogenetic Services Antibody Strip Tests	Cry1Ab, PAT, BAR, Cry3Bb, Cry9C, Cry1F, Cry1Ac, Cry2A, CP4-EPSPS	Corn, cotton, soybean	0.1%-1%
Romer Labs AgraStrip® GMO Test Kits	Cry1Ab, Cry1Ac, Cry3Bb1, Cry1Ab, Cry9C, Cry1F, Cry34Ab1, Cry3A, PAT, CP4-EPSPS	Corn and other matrices	0.1%-1%
EnviroLogix GMO Test Kits	Cry1Ab, Cry1Ac, Cry2Ab, Cry 3Bb, Cry 9C, Cry 1F, Cry34, Cry3A, Cry 2A, Cry2Ac, PAT/bar, CP4-EPSPS	Corn, cotton, soybean, canola, alfalfa, rice	0.1%-1%
Agdia GMO Test Kits	Cry1Ab, Cry1Ac, Cry1F, Cry34Ab1, Cry2A, Cry3Bb1, NPTII, PAT/bar, CP4-EPSPS	Cotton, soybean, corn, sugar beet, flex	0.1% -1%
SDIX GMO Chek Tests	Cry1Ab, Cry1Ac, Cry1F, Cry34Ab1, Cry2Ab, Cry3Bb1, Cry9C, PAT,	Corn, canola, cotton, soybean, alfalfa, sugar	0.1% - 5%

	CP4-EPSPS	beet, rice	
DesiGen GMO Kits	Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab, CP4-EPSPS	Cotton, corn, soybean	Below 1%

EnviroLogix Inc. has recently designed QuickComb Kit for presence of certain transgenic proteins (Cry1Ac, Cry2A and CP4-EPSPS) in GM corn bulk grain, which also gives the quantitative results. Agdia Inc. has developed ImmunoStrip test for the simultaneous detection of Bt-Cry1F, Bt-Cry1Ac and CP4 EPSPS transgenic proteins. Strategic Diagnostics Inc. (SDIX) has developed BR test kit for simultaneous detection of Cry1Ac and CP4-EPSPS proteins. In India, the Bt-Express dipstick kit was developed at the Central Institute for Cotton Research (CICR), Nagpur to assist regulators, farmers, seed industry and researchers in identifying Bt-cotton.

For many years, the dipstick format was very popular and was implemented in various tests. However, recent market needs to have created higher demands, requiring dipstick tests to become more than qualitative tests. The evolution of dipstick tests toward true quantitative formats is an area of active research and development, especially for tests of diseases, microbial contamination, and allergens. For detection of GM produce, dipsticks are currently limited to few products, but the strips that can simultaneously detect multiple proteins and employing for a broad range of GM materials are being developed to reduce testing time and cost as well.

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