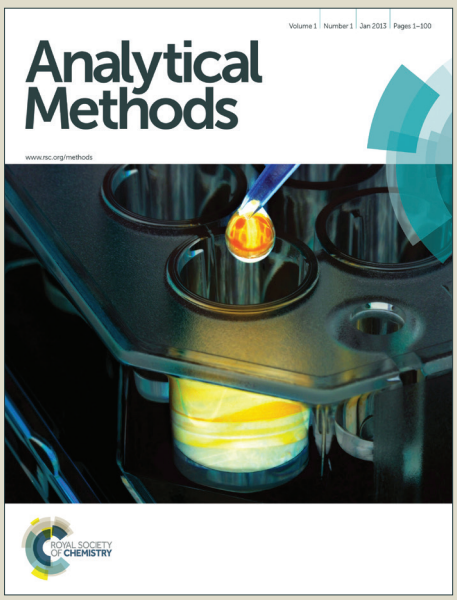
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Food Analytical

A novel immunocromatographic strip test for rapid detection of Cry1Ac and Cry8Ka5 proteins in genetically modified crops

Vanessa O. Santos[1,4], Patrícia B. Pelegrini[4], Fernanda Mulinari[2], Rodrigo S. Moura[3], Ludimila P.V. Cardoso[3], Samira Bührer-Sékula[3], Robert N. G. Miller[5], Eduardo R. C. Pinto[4] and Maria Fátima Grossi-de-Sá [4,6 *]

[1] Department of Biochemistry, Program of Biochemistry and Molecular Biology, Federal University of Rio Grande do Norte, Av. Salgado Filho, S/N, 59078-970 Natal, RN, Brazil.

[2] Pioneer Union for Social Insertion, BR020 Km 12, 70390-125, Planaltina, DF, Brazil.

[3] Tropical Pathology and Public Health Institute, Federal University of Goiás, R. 235 S/N, 74605-050, Goiânia, GO, Brazil.

[4] EMBRAPA Genetic Resources and Biotechnology PqEB - Av. W5 Norte, P.O. Box 02372, 70770-917, Brasília, DF, Brazil.

[5] Department of Cellular Biology, University of Brasília, 70910-917, Brasília, DF, Brazil.

[6] Catholic University of Brasilia, SGAN 916, Av. W5, 70790-160, Brasília, DF, Brazil.

*Corresponding author: Maria Fatima Grossi-de-Sá

Tel: +55 61 3448 4902

E-mail: fatima.grossi@embrapa.br

Abstract

The cultivation of genetically modified (GM) crops has grown rapidly worldwide. This has led to regulatory authorities implementing strict procedures to monitor and verify the presence and abundance of GM varieties in agricultural crops. Immunochromatographic strip tests have been employed for detection of transgenic proteins expressed in GM crops as rapid, reliable and cost-effective screening tools. In this study, we developed a novel and sensitive strip test assay, based on a sandwich format, for the identification of Cry1Ac and Cry8Ka5 transgenic proteins. We generated two monoclonal antibodies (mAb), namely 1B1 and 5H4, that bind with high specificity to Cry1Ac and Cry8Ka5 proteins. For development of strip tests, colloidal gold particles were coated with 1B1 mAb and used as detector reagent. The **5H4 mAb** was sprayed at the test line of a nitrocellulose membrane to serve as a capture reagent and anti-species specific antibody were used at the control line. The strip test developed was capable of detecting **0.06µg of Cry1Ac** and Cry8Ka5 proteins. For validation of strip test, GM and non-GM cotton leaf samples were employed. The results indicated that the strip test was capable of distinguishing between GM and non-GM cotton samples, offering potential for use as a rapid and cost-effective screening tool for insect-resistant GM crops expressing Cry1Ac and Cry8Ka5 proteins to control lepidopterans and coleopteran pests, respectively.

Keywords: Strip test; GM crop detection; Cry1Ac protein; Cry8Ka5 protein; immunoassay.

Introduction

Genetic transformation has become an important tool for crop improvement[1]. The application of engineering to food and feed crops is widely acknowledged as a useful tool for addressing global agricultural challenges of population growth and climate change[2]. Such agricultural biotechnology has been widely adopted by growers, with GM crop to date with insect resistance or herbicide tolerance traits requiring lower inputs and allowing flexibility in crop management strategies, whilst maintaining or increasing crop yield and quality [3,4].

GM insect-resistant crops have been developed using specific genes isolated from the naturally occurring soil bacterium *Bacillus thuringiensis* (Bt)[5]. These genes encode specific insecticidal proteins known as Cry proteins[6]. The introduction of genetically engineered crops incorporating these insecticidal proteins has provided growers with an opportunity to reduce insecticide application required for management of lepidopteran, coleopteran and dipteran insect-pests[7,8]. To date, Bt cry genes have been widely applied in GM maize and cotton development[9]. For example, in Brazil it is now possible to find at least 12 varieties of Bt cotton released for cultivation that express the Cry1Ac protein alone or fused to Cry 2Ab2 or Cry1F proteins[10]. Now, these GM cotton varieties mainly target the cotton bollworm *Helicoverpa armigera.* In the case of the major coleopteran cotton boll weevil *Anthonomus grandis*, the mutant toxin Cry8Ka5, which offers increased activity against this pest, has recently been proposed for incorporation into this agribusiness commodity in Brazil[8, 11.]

Given the rapid development of GM crops and their increased presence in food and feed, public concern over traceability, food safety and potential ecological

contamination is becoming widespread[12]. As such, there is an increasing demand for analytical methods for detection of introduced gene encoding DNA or their expressed protein(s) in transgenic plants[1,9]. Moreover, precise and accurate detection methods are a prerequisite for reliable control of GM crops in the agricultural market[13]. Various methodologies have been employed to detect the presence of GM materials in food[15-20]. Recently, the database GMO Detection methods (GMDD) was developed, which summarizes all GMO detection methods developed to date[20]. Commonly employed DNA-based methods include PCR, real-time PCR and DNA chip technology[18,19], while protein-based methods include imuno-PCR[14], mass spectrometry[15,16] and near infrared (NIR) spectroscopy[17]. Although these methods are very accurate and sensitive, they are not cost-effective for large-scale analyses. While less expensive protein-based methods, such as enzyme linked immunosorbent assay (ELISA) and Western blot, also offer accuracy and sensitivity, these methods still have limitations, in that they require trained personnel and are unsuitable for on-site testing[22].

The immunochromatographic (IC) strip test is a well-established diagnostic tool[23]. This technology offers advantages in terms of speed, simplicity and cost-effectiveness when compared to the abovementioned detection methods[21]. Moreover, IC strip tests also offer convenience for on-site testing under field conditions by untrained personnel[24]. Although several IC strips are now commercially available for detection of transgenic events, no detection kits are available for GM crops carrying the Cry8ka5 protein. Thus, the goal of this study was to develop an IC strip for the simultaneous detection of the Cry1Ac protein, which is found in Bt crops approved for cultivation, and the Cry8Ka5 mutant protein, which has been employed by our group in the development of transgenic cotton.

Materials and Methods

Reagents and materials

Goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP), complete and incomplete Freund's adjuvants, chloroauric acid ($HAuCl_4.3H_2O$), sodium citrate, polyethylene glycol 4000 (PEG 4000), hypoxanthine-aminopterine-thymidine (HAT) medium, 4-nitrophenyl phosphate and mouse monoclonal antibody ISO2-1 kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI1640 medium with L-glutamine and HEPES, fetal bovine serum (FBS), penicillin, streptomycin and rabbit anti-mouse immunoglobulin IgG were purchased from Thermo Fisher Scientific (Rockvford, IL, USA). The HiTrap protein G HP affinity column was purchased from GE Healthcare (Uppsala, Sweden). Gelatin (blotting grade), 4-(4-amino-3,5-dimethylphenyl)-2,6-dimethylaniline (TMB) and Protein assay were obtained from Bio Rad Laboratories (Hercules, CA, USA). High-flow nitrocellulose membrane, sample pad, conjugate pad and absorbent pad were obtained from Millipore (Bedford, MA, USA). High Binding Flat-bottomed polystyrene microplates were obtained from Costar (Corning, MA, USA), and cell culture flasks and plates from TPP (St. Louis, MO, USA). Murine myeloma Sp2/0-Ag14 cell lines were kindly provided by Dr. Sandra Farias from UFRGS, Rio Grande do Sul, Brazil.

Instruments

A XYZ 3050 Biostrip Dispenser and CM 4000 Cutter were purchased from Bio-Dot (Irvine, CA, USA). A Microplate Reader Spectra Max M2 was obtained from Molecular Devices Corp. (Sunnyvale, CA, USA).

Preparation of the Bt Cry1Ac and Cry8Ka5 toxins

The Cry1Ac protein was produced by heterologous expression in *E.coli* JM109, transformed with the recombinant plasmid pKK223-3 containing the truncated *cry1A*c gene of *B. thuringiensis subsp hurstaki* (HD73), according to the reported method[25]. The activation of Cry1Ac toxin was performed with trypsin 1:50 (w/w) for 2 h, at 37ºC. Cry8Ka5 protein production was performed by heterologous expression in *E.coli* BL 21(DE3) containing the mutant gene inserted into plasmid pET101/D TOPO, as described by Oliveira[8].

All toxin samples were purified, identified by 12% SDS-PAGE, quantified via the Bradford method[26] using BSA as a standard, and stored at -80ºC.

Production of monoclonal antibodies (mAbs)

Immunization

The Cry 1Ac protein was used as an immunogenic molecule for obtaining mAbs. Cry1Ac protein (250µg) was added to 0.25 mL sterilized PBS and emulsified with an equal volume of Freund´s complete adjuvant. The solution was applied via intraperitoneal injections in ten 8-week-old female Balb/c mice, during a three-week interval. Subsequent immunization (boosters) were applied with incomplete Freund's adjuvant. Later, blood samples were removed from the caudal vein of the mice, at intervals after each booster, and then assayed by indirect ELISA for antibody titration. The mouse with the highest serum titration received a final intraperitoneal injection three days before cell fusion.

Experimental procedures were carried out strictly in accordance with the "Administrative Rules for Laboratory Animals in Brasília (DF) state'' (2011), and were approved by The Animal Care and Use Committee of EMBRAPA Genetic Resources and Biotechnology (Brasília, DF, Brazil).

Cellular fusion and screening of hybridomas

Cell fusion procedures were performed according to the reported method[27], with modification. Briefly, spleen cells from the immunized mice were isolated and fused with Sp2/0- Ag14 murine myeloma cell lines at a ratio of 1:10 in the presence of 1mL PEG 4000 solution (0.8 g mL^{-1}). The fused cells were diluted with fresh HAT medium and distributed into a 96-well culture plate. After ten days, hybridoma supernatants were analyzed by ELISA for the presence of antibodies against activated Cry1Ac protein. Culture supernatants from the cells with highest absorption value were transferred to 24-well culture plates and again tested. Only clones that maintained higher absorption in ELISA tests were chosen for further selection. Cell suspensions from each well of the 24-well culture plate were diluted in order to obtain one cell per well, and distributed into a 96-well culture plate. Wells containing a single hybridoma were retained for antibody production and further characterization. These cloned hybridoma cells were introduced via intraperitoneal injection into Balb/c mice pretreated with injection of 0.3 mL pristane. Resulting ascites were purified using a protein A affinity column.

ELISA assays

96 well microplates were coated with 1 µg of Cry1Ac or Cry8Ka5 protein in coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) and incubated at 4ºC overnight.

Microplates were washed three times with PBST buffer (PBS containing 0.05% *(v/v)* Tween 20, pH 7.4). To block remaining sites on the wells, a blocking solution (PBST and 3% *(w/v)* gelatin) was used for 1 h at 37ºC. After subsequent washing, 100 µL of 1:2000 dilution of each mAbs were added and incubated for 1 h at 37ºC. The plate was repeatedly washed three times to remove unbound antibodies and then incubated with 1:5000 dilution of goat anti-mouse IgG-HRP prepared in PBST (containing 1% gelatin). Microplates were incubated for 1h at 37ºC, 100µL of TMB solution added to each well, and then plates incubated for 10 min at room temperature. The reaction was stopped by the addition of 50 µL 2 M sulfuric acid and absorbance values at 450 nm then determined.

Mapping the binding region of mAbs to Cry1Ac protein

The synthetic peptides (2.5µM): PT3b (PPRQGFSHRLSHV), PT4c (LGQGEYRTLSST), PT4d (IIRAPMFSWIHRSAE), PT5d (GTEFAYGTSPNL) and PT5e (FRRELTLTVLDI) were produced by the company GenScript (USA). These peptides were coupled to BSA according to the reported method[28] , then incubated on a 96 well microplate overnight at 4ºC. The same way procedures were followed as described in ELISA assays.

Sandwich ELISA

96 well microplates were coated with 100 µL of capture antibody (mAb) at 1:2000 dilution in coating buffer and incubated at 4ºC overnight. Following three repeat washes with PBST, each well was filled with 200 µL of blocking buffer and incubated at 37ºC for 1 h. Afterwards, 1µg Cry1Ac protein was added and incubated at 37ºC for 1h. Following washing, 100 µL of the detection antibody (mAb) at 1:2000 dilution was added to each well and incubated at 37ºC for 1h. Washes were conducted as in the

previous step, 100 µL goat anti-mouse IgG- HRP (1:5000 dilution in PBST containing 1% gelatin) was then added to each well and incubated at 37ºC for 1h. Plates were washed again, and 100-µL TMB solution was finally added to each well. After color developing at room temperature for 10 min, the reaction was stopped with 50 µL of 2M H_2SO_4 and the absorbance value determined at 450 nm.

Characterization of mAbs

Isotypes of mAbs were obtained using an isotyping kit according to the manufacturer's instructions. Analysis of the cross-reactivity (CR) of antibodies was performed by ELISA, using the Cr8Ka5 recombinant protein as antigen. The ELISA assay was employed as described previously.

Preparation of colloidal gold labelling of the anti-Cry mAb

Colloidal gold with a mean particle diameter of 40 nm was produced by the reduction of gold chloride with 1% sodium citrate, as previously described [29]. The probe was prepared as previously reported [30] with minor modifications. Prior to conjugation, the optimal pH and amount of antibody for conjugation were determined. Briefly, 150 µg of purified 1B1 mAb was added drop-wise onto 10 mL of a colloidal gold solution (pH 9.0) with gentle stirring. The mixture was agitated for 1 h at room temperature using an overhead shaker. BSA was added to block any remaining free binding sites on the surface of the gold particles, and the solution was again incubated for 30 min at room temperature. Unbound proteins were removed by centrifugation (18,500 x g for 30 min, 4 ºC), the pellet washed with deionized water and the mixture again centrifuged. After

removal of the supernatant, the pellet was dissolved in 2 mL of BSA solution at 2% and stored at 4 ºC until use.

Preparation of the immunochromatographic (IC) test strip

The test strip was prepared by applying **5H4 mAb (0.5 µg µL^{-1})** and **anti-mouse IgG (0.5 µg µL^{-1})** to the nitrocellulose membrane at the test and control lines, respectively. With test and control lines separated by a distance of 5 mm, the reagents were applied in a dot format using a BioDot XYZ 3050 micro-brush at **1 µL cm-^{1}. The total volume dispensed into the test and control line were 0.5 µL of solution. Therefore, each strip test contained 0.25 µg of 5H4 mAb and anti-mouse IgG into test and control line, respectively.** The coated membrane was then dried at 37 ºC for 30 min. Subsequently, the membrane was blocked with 2% BSA solution to prevent nonspecific adsorption and stored in a desiccator. **For the conjugate pad, the airbrush equipment was calibrated to release 10 µL cm^{-1} of 1B1 mAb-gold conjugate. The initial solution of conjugate used as a detection reagent was at a concentration of 0.075 µg µL^{-1}. For each strip test, the total volume dispensed was 5 µL, corresponding of 0.375 µg of 1B1 mAb**. After, the conjugate pad was then dried at 37 ºC for 30 min. The coated membrane, conjugate pad and absorbent pad were assembled and cut lengthways (5 mm x 70 mm) using a guillotine cutter. The sample pad (absorbent paper) and conjugate pads (glass-fiber membrane) were previously treated with blocking solutions (20 mM phosphate buffer, pH 7.4, containing 2% *(w/v)* BSA, 2.5% *(w/v)* sucrose, 0.3% *(w/v)* polyvinylpyrrolidone and 0.02% *(v/v)* sodium azide) and dried at 37 ºC overnight.

IC strip *in vitro* test with Bt Cry1Ac and Cry8ka5 proteins

Purified Cry1Ac and Cry8Ka5 protein samples **(2.5 µg mL^{-1})** were used to determine the sensitivity of the strips. The samples were prepared in 0.05 M carbonate-bicarbonate, pH 9.6, buffer and 200µL of each sample, equivalent at **a final concentration of 0.5 µg,** were dispensed into wells of a micro plate. Afterwards, each individual strips was dipped into test samples and left for 10 min.

IC strip assay in cotton samples

Cotton leaf samples for GM (Bollgard®), GM (developed by our team) and non-GM (Coker-312) were used to confirm the accuracy of the developed strip test. The GM and non-GM leaf disc samples were ground to a fine powder (100 mg) and homogenized with 1mL of extraction buffer (PBS with 0,05% *(v/v)* Tween-20 and 1% PVP-40 and 0.032mg.mL^{-1} trypsin), according to the reported method[31]. The homogenate was agitated for 30 min and incubated at 37ºC for 1h. After, 22µL of **10mM phenylmethanesulfonylfluoride (PMSF)** was added as the stop solution. These extracts were directly used for strip test evaluation. 200 µL of each sample was dispensed into micro plate wells, strips dipped into homogenates and incubated at room temperature for 10 min.

Statistical analyses

Data were analyzed using GraphPad InStat™ (GraphPad software, V2.05). ANOVA analyses were performed using the Bonferroni posttest and Tukey's multiple comparison tests with confidence intervals of 95%. Values of $p<0.01$ and $p<0.05$ were considered statistically significant.

Results and Discussion

Recombinant proteins production

SDS-PAGE profiles of Cry1Ac and Cry8Ka5 recombinant proteins after expression and purification are shown in Fig.1. The toxins presented the expected molecular mass (near 70 kDa). The Image Master 2D platinum (v.7.0 GE Healthcare) software was used to estimate the relative percentage of purity of each protein obtained. For both Cry1Ac and Cr8Ka5 proteins, different batches following expression and purification presented relative purities, which ranged from 75% up to 95%. SDS-PAGE methodology is widely used by chemical companies for analysis of purity of commercialized proteins[11].

Production of Monoclonal Antibodies (mAbs)

Antisera from immunized mice showed higher titer values in ELISA assays using Cry1Ac protein as antigen than in non-immunized mice. After fusion, cloning and ascites purification, five mAbs were obtained, identified as 1B1, 1B5, 5H4, 2E3 and 3C10. The interaction of each mAb with Cry1Ac or Cry8Ka5 proteins was analyzed by ELISA (Fig.3). The results showed that all mAbs were highly specific to Cry1Ac and were able to recognize Cry8Ka5, with the exception of mAb 2E3. Therefore, this mAb not was used in subsequent assessments. These results suggest that the specific mAbs are binding to conserved structures among these Cry toxins, as described previously[34]. Previous studies reported a monoclonal antibody produced against a Cry1Ab lepidopteran-specific toxin that also showed cross-reaction with a Cry9 coleopteran-specific toxin[35.] Several mAbs produced against peptide sequences from Cry toxins have been used to elicit the binding site of these proteins with their specific receptors[32,33,36]. It

is important to increase knowledge about the mode of action of Cry toxins for target insects. In this study, specific synthetic peptides of the binding region of the cadherin-like receptor from *Helicoverpa armigera (*HaCad) to the Cry 1Ac protein (data no shown) were used for epitope mapping of mAbs produced. For this assay, the Cry1Ac protein was not previously activated because the PT5e peptide sequence is localized in the N-terminal domain found on truncated Cry 1Ac protein. As shown in Fig. 3, of the five peptides tested, only the PT4d and PT5d sequences to mAbs 1B1 and 5H4 showed a statically significant difference ($P<0.05$). These finding suggest that the 1B1 and 5H4 mAbs recognized different binding site on the Cry1Ac protein. To confirm these resulted a sandwich ELISA assay was performed. Pairwise mAbs combinations are shown in Fig.4. When the combination 5H4 mAb capture antibody and 1B1 mAb as second antibody was used, an increase in pairing occurred ($P<0.05$) greater than observed in other mAbs combinations. Hence, these two mAbs were selected and used for preparation of the strip test.

Development and optimization of the strip test

In this study, we developed a test strip based on a sandwich immunoassay format for qualitative detection of Cry1Ac and Cry8Ka5 proteins found in transgenic plants (Fig.5). The system in sandwich format employs two different antibodies that bind to distinct epitopes of the analyte. A labeled antibody is placed onto a glass-fiber membrane (conjugate pad) to serve as detector reagent and another antibody is sprayed at the test line of the nitrocellulose membrane to serve as capture reagent. A second antibody specific to recognize the first antibody is used to produce a control signal [21].

As shown in Fig.6, the strip tests were placed into wells of micro plates carrying test samples and the test results interpreted as positive or negative based upon visual inspection. As the sample flowed sequentially through the detection antibody (1B1 mAb) and the capture antibody (5H4 mAb), the Cry1Ac protein (Fig.6A) or Cry8Ka5 (Fig.6B) accumulated on the test line, to reveal a visible red line. A second red line was also observed on the control line, indicating correct test performance. A solution containing only extraction buffer without trypsin was used as negative control. With this solution, no color developed on the test line, indicating the absence of Cry1Ac or Cry8Ka5 proteins. These results indicated that the developed strip test enabled specific detection of Cry1Ac or Cry8Ka5 in GM samples**, showing high sensitivity levels**.

Validation of strip test using GM cotton samples

Bollgard® cotton (positive control) and Coker 312 (negative control) samples were previously identified by a commercial strip test produced by Envirologix Inc. (data not shown). Cry1Ac and Cry8Ka5 toxin concentration levels from GM cotton leaf samples were estimated using a previous established standard curve (Fig.7A). The standard concentrations of Cry1Ac and Cry8Ka5 toxins (0 to 1 μg) resulted in a standard curve with good linearity of R^2= 0.9714 and 0.9862, respectively. The cotton plants developed by our team were analyzed by PCR assays (data not shown) and two positive plants (named plant 50 and 217) were further characterized by ELISA. As shown in Fig.7B, only plant 50 showed a statically significant difference ($P<0.05$) to non-GM cotton plant. Therefore, this plant was chosen for use in determining the accuracy of the strip test.

According to previous study reports [37], the level of expression of the Cry1Ac protein in terminal leaf tissues was estimated at 2.98 ng mg^{-1} or 2.98µg g^{-1} dry tissue**. In this study, we used 100 mg (dry tissue) of total protein extraction from GM and non-GM cotton leaves. After extraction procedure, the estimate amount of the Cry1Ac and Cry8Ka5 transgenic proteins in the sample was of 0.298 µg mL^{-1}. For evaluation of the strip test, an aliquot of 200 µL of extract was used, giving an approximate concentration of 0.06 µg for Cry1Ac and Cry8Ka5.**

As show in Fig.8, two red lines were clearly visualized in the test and control line when all GM samples were applied to the strip test. This means that the strip test developed in this study reacts to Cry8Ka5 and Cry1Ac proteins found in these GM cotton samples (Fig8A and Fig.8B, respectively). As expected, only a strong red line on the control line was observed when the non-GM samples (Coker 312) were applied to the strip test.

Currently, strip tests are commercially available to detect *Bt* transgenic proteins in GM cotton events used commercially to control lepidopterans [22]. These events include Bollgard® (expressing the Cry1Ac protein), Bollgard II® (expressing the Cry1Ac and Cry2Ab proteins) and Widestrike® (expressing Cry1Ac and Cry1F proteins). Now, however, no strip tests are available for detection of *Bt* Cry8Ka5 protein, which is used for the developed Brazilian GM cotton effective against the cotton boll weevil coleopteran insect pest, which is particularly harmful in Latin America [8].

Conclusion

In the present study, we developed a qualitative immunocromatographic strip test for detection of Cry1Ac and Cry8Ka5 proteins found on insect-resistant GM crops. Results showed that the strip test was sufficiently sensitive and accurate for detection of these

proteins on GM cotton crops. Moreover, these results were obtained within 10 min without the need for expensive equipment or reagents. The strip test is applicable for use directly in the field, as a rapid and cost-effective screening tool for Cry1Ac or Cry8Ka5 protein detection in GM crops.

Acknowledgements

The authors thank CNPq (Grant number: 350165/2008-4), CAPES (Grant number: 1113634) and EMBRAPA Genetic Resources and Biotechnology (Grant number: 02.09.01.013.00.03) for financial support for this study.

All authors declare that they have no conflict of interest.

REFERENCES

[1] E. Anklam, F. Gadani, P. Heinze, H. Pijnenburg, G. Van Den Eede, *Eur. Food Res.Technol.,* 2002, 214, 3-26.

[2] P. D. Brune, A. H. Culler, W. P. Ridley, K. Walker, *J. Agric. Food Chem*., 2013,61, 8243-8247.

[3] C. James, *Global Status of commercialized Biotech/GM crops: 2013*. ISSA Brief 43, ISAAA., N.Y, 2013.

[4] G. Brookes, P. Barfoot, *GM Crops & Food: Biotechnology in Agriculture and the Food Chain.,* 2013, 4:1, 74-83.

[5] J. Li, J. Carroll, D.J. Ellar, *Nature.,* 1991, 353, 815–821

[6] S. Atsumi, Y. Inoue, T. Ishizaka, E. Mizuno, Y. Yoshizawa, Madoka Kitami, R. Sato, *FEBS journal.,* 2008, 275, 4913-4926.

[7] R. V. Gunning and G.D. Moores, *Pesticide Biochemistry and Physiology*., 2010, 97, 55-59.

[8] G. R. Oliveira, M. C.M. Silva, W. A. Lucena, E. Y.T. Nakasu, Alexandre A.P. Firmino, M. A. Beneventi, D.S.L.Souza, J. E.Gomes-Jr, J. D.A de Souza-Jr, D. J. Rigden, H. B. Ramos, C. R. Soccol, M.F.Grossi-de-Sá, *BMC Biotechnology.,* 2011, 11, 85-97.

[9]J. W. Stave, *Journal of AOAC International.,* 2002, 85, 780-786.

[10] CNTBIO, http://www.ctnbio.gov.br/index.php/content/view/14783.html.(accessed September 2014).

[11] D. F. Farias, M. P. Viana, G. R. Oliveira, V. O. Santos, C. E. M. Pinto, D. A. Viana, I. M. Vasconcelos, M. F.Grossi-de-Sá, A. F.U. Carvalho, *Food and Chemical Toxicology.,* 2015, 81, 81-91.

[12] X. Wang, D. Teng, Q. Guan, F. Tian, J. Wang, *Food Chemistry.,* 2015, 173, 1022-1029.

[13] D. Morisset, T. Demsar, K. Gruden, J. Vojvoda, D. Stebih, J. Zel, *Nature Biotechnology.,* 2009, 27, 700-701.

[14] R.C. Allen, S.Rogelj, S.E. Cordova, T.L. Kieft, *Immunol Meth.,* 2006, 308, 109-115.

[15] W.C. Byrdwell, W.E. Neft, J. Liq. *Chrom. Rel. Technol.,* 1996, 19, 2203-2225.

[16] M. F. Ocana, P. D. Fraser, R. K. Patel, J.M. Halket, P.M. Bramley, *Mass Spectrom.,* 2007, 21, 319-328.

[17] L. J. Xie, Y.B. Ying, H.Y. Yu, *Anal. Chim. Acta.,* 2007, 584, 379-384.

[18] K.S. Kumar, S.H. Kang, *Electrophoresis.,* 2007, 28, 4247-4254.

[19]S. Leimanis, M. Hernandez, F. Boyer, M. Burns, S. Bruderer, T. Glouden, N. Harris, O. Kaeppeli, P. Philipp, M. Pla, P. Puigdomenech, M. Vaitilingom, Y. Bertheau, J. Remacle, *Plant Mol Biol.,,* 2006, 61, 123-139.

[20] W. Dong, L. Yang, K. Shen, B. Kim, G.A. Kleter, H. J. Marvin, R.Guo, W. Liang, D. Zhang, *BMC Bioinformatics.,* 2008, 9, 260-267.

[21] B. Ngom, Y. Guo, X. Wang, D. Bi, *Anal Bioanal Chem,* 2010, 397, 1113-1135.

[22] R. Kumar, R. P. Sinha, *International Journal pf Pharma and Bio Sciences.,* 2011, 2, 111-121.

[23] G. A. Posthuma-Trumpie, J. Korf, A. van Amerongen, *Anal Bioanal Chem.,* 2009, 393, 569-582.

[24] R. Kumar, C. K. Singh, S. Kamle, R. P. Sinha, D. N. Kachru, *Food Chemistry.,* 2010, 122, 1298-1303.

[25] D . F. Farias, M. P. Viana, G. R. Oliveira, M. A. Beneventi, B. M. Soares, C. Pessoa, I. P. Pessoa, L. P. Silva, I. M. Vasconcelos, M. F. Grossi-de-Sá, A. F. U. Carvalho, *Biomed Research International.,* 2014, DOI: /10.1155/2014/810490.

[26] M. M. Bradford, Analytical Biochemistry., 1976, 72, 248-254.

[27] G. Köhler, C. Milstein, Nature., 1973, 256, 495-497.

[28] S. S. Lateef, S. Gupta, L. P. Jayathilaka, S. Krishnanchettiar, J-S, Huang and B-S, Lee, *Journal of Biomolecular Techniques.,* 2007, 18, 173-176.

[29] M. A. Hayat, *Colloidal Gold: Principles, Methods and Applications,* Academic Press, 1989, New York.

[30] L. Biing-Hui, H. Yu-Tien, L. Chuan-Chen, Y. Feng-Yih, *Food Control.,* 2013, 30, 184-189.

[31] G. Shan, S. K. Embrey, B. W. Schafer, *J. Agric. Food Chem.,* 2007, 55 , 5974-5979.

[32] S. Atsumi, E. Mizuno, H. Hara, K. Nakanishi, M. Kitami, N. Miura, H. Tabunoki, A.Watanabe, R. Sato, *Appl Environ Microbiol.,* 2005, 71, 3966–3977

[33] H. Hara , S. Atsumi, K. Yaoi, K. Nakanishi, S. Higurashi, N. Miura, H. Tabunoki, R. Sato, *FEBS Lett.,* 2003, 538, 29–34.

[34] E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J.Feitelson, D.R. Zeigler, D. H. Dean, *Microbiology and Molecular Biology Reviews.,* 1998, 62, 775-806.

[35] U. Walshus, S. Witt, C. Wittman, *Food and Agricultural Immunology.,* 2002, 14, 231-240.

[36] R. Xie, M. Zhuang, L.S. Ross, I. Gomez, D.I. Oltean, , A. Bravo, M. Soberon, S.S. Gill, *J Biol Chem.,* 2005, 280, 8416–8425.

[37] M. W. Siebert, T. G. Patterson, G. J. Gilles, S. P. Nolting, L. B. Braxton, B. R. Leonard, J. W. Van Duyn, R. B. Lassiter, *Journal of Economic Entomology,* 2009, 102, 1301-1308.

Figure Legends

Fig.1. SDS-PAGE profiles of the heterologous expression products of Cry1Ac and Cry8Ka5 proteins. Lane 1: molecular mass marker (BenchMark™ Pre-Stained Protein Ladder, Invitrogen); lane 2: Before purification of Cry1Ac protein; lane 3: Before purification of Cry8Ka5 protein; lane 4: Cry 1Ac toxin after trypsinization and dialysis; lane 5: Cry8Ka5 toxin after purification on the chromatographic affinity (Ni-NTA resin).

Fig. 2. Evaluation of the cross interaction of the mAbs *anti*-Cry1Ac against Cry8Ka5 recombinant protein by ELISA. High binding EIA/RIA microplates were coated with 1µg of Cry1Ac or Cry8Ka5 protein and kept overnight at 4 ºC. The plates were blocked with 3% gelatin in PBST. An aliquot of 100 µL of each mAbs (1:2000) was added for 1 h at 37 ºC. The binding was identified using a goat anti-mouse IgG-HRP (1:5000) and developed using 100 µL of 4-(4-amino-3,5-dimethylphenyl)-2,6-dimethylaniline (TMB). The reaction was stopped by the addition of 50 µL of 2M sulfuric acid, and the absorbance was measured at 450 nm. All statistical tests were performed using a two-way ANOVA. **** denotes $p < 0.01$.

Fig. 3. Epitopes mapping of mAbs by ELISA using Cry1Ac toxin synthetic peptides. The peptides coupled with BSA protein (1µg) were coated on High binding EIA/RIA microplates and kept overnight at 4 ºC. The plates were blocked with 3% gelatin in PBST. An aliquot of 100 µL of each mAb (1:2000) was added for 1 h at 37 ºC. The binding was identified using a goat anti-mouse IgG-HRP (1:5000) and developed using 100 µL of 4-(4-amino-3,5-dimethylphenyl)-2,6-dimethylaniline (TMB). The reaction was stopped by the addition of 50 µL of 2M sulfuric acid, and the absorbance was measured at 450 nm. All statistical tests were performed using a two-way ANOVA. ** denotes $p < 0.05$.

Fig.4. Evaluation of interaction of mAbs by Sandwich ELISA. High binding EIA/RIA microplates were coated with 100 µL of capture antibody (mAb) at 1:2000 dilution in coating buffer at 4ºC overnight. The plates were blocked with 3% gelatin in PBST. Then, 1µg Cry1Ac protein was added and kept at 37ºC for 1h. An aliquot of 100 µL of the detection antibody (mAb) at 1:2000 dilution was added to each well and incubated at 37ºC for 1h. Binding was identified using a goat anti-mouse IgG-HRP (1:5000) and developed using 100 µL of 4-(4-amino-3,5-dimethylphenyl)-2,6-dimethylaniline (TMB). The reaction was stopped by the addition of 50 µL of 2M sulfuric acid, and the absorbance was measured at 450 nm. All statistical tests were performed using a two-way ANOVA. **** denotes $p < 0.01$.

Fig.5. The schematic image of the assembled strip test and the principle of detection using sandwich immunoassay format (A) Samples with Cry1Ac or Cry8Ka5 protein. (B) Samples without Cry1Ac or Cry8Ka5.

Fig.6. Immunochromatographic strip test for Cry1Ac and Cry8Ka5 protein detection. Tests were evaluated using 0.5µg of Cry1Ac toxin (A) and 0.5µg of Cry8Ka5 toxin (B). (-) Corresponds to the negative control of 0.05 M carbonate-bicarbonate without the *Bt* proteins, pH 9.6 buffer (200µL).

Fig. 7. Evaluation of accuracy of strip test using GM cotton leaf samples. A- Standard curve for Cry1Ac and Cry8Ka5 (0-1µg) toxins using 1B1mAb. B- Detection of Cry1Ac protein (Bollgard® GM cotton) used as positive control and Cry8Ka5 protein (GM 50 and GM 217 cotton plant developed by our team). Non-GM (Coker 312) was used as negative control *** All statistical tests were performed using a two-way ANOVA. *** denotes $p < 0.05$.

Fig. 8. Evaluation of the strip test for detection of Cry8Ka5 and Cry1Ac toxins in GM and non-GM cotton leaf samples. (A) GM cotton leaf samples (developed by our team) containing the Cry8Ka5 toxin (B) Bollgard ® cotton leaf samples containing the Cry1Ac protein. Non-GM cotton leaf samples (Coker 312).

Figure 1

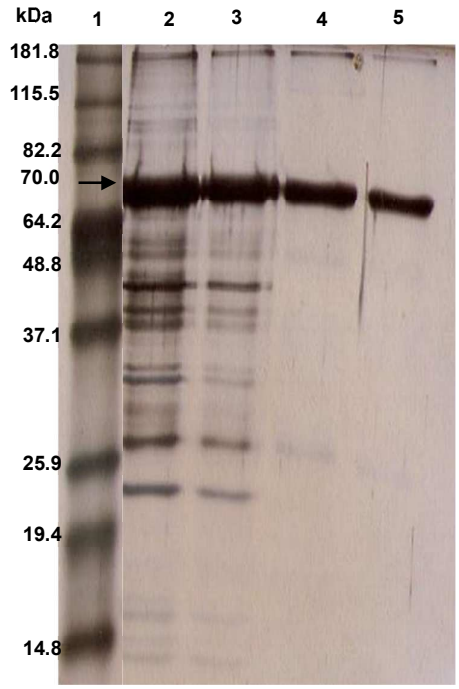


Figure 2

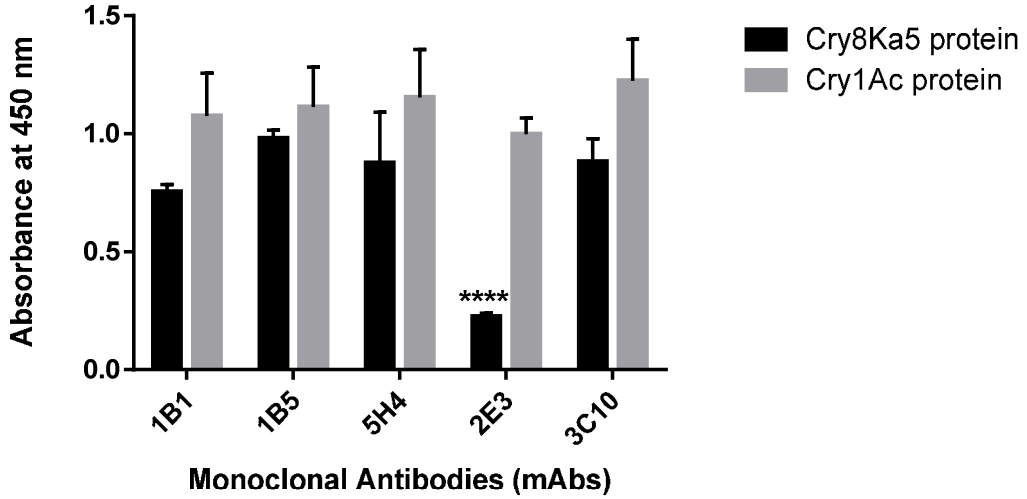


Figure 3

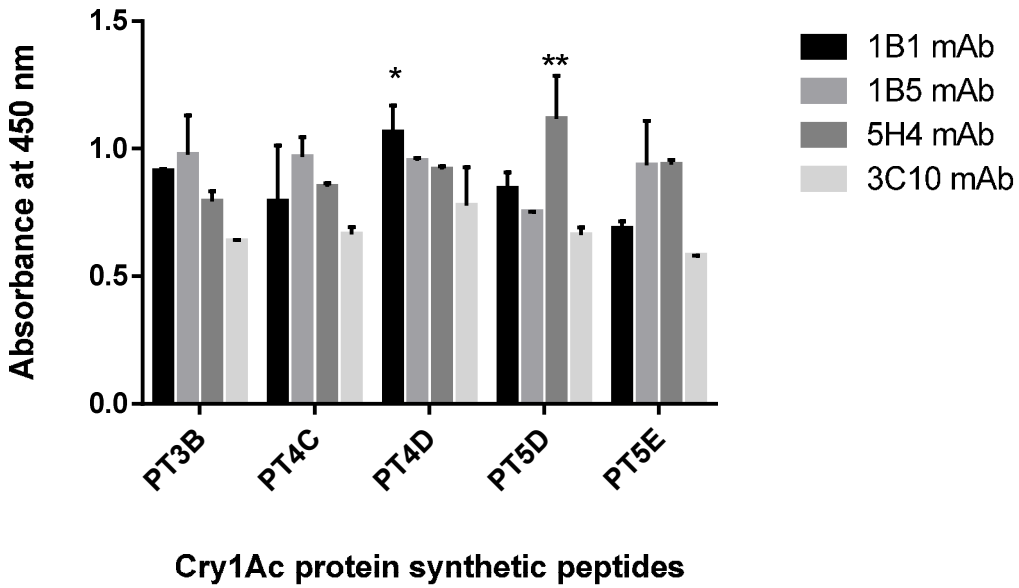


Figure 4

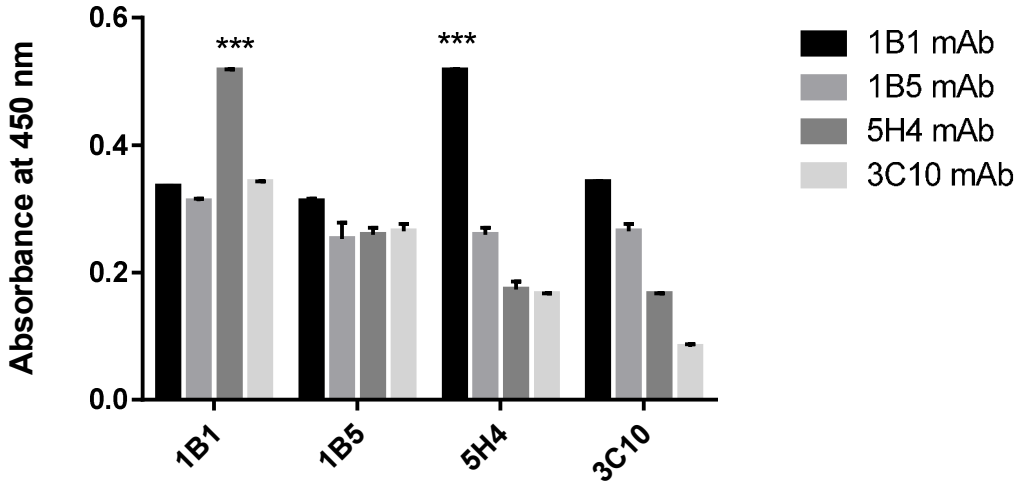


Figure 5

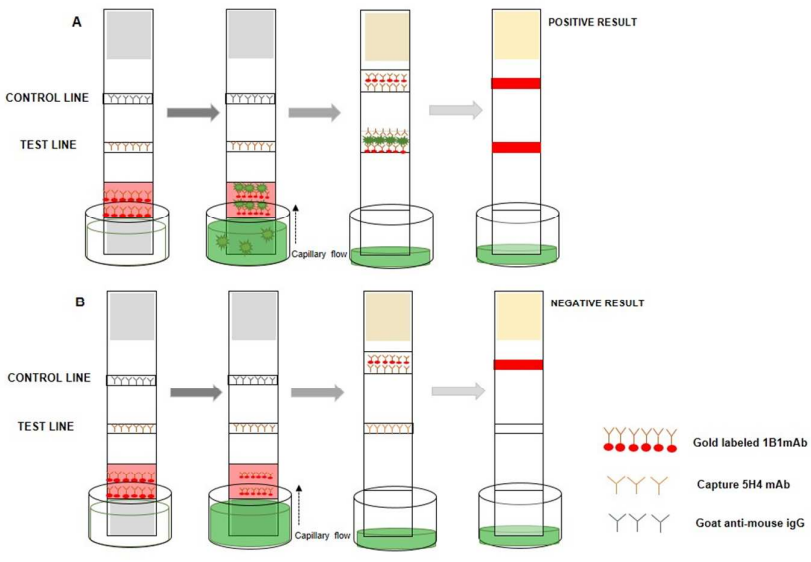
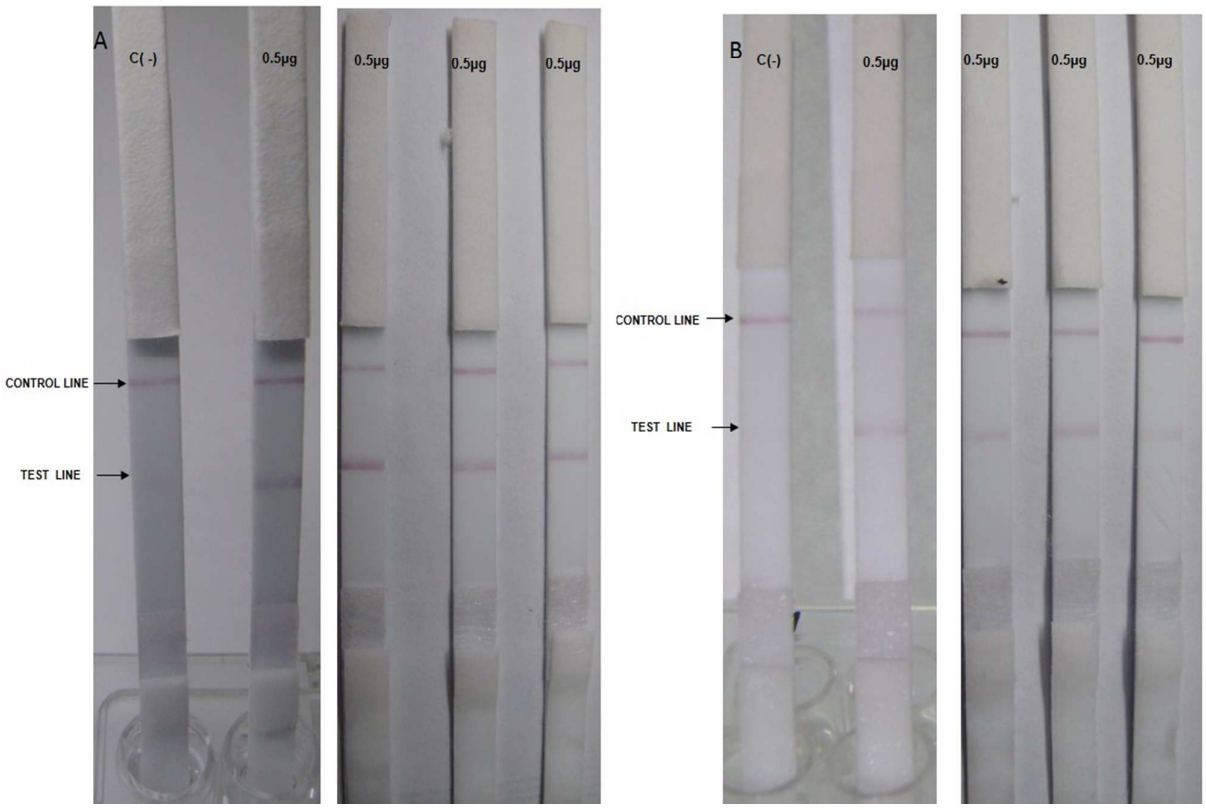


Figure 6

581



582

583

584

585

586

587

588

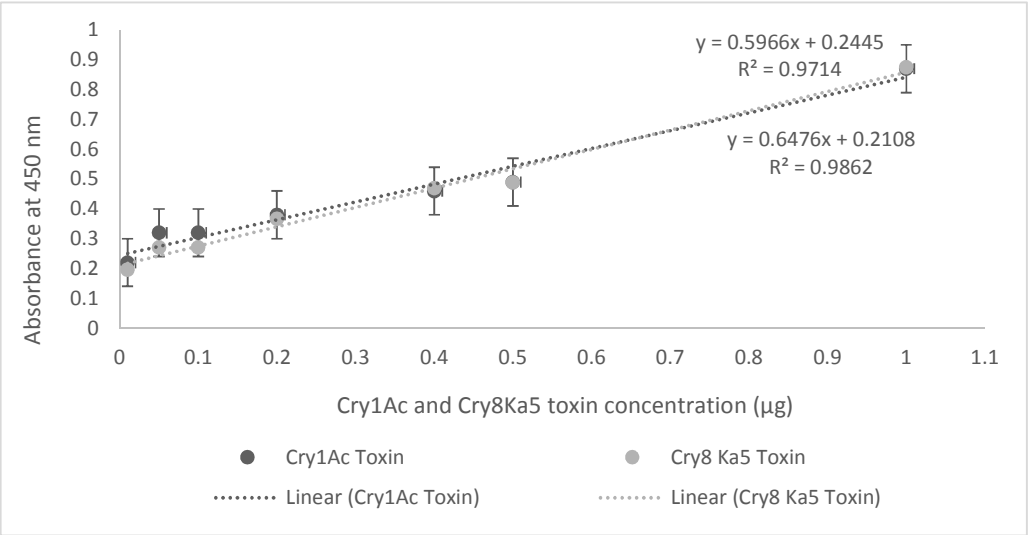
589

590

591

592 Figure 7

A



B

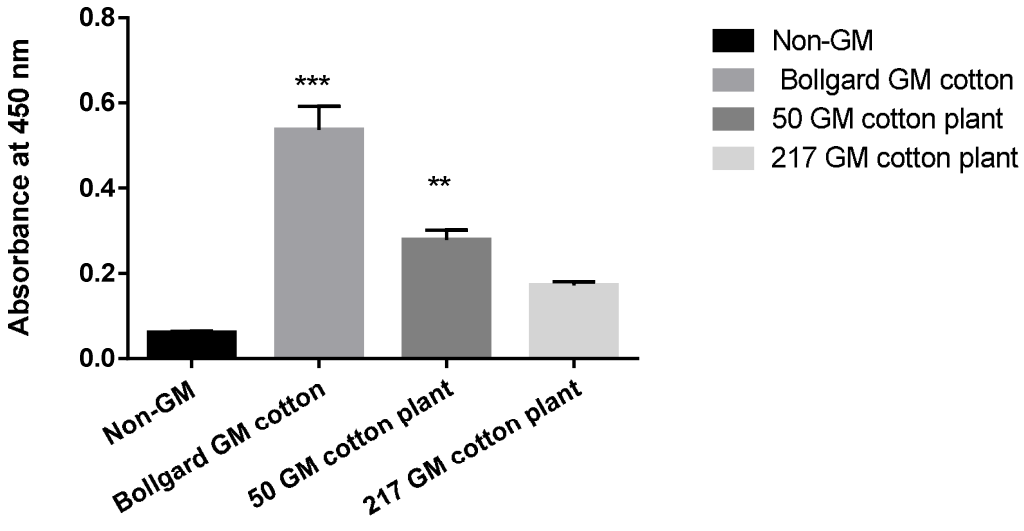
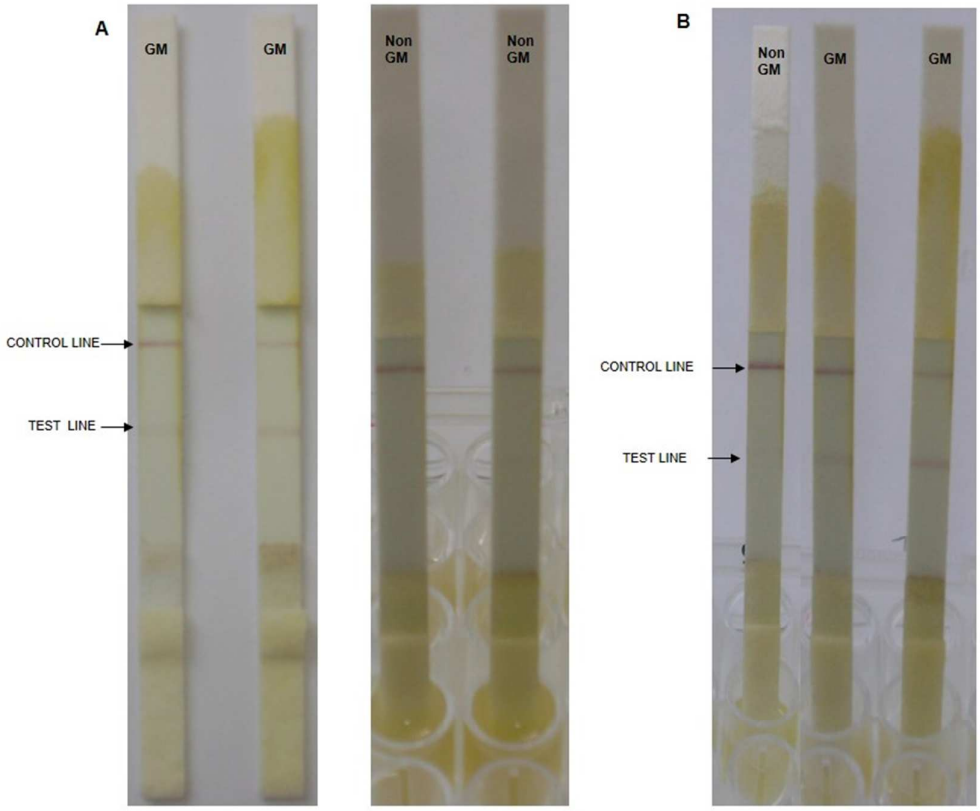


Figure 8



629

630

631

632

633

634

635

636

637

638

639 Table of contents