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(54) Title: REAGENTS, METHODS AND KITS FOR DETECTING *BACILLUS THURINGIENSIS* PROTEINS

(57) Abstract: Epitopes, antibodies, methods and kits for the detection of Bt proteins in a sample are provided. The proteins to be detected are the Bt proteins known to those skilled in the art as the Cry1 Ab and Cry1 Ac proteins. The epitopes are antigenic peptides of these Bt proteins and are immunoreactive with the monoclonal antibodies 113L2 and 113I1. The epitopes are isolated or synthesized and administered to animals to produce anti-Bt monoclonal and polyclonal antibodies having superior sensitivity for Bt proteins. The antibodies are useful in immunoassay methods for the detection of genetically modified organisms that have been engineered to include a Bt gene.

"REAGENTS, METHODS AND KITS FOR DETECTING *BACILLUS THURINGIENSIS* PROTEINS"

Field of the Invention

This relates to the field of immunology and more specifically relates to immunoassay methods, epitopes, and antibodies for the detection of *Bacillus thuringiensis* proteins.

Background of the Invention

Immunoassays are analytical methods that utilize immunologic techniques to detect chemicals or biomolecules in a sample. Immunoassays have been used to detect substances in many different kinds of samples in many different markets. Certain immunoassays have been developed to provide rapid, reliable, inexpensive tests that can be run by untrained individuals in the field, home, or other non-laboratory settings. One type of immunoassay, referred to as an immunochromatographic or lateral flow strip test, has been successfully developed into a 'one-step' test that is employed for on-site analyses. The over-the-counter home pregnancy test is an example of a simple, one-step immunochromatographic strip test.

Modern biotechnology methods are being used to genetically modify plants. These genetically modified plants, and the seeds, grain and food derived from them, all may contain novel recombinant proteins. For example, commercial crops (such as corn, cotton, soybean, canola, potatoes, and others) are being genetically modified so that they are resistant to insects and herbicides or have improved agronomic or nutritional characteristics. It has been estimated that approximately 50% of the U.S. soybean crop and 30% of the 1999 corn crop was genetically enhanced. At this time, the major genetic trait engineered into soybean is resistance to the ROUNDUP™ herbicide, and

the major trait engineered into corn is resistance to insects. Insect resistance has also been engineered into cotton and potatoes.

Resistance to insects in plants has been accomplished primarily by genetic engineering techniques in which specific genes isolated from the soil microorganism *Bacillus thuringiensis* (Bt) are introduced into the genome of the plant needing insect protection. Bt is a naturally occurring soil bacterium that produces a protein that is toxic to some insects. These bacterial genes are referred to by those skilled in the art as *cry* genes. The insertion of a *cry* gene into the genetic makeup of a plant protects against certain insects throughout the life of the plant. The *cry* genes produce Cry proteins, which are toxic to very specific classes of insect pests but not humans or other animals. The recombinant Cry proteins produced by the *cry* genes in plants include Cry1Ab, also referred to by those skilled in the art as either Novartis Bt11 (Novartis, Research Triangle Park, NC) or Monsanto Mon810 (Monsanto, St. Louis, MO), and Cry1Ac, also referred to by those skilled in the art as Dekalb DK493 (Dekalb Genetics Corporation, Dekalb, IL). Harmful insects, such as corn borers, that eat these genetically modified plants ingest the recombinant protein and die, thereby dramatically reducing the requirement for chemical insecticide applications. The overwhelming majority of the genetically modified corn on the market today contains a gene resulting in the production of the Bt Cry1Ab protein in the tissues of the plant including seed and grain. Food fractions prepared from such grain may also contain these recombinant proteins.

While the concentration of the Bt Cry proteins in these plants is sufficient to kill harmful insects, it is very low (parts per million to parts per billion) and therefore difficult to detect. However, it has become extremely important to determine the presence of such proteins in plants and food products for regulatory, environmental, safety, and world trade issues. Immense quantities of grain and seed are harvested, transported, mixed, stored, distributed and traded throughout the world. Therefore, it is important to have tests that can detect these recombinant proteins in order to address these issues. It is highly desirable to have a rapid, simple and inexpensive method that can be used to test grain samples at many points along the distribution channel including trucks, elevators, barges, ships, etc. A competitive or non-competitive immunoassay method utilizing a chromatographic strip test is ideally suited for this purpose.

Strip tests, also known as lateral flow devices, are comprised of multiple porous components, membranes and filters, through which liquid sample is drawn by capillary action. Analyte in the sample reacts with the test reagents contained within the test strip as it traverses the length of the strip. To detect an analyte (such as a genetically engineered protein or mycotoxin) in grain or seed (e.g., corn, soybean, rice, wheat, etc.), the grain is ground into a powder and the protein extracted from the powder with a liquid that is then separated from the solid material and assayed using the test. The liquid is applied to the chromatographic strip, and the analyte migrates toward the distal end of the strip. As it migrates down the strip, the analyte reacts with reagents applied to or immobilized on the strip causing a detectable signal product. Detection of the signal indicates the presence of the analyte in the sample.

Recently the European Union passed legislation requiring that all foodstuffs containing genetically modified organisms (GMO) over a certain threshold concentration be labeled as containing GMO. Japan, Brazil, Korea, New Zealand, Australia, and other countries have passed, or are considering, similar legislation. A key feature of this legislation is the detection of genetically modified ingredients, such as grain, in the presence of non-genetically modified grain at specified threshold concentrations ranging from 1 to 5%. The detection of low concentrations of recombinant protein in containers of bulk grain containing only a few percent of genetically modified grain requires the utilization of a very sensitive test method.

What is needed is a sensitive, simple, reliable and cost-effective method for determining the presence of Bt proteins, such as the Bt Cry1Ab or Bt Cry1Ac proteins, in genetically modified crops, seeds, grain and food fractions, that is capable of detecting the protein in commercial products at concentrations that are economically relevant and suitable for regulatory review purposes.

Summary of the Invention

Epitopes, antibodies, methods, and kits for the detection of *Bacillus thuringiensis* (Bt) proteins in a sample are provided. The preferred proteins to be detected are the Bt proteins known to those skilled in the art as the Cry1Ab and Cry1Ac proteins. The epitopes are antigenic peptides of these Bt proteins and are preferably immunoreactive with the monoclonal antibodies 113L2 and

113I8. The epitopes are isolated or synthesized and administered to animals to produce anti-Bt monoclonal and polyclonal antibodies having superior sensitivity for Bt proteins. The antibodies are useful in immunoassay methods for the detection of genetically modified organisms that have been engineered to include a Bt gene. The preferred antibodies are the monoclonal antibodies 113L2 and 113I8.

The methods are immunoassays, preferably ELISA or lateral flow device (LFD) assays, employing antibodies described herein and are capable of detecting low concentrations of Bt protein in genetically enhanced crop samples. The antibodies are immunoreactive with epitopes, preferably the Cry1Ab or Cry1Ac protein epitopes, or a common epitope, preferably on both the Cry1Ab and Cry1Ac proteins, and react minimally with other proteins that may be present in the sample, thus providing for an accurate determination of the presence of a genetically modified organism in a sample, such as a grain sample.

The epitopes, antibodies, or both, are collectively assembled in a kit with conventional immunoassay reagents for detection of Bt protein. The kit may optionally contain both monoclonal and polyclonal antibodies and a standard for the determination of the presence of Bt protein in a sample.

It is therefore an object of the present invention to provide epitopes, antibodies, immunoassay methods, and kits for the detection of Bt protein in a sample, particularly an agricultural sample.

It is a further object of the present invention to provide a highly sensitive immunoassay for Bt protein.

It is a further object of the present invention to provide an epitope for the production of antibodies highly specific for Bt protein.

It is a further object of the present invention to provide high affinity antibodies for the Bt proteins Cry1Ab and Cry1Ac that exhibit minimal crossreactivity with other proteins.

These and other objects of the present invention will become apparent after reading the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Drawings

Figure 1 is a graph comparing the monoclonal antibodies 87Ab1.1, 113I8, 113L2, 113L10, 113L11, 113L12, 113L13 as capture reagents in the

Cry1Ab enzyme immunoassay described herein in combination with the polyclonal antibody R350-351.

Figure 2 is a graph and table comparing the Bt11 extract, Mon810 extract, Non-genetically modified (Non-GMO), and buffer using the monoclonal antibodies of Figure 1 with R350-351 at 100ng/ml.

Figure 3 is a graph showing signal-to-noise ratio of GMO corn RAC extract by ELISA.

Figure 4 is a graph showing the immunological reactivities of GMO corn RAC extract by ELISA.

Figure 5 is a graph showing optical density versus % MON810 corn seed for detection of transgenic MON810 corn seed by ELISA.

Figure 6 is a graph showing evaluation of antibody-gold preparations with extract of MON810 (Cry1Ab).

Figure 7 is a graph showing evaluation of antibody-gold preparations with extract of DK493 corn (Cry1Ac).

Figure 8 is a graph and table showing the direct bind on Cry1A(c) protein of various monoclonal antibodies.

Figure 9 is a graph and table showing the direct bind on the Cry1A(b) protein of various monoclonal antibodies.

Figure 10 is a graph and table showing the results of an epitope mapping experiment with various monoclonal antibodies.

Detailed Description of the Disclosed Embodiments

Epitopes, antibodies, methods, and kits for the detection of *Bacillus thuringiensis* (Bt) proteins in a sample are provided. The preferred proteins to be detected are the Bt proteins known to those skilled in the art as the Cry1Ab and Cry1Ac proteins. The epitopes are antigenic portions or peptides of the Bt proteins, preferably of the Cry1Ab or Cry1Ac Bt proteins or both the Cry1Ab or Cry1Ac Bt proteins, and are preferably immunoreactive with the monoclonal antibodies 113L2 and 113I8, produced by hybridomas which have been deposited with the American Type Culture Collection, Rockville, MD.

The hybridoma (113L2.1) producing monoclonal antibody 113L2 was deposited as ATCC Patent Deposit Designation PTA-1052 on December 14, 1999. The hybridoma (113I8.1) producing monoclonal antibody 113I8 was deposited as ATCC Patent Deposit Designation PTA-1063 on December 15, 1999. Anti-Bt monoclonal and polyclonal antibodies having similar or

superior sensitivity for Bt proteins are produced by the immunization of an animal with Bt proteins, such as Cry1Ab and Cry1Ac, isolation of antibodies that react with the epitopes, and the collection and purification of the antibodies from a biological fluid such as blood in accordance with methods well known to those skilled in the art.

Immunoassay methods containing the antibodies immunoreactive with the epitopes are useful for the detection of genetically modified organisms that have been engineered to include a Bt gene. The immunoassays are capable of detecting low concentrations of Bt protein in genetically enhanced crop samples. The antibodies are immunoreactive with epitopes, preferably Cry1Ab or Cry1Ac epitopes, or a common epitope, such as on both the Cry1Ab and Cry1Ac proteins, and react minimally with other proteins that may be present in the sample, thus providing for an accurate determination of the presence of a genetically modified organism in a sample, such as a grain sample. The preferred antibodies are monoclonal antibodies 113L2 and 113I8, produced by hybridomas which were deposited with the American Type Culture Collection as described above. A second preferred antibody is monoclonal antibody 87AB1, which is specifically immunoreactive with Cry1Ab, but is not immunoreactive with Cry1Ac as described in more detail below. Monoclonal antibody 87AB1 is produced by hybridoma 87AB1.1, which was deposited with the ATCC as ATCC Patent Deposit Designation PTA-1051 on December 14, 1999.

The antibodies are collectively assembled in a kit with conventional immunoassay reagents for detection of Bt protein. The kit may optionally contain both monoclonal and polyclonal antibodies and a standard for the determination of the presence of Bt protein in a sample. The kit containing these reagents provides for simple, rapid, on site detection of Bt protein.

During the development of a strip test to detect the Bt Cry1Ab protein in genetically enhanced crops (such as corn and cotton), great difficulty was encountered achieving the sensitivity and time-to-result specifications that were required of the commercial product. Many different monoclonal antibodies were evaluated and several candidates were identified, including monoclonal antibodies 113L2 and 113I8, that had superior sensitivity when compared to other antibodies (87AB1, 87AI9 and 113L11). During the course of the test development program, these antibodies were tested for crossreactivity to a structurally related Bt protein, referred to those skilled in the art as Cry1Ac. Cry1Ac has also been introduced into corn and cotton

plants using genetic engineering techniques. Therefore, it is important to know whether a test product has the ability to detect this protein. Surprisingly, all of the high sensitivity Cry1Ab antibodies (113L2 and 113I8) crossreacted significantly with Cry1Ac, while the low sensitivity Cry1Ab antibodies (87AB1, 87AI9 and 113L11) did not. The epitopes recognized by these classes of antibodies were demonstrated to be spatially distinct by virtue of antibody competition analysis (epitope mapping). It was further demonstrated that these antibodies have superior sensitivity in multiple immunoassay formats (ELISA and strip test, or lateral flow device).

Taken together, these results demonstrate that an epitope or epitopes, defined as immunoreactive with monoclonal antibodies 113L2 and 113I8, are present on both Cry1Ab and Cry1Ac proteins that elicit high sensitivity antibodies required for detection of low concentrations of these proteins in genetically engineered crop tissues, such as, but not limited to, leaf, stem, seed, stalk, root, and the like, or products derived from such crops, such as food fractions. The epitopes are useful for producing antibodies, tests and kits having the superior sensitivity required of successful commercial products.

Antibodies

Epitopes having the characteristics set forth above are used for the production of both monoclonal or polyclonal antibodies reactive toward Bt protein. The preferred antibody is a monoclonal antibody, due to its higher specificity for analyte.

Monoclonal antibodies are generated by methods well known to those skilled in the art. The preferred method is a modified version of the method of Kearney, *et al.*, *J. Immunol.* 123:1548-1558 (1979). Briefly, animals such as mice or rabbits are inoculated with the immunogen in adjuvant, and spleen cells are harvested and mixed with a myeloma cell line, such as P3X63Ag8,653. The cells are induced to fuse by the addition of polyethylene glycol. Hybridomas are chemically selected by plating the cells in a selection medium containing hypoxanthine, aminopterin and thymidine (HAT). Hybridomas are subsequently screened for the ability to produce anti-Bt monoclonal antibodies. Hybridomas producing antibodies are cloned, expanded and stored frozen for future production.

The antibody may be labeled directly with a detectable label for identification and quantitation of Bt protein. Labels for use in immunoassays are generally known to those skilled in the art and include enzymes,

radioisotopes, and fluorescent, luminescent and chromogenic substances including colored particles such as colloidal gold and latex beads.

Alternatively, the antibody may be labeled indirectly by reaction with labeled substances that have an affinity for immunoglobulin, such as protein A or G or second antibodies. The antibody may be conjugated with a second substance and detected with a labeled third substance having an affinity for the second substance conjugated to the antibody. For example, the antibody may be conjugated to biotin and the antibody-biotin conjugate detected using labeled avidin or streptavidin. Similarly, the antibody may be conjugated to a hapten and the antibody-hapten conjugate detected using labeled anti-hapten antibody. These and other methods of labeling antibodies and assay conjugates are well known to those skilled in the art.

Immunoassays

A highly sensitive immunoassay employing the antibodies prepared from the epitopes described above is provided. The preferred immunoassays are ELISA assays and strip test or lateral flow device (LFD) assays.

The immunoassay is useful for detecting the presence or amount of Bt in a variety of samples, particularly agricultural samples such as plant material, particularly agricultural samples. The sample may be obtained from any source in which the Bt proteins are accessible to the antibody. For example, the sample may be any plant tissue or extract including root, stem, stalk, leaf, or seed or products derived from such crops, such as food fractions. Preferably the sample is dried, ground, or powdered prior to analysis.

The antibody and assay conjugates may be employed in any heterogeneous or homogeneous, sandwich or competitive immunoassay for the detection of Bt protein. Either the antibody is labeled with a detectable label or coupled to a solid phase. Methods for coupling antibodies to solid phases are well known to those skilled in the art. In accordance with the immunoassay method, the sample containing the analyte is reacted with the antibody for a sufficient amount of time under conditions that promote the binding of antibody to Bt protein in the sample. It will be understood by those skilled in the art that the immunoassay reagents and sample may be reacted in different combinations and orders. A physical means is employed to separate reagents bound to the solid phase from unbound reagents such as filtration of particles, decantation of reaction solutions from coated tubes or wells, magnetic separation, capillary action, and other means known to those skilled

in the art. It will also be understood that a separate washing of the solid phase may be included in the method.

The concentration of Bt protein in the sample is determined either by comparing the intensity of the color produced by the sample to a color card or by using a reflectometer.

The resulting reaction mixture, or combination of antibody and sample, is prepared in a solution that optimizes antibody-analyte binding kinetics. An appropriate solution is an aqueous solution or buffer. The solution is preferably provided under conditions that will promote specific binding, minimize nonspecific binding, solubilize analyte, stabilize and preserve reagent reactivity, and may contain buffers, detergents, solvents, salts, chelators, proteins, polymers, carbohydrates, sugars, and other substances known to those skilled in the art.

The reaction mixture solution is reacted for a sufficient amount of time to allow the antibody to react and bind to the analyte to form an antibody-analyte complex. The shortest amount of reaction time that results in binding is desired to minimize the time required to complete the assay. An appropriate reaction time period for an immunochromatographic strip test is less than or equal to 20 minutes or between approximately one minute and 20 minutes. A reaction time of less than five minutes is preferred. Most preferably, the reaction time is less than three minutes. By optimizing the reagents, binding may be substantially completed as the reagents are combined.

The reaction is performed at any temperature at which the reagents do not degrade or become inactivated. A temperature between approximately 4°C and 37°C is preferred. The most preferred reaction temperature is ambient or room temperature (approximately 25°C).

Immunoassay Kit

An immunoassay kit for the detection of Bt protein in a sample preferably contains one or more antibodies prepared using the epitopes described above. The antibodies may be immobilized on a chromatographic test strip or LFD or contained in an assortment of reagent containers in lyophilized or solubilized form.

The kit may additionally contain equipment for obtaining the sample, a vessel for containing the reagents, a timing means, a buffer for diluting the sample, and a colorimeter, reflectometer, or standard against which a color change may be measured.

In a preferred embodiment, the reagents, including the antibody are added to a chromatographic strip and dried. Addition of aqueous sample to the strip results in solubilization of the dried reagents, causing them to react as the sample diffuses and migrates down the strip.

The epitope, antibodies, immunoassay methods, and kits described above will be further understood with reference to the following non-limiting examples.

Example 1: Assay for Cry1Ab in Microtiter Plate Format Using Monoclonal Antibodies

An immunoassay was performed for the detection of Cry1Ab as follows:

Plate coating procedure

Monoclonal antibodies isolated from mice immunized with Cry1Ab protein were prepared at 2.5 µg/ml in phosphate buffered saline (PBS) for coating according to Table 1 below. An aliquot of 100 µl per well was added to Nunc MAXISORP™ wells (C12), sealed with plate sealer, and incubated overnight at 4°C.

Table 1

Anti-Cry1Ab Monoclonal Antibodies	concentration mg/ml	µl for 12 ml at 2.5 µg/ml
113L2	0.543	0.055
113L10	0.386	0.078
113L11	0.751	0.040
113L12	0.625	0.048
113L13	1.060	0.028
113I8	0.391	0.077

The following day, the contents of the wells were discarded and blocked with 1% bovine serum albumin (BSA) in PBS with 0.1% Tween 20.

Samples

Leaf Cry1Ab positive samples (Novartis, Research Triangle Park, NC) were prepared by grinding 500 mg of leaf in mortar with a pestle, then adding 10 ml of TRAITCHECK™ buffer (Strategic Diagnostics, Inc., Newark, DE). The sample was spun in microfuge tubes to clear (15K for 5 minutes).

Extracts of BT11, Mon810, and non-GMO were obtained from Strategic Diagnostics, Inc. personnel.

Procedure

Wells were washed three times with plate washer.

100 µl of sample were added to wells and incubated 1 hour at 37°C.

Wells were washed six times with plate washer.

100 µl of polyclonal anti-Cry1Ab was added at 1000, 333, or 100 ng/ml in BSA blocking buffer.

R350-351 (SDI)

Rabbit #1 (853)

Rabbit #2 (854)

Rabbit #3 (855)

Reactants were incubated 1 hour at 37°C and washed six times with plate washer.

100 µl per well of horse radish peroxidase (HRP) Mouse anti-rabbit (Jackson) at 1/4000 in BSA blocking buffer was added.

Plates were washed six times with plate washer.

Tetramethylbenzidine (TMB, KPL) was added and plates read at 650 nm after 20 minutes.

The results are shown in Figures 1 and 2.

Conclusion

The monoclonal antibodies 113L2, 113L13 and 113I8 all provide significantly better results than 87Ab1.1 with both leaf extracts as well as corn extracts.

Example 2: Analysis of GMO Corn Using ELISA

An enzyme linked immunoassay was used to analyze a corn sample for the presence of genetically modified organism (GMO) corn.

GMO Sample (Mon810 or Bt11) Preparation

1. Create a desirable percentage of GMO to non-GMO using kernel to kernel ratios:

0% GMO = 200 non-GMO

0.5%GMO = 1 GMO + 199-nonGMO

1% GMO = 2 GMO + 198 non-GMO

2% GMO = 4 GMO + 196 non-GMO

4% GMO = 8 GMO + 192 non-GMO

8% GMO = 16 GMO + 184 non-GMO

16% GMO = 32 GMO + 168 non-GMO

32% GMO = 64 GMO + 136 non-GMO

2. Add samples to Mason jars and grind using a Waring blender. A fine powder is obtained by further grinding with a coffee mill.

3. From each percentage to be tested, add 0.4 gram of the powder to a 2 mL microcentrifuge vial. Then transfer 1 mL of 10 mM PBS-0.05% Tween 20 buffer (PBST) (Ph7.2) to the vial and vortex vigorously for approximately 20 seconds.

4. Let vial incubate at room temperature for five minutes and centrifuge at 5,000 rpm for five minutes.

Microtiter plate Preparation

1. Add 100 µL of 3 µg/mL of 87Ab1.1 or L-2 monoclonal antibody in 50 mM sodium carbonate coating buffer (pH 9.6) to each well of microtiter plate.

2. Incubate microtiter plate overnight at 4°C.

3. Pour out coating solution and block each well of microtiter plate with 200 µL of blocking solution [10 mM Tris buffer containing 0.02% (w/v) sodium caseinate, 5% (w/v) sucrose; pH 8.3].

4. Incubate microtiter plate at 37°C for two hours.

5. Pour out blocking solution and blot remaining liquids from microtiter plate with dry paper towel.

6. Allow microtiter plate to stand in dry room overnight.

Assay Procedure

1. Pipette 100 µL of supernatant from microcentrifuge vial and deliver to sample well of microtiter plate.

2. Incubate microtiter plate at room temperature for 15 minutes.

3. Aspirate and wash microtiter plate two times each way (with reverse direction).

4. Pipette 100 µL of MR122-biotin conjugate (1:3200 dilution in PBST) to each sample well of microtiter plate and allow incubation to proceed at room temperature for 15 minutes.

5. Aspirate and wash microtiter plate two times each way (with reverse direction).

6. Add 100 µL of streptavidin HRP conjugate (1:64000 dilution in PBST) to sample well of microtiter plate and incubate at room temperature for 15 minutes.

7. Aspirate and wash microtiter plate two times each way (with reverse direction).

8. Add 100 µL of TMB substrate to each well of microtiter plate and allow color reaction to proceed at room temperature for 20 minutes.

9. Stop the reaction with 100 µL of stop solution [0.5% (v/v) sulfuric acid].

10. Read the optical density (O.D.) of microtiter plate at 450 nm with subtraction of 650 nm.

The results are shown in Figures 3 and 4.

The results of the detection of transgenic MON810 corn seed by ELISA using the monoclonal antibodies 87AI9 and 87AB1.1 are shown in Figure 5

Example 3: Analysis of GMO Corn Using Strip Test

An immunochromatographic strip test was used to analyze a corn sample for the presence of genetically modified organism (GMO) corn.

Procedure

Extracts of corn were prepared by grinding 39 grams of corn to a fine powder. 10 grams of powder was added to a 50 ml centrifuge tube along with 40 ml of TRAITCHECK™ buffer (0.1% Tween, 0.1 M phosphate, pH 7.4, Strategic Diagnostics, Inc., Newark, DE) and shaken for 15 minutes at room temperature. Large particulates were removed by centrifugation at 3000 x g for 10 minutes and the supernatant removed for assay. Extracts were further diluted as indicated in TRAITCHECK™ buffer for assay.

Corn samples for this test consisted of Novartis Bt11 (Cry1Ab), Monsanto Mon810 (Cry1Ab), and Dekalb DK493 (Cry1Ac) (Dekalb Genetics Corporation, Dekalb, IL).

Assay

Three centimeter wide by 35 cm long nitrocellulose strips (Millipore SXHF) were sprayed with rabbit polyclonal anti-Cry1Ab at 2 µg/cm at a distance of 1.25 mm from the bottom of the strip. Strips were mounted onto plastic backing with a wicking pad positioned on one edge and cut into 5.5 mm wide pieces.

Colloidal gold particles were prepared by adding 2.5 µg of antibody for each to 1 OD_{520} of 40 nm colloidal gold (British Biocell International). After a 10 minute incubation, the gold was stabilized by the addition of bovine serum albumin and excess non-bound antibody removed by washing by centrifugation.

100 µL of dilutions of each extract were placed in wells of 48 well plates. 20 µL of colloidal gold at 2.0 OD_{520} was added to each well, quickly

mixed and one of the anti-Cry1Ab nitrocellulose strips added to each well. Solutions were allowed to wick up the strips for 10 minutes at which time the strips were removed and scored for color intensity relative to gradations of red on a color card.

The results are shown in Figures 6 and 7.

Example 4: Direct Bind Titration of Monoclonal Antibodies

An experiment was performed for the direct bind titration of monoclonal antibodies to Cry1Ab over various antigens.

Antigens

1. Novartis Cry1Ab 15Feb99 2.27 mg/mL (J. Stein)
2. Novartis Cry1Ab 23Jan99 2.0 mg/mL (M. Yarnell)
3. Mon Cry1Ab (Tryptic Digest) 1.8 mg/mL
4. Mon HD73 (Cry1Ac) 1.14 mg/mL
5. Cry1Ab 33680 23Jan98 9.89 mg/mL
6. Bt Cry1Ab CBI-03 02/02/99 0.55 mg/mL

1. Two plates were coated with each antigen at 1.0 μg/mL on 0.1 M Carb pH 9.6 for one hour. Dump contents.

2. Block one hour with 200 μL PCT (PBS, 1% casein, pH 7.5), wash two by three times with PT (PBS, 0.05% Tween 20, pH 7.5).

3. Titrate monoclonal antibodies on plates with each coating antigen. Incubate one hour at 37°C. Titer in PCT. Wash as above.

4. Add 1:3000 dilution Ra-anti-Ms LN:90547008R in PCT to monoclonal antibody plates. Incubate 1 hour at 37°C or over night at 4°C. Wash two by three times with PT.

5. Add 100 μL/well tetramethylbenzidine; incubate until sufficient color, read at OD_{650}.

The results are shown in Figures 8 and 9.

Example 5: Cry1Ab Epitope Mapping

An experiment was performed to map the Cry1Ab epitope.

1. Coat two NUNC MAXISORP™ plates at 5 μg/mL (100 μL/well) PAb R350-351 in 0.1 M carbonate. Incubate one hour at 37°C.

2. Dump plates and pat dry.

3. Block with PCT (PBS, 1% casein, pH 7.5).

4. Incubate 30 minutes or ore at 37°C. Wash three times with PT (PBS, 0.05% Tween 20, pH 7.5)

5. Add 100 μL/well of Bt11 corn seed extract at 1:100 dilution in PCT.

6. Incubate one hour at 37°C. Wash.

7. Titrate monoclonal antibodies down plates at μL/well (starting concentration 20 μg/mL) and 1:3 down in PCT.

8. Incubate one hour at 37°C. Wash.

9. Add 0.2 μg/mL dilution of 87AI9-Biotin Conjugate at 100 μg/well in PCT.

10. Incubate one hour at 37°C. Wash.

11. Add 1:2000 dilution of Streptavidin-horse radish peroxidase conjugate in PCT.

12. Incubate one hour at 37°C. Wash.

13. Add 100 μg/well of tetramethylbenzidine.

The results are shown in Figure 10.

All references cited herein are hereby incorporated by reference.

Modifications and variations of the present epitopes, antibodies, methods and kits for detecting Bt protein will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

What is claimed is:

1. A method for detecting a *Bacillus thuringiensis* (Bt) protein in a genetically modified sample comprising reacting the sample with an antibody immunoreactive with an antigenic Bt epitope and detecting the binding of the antibody to the epitope.

2. The method of Claim 1 wherein the method is selected from the group consisting of an ELISA assay and a lateral flow device assay.

3. The method of Claim 1 wherein the sample is a genetically modified agricultural product.

4. The method of Claim 1 wherein the sample is selected from the group consisting of a leaf, stem, stalk, and seed.

5. The method of Claim 1 wherein the Bt protein is selected from the group consisting of a Cry1Ab protein and a Cry1Ac protein.

6. The method of Claim 1 wherein the antibody is a monoclonal antibody selected from the group consisting of 113L2, 113I8, and 87AB1.

7. An antibody for the detection of a *Bacillus thuringiensis* (Bt) protein, wherein the antibody is immunoreactive with an antigenic epitope of the Bt protein.

8. The antibody of Claim 7 wherein the Bt protein is selected from the group consisting of a Cry1Ab protein and a Cry1Ac protein.

9. The antibody of Claim 7 wherein the antibody is specifically immunoreactive with an epitope common to both a Cry1Ab and a Cry1Ac Bt protein.

10. The antibody of Claim 9 wherein the antibody is a monoclonal antibody selected from the group consisting of 113L2 and 113I8.

11. The antibody of Claim 7 wherein the antibody is immunoreactive with a Cry1Ab Bt protein.

12. The antibody of Claim 11 wherein the antibody is a monoclonal antibody designated 87AB1.

13. An isolated epitope of a *Bacillus thuringiensis* (Bt) protein wherein the epitope is common to both a Cry1Ab and a Cry1Ac Bt protein.

14. The epitope of Claim 13 wherein the epitope is immunoreactive with a monoclonal antibody selected from the group consisting of 113L2 and 113I8.

15. An isolated epitope of a *Bacillus thuringiensis* (Bt) protein wherein the epitope is present on a Cry1Ab protein, but is absent on a Cry1Ac protein.

16. The epitope of Claim 15 wherein the epitope is immunoreactive with a monoclonal antibody designated 87AB1.

17. A kit for detecting a recombinant *Bacillus thuringiensis* (Bt) protein in a genetically modified agricultural sample comprising a buffer and an antibody, wherein the antibody is immunoreactive with an antigenic epitope of the Bt protein.

18. The kit of Claim 17 further comprising a chromatographic test strip, wherein the antibody is immobilized on the test strip.

19. The kit of Claim 17 wherein the Bt protein is selected from the group consisting of a Cry1Ab protein and a Cry1Ac protein.

20. The kit of Claim 17 wherein the antibody is a monoclonal antibody selected from the group consisting of 113L2, 113I8, and 87AB1.

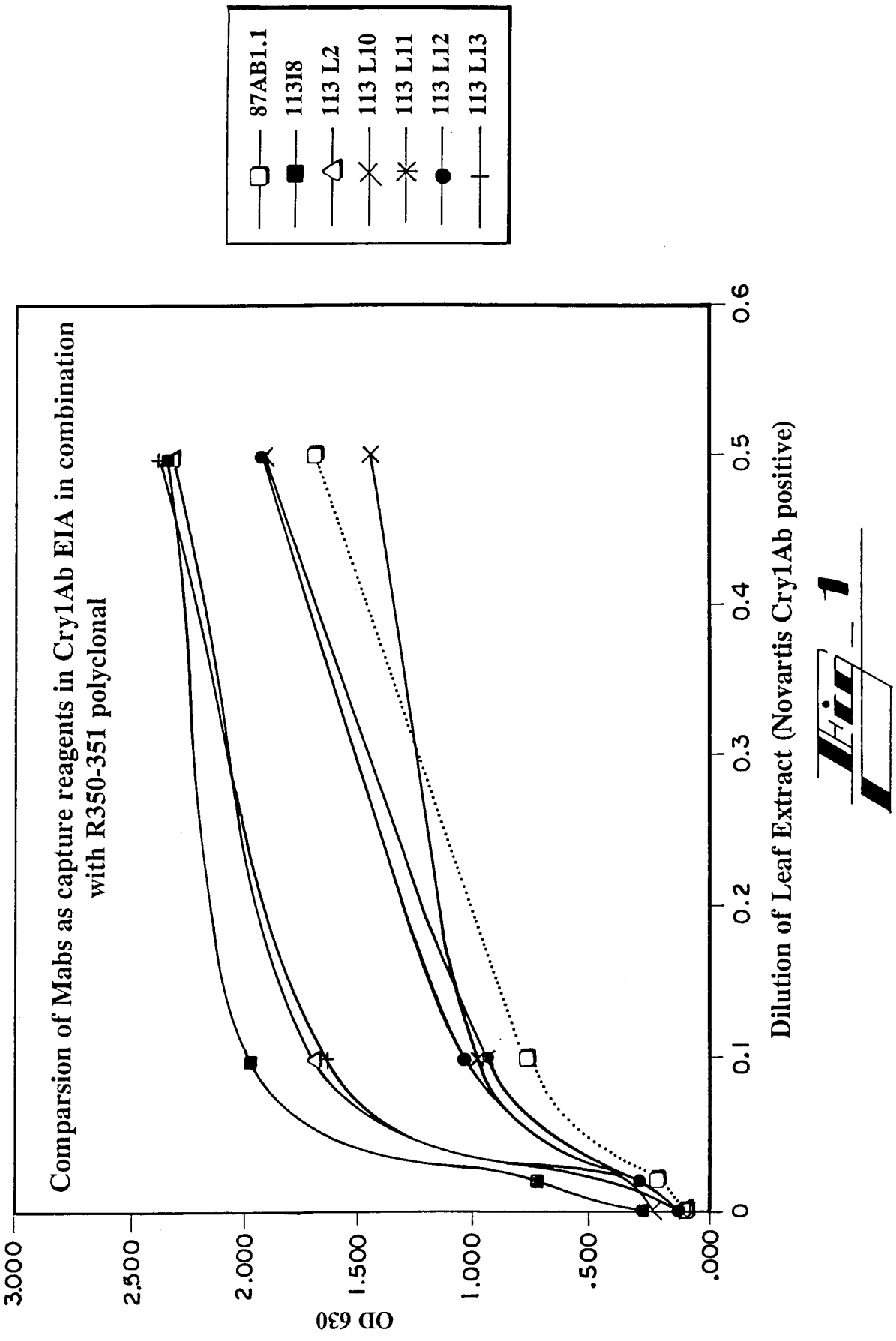
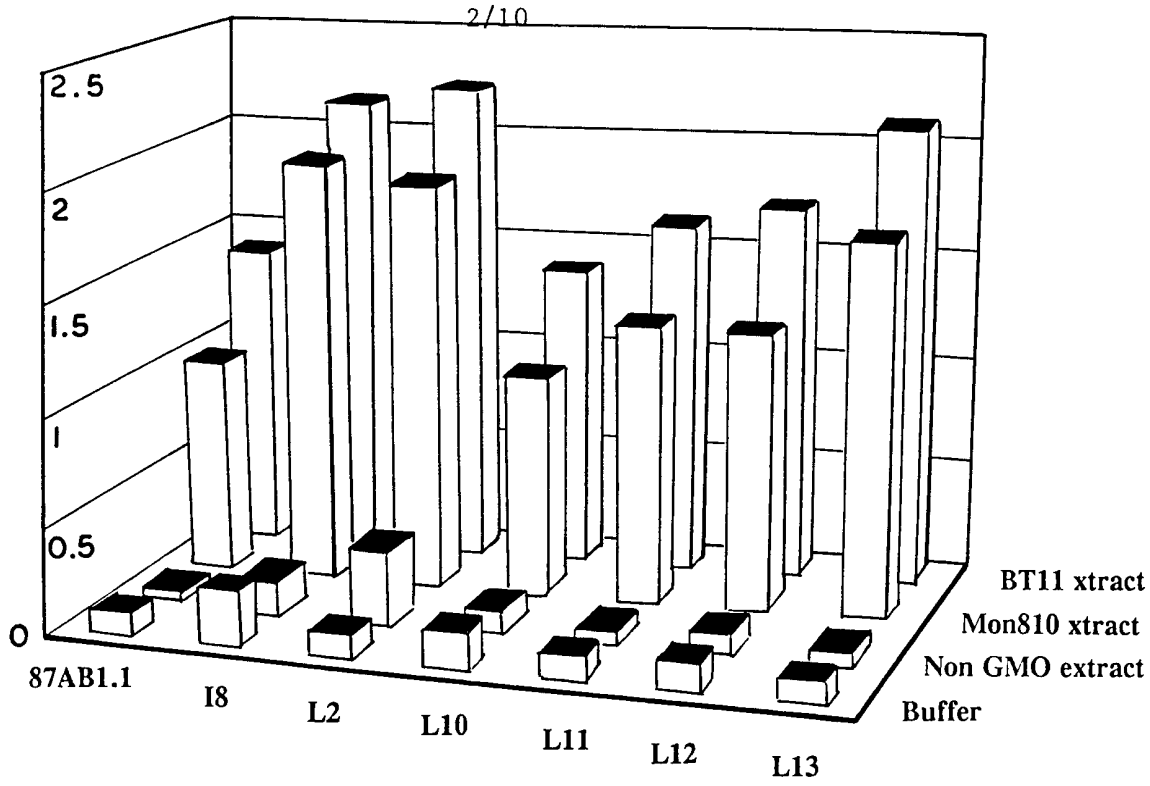

87AB1.1
113I8
113 L2
113 L10
113 L11
113 L12
113 L13
Comparsion of Mabs as capture reagents in Cry1Ab EIA in combination with R350-351 polyclonal
OD 630
3.000
2.500
2.000
1.500
1.000
.500
.000
0
0.1
0.2
0.3
0.4
0.5
0.6
Dilution of Leaf Extract (Novartis Cry1Ab positive)

Fig. 1



Comparison of extracts using monoclonal capture along with R350-351 at 100 ng/ml

Extract	87AB1.1	I8	L2	L10	L11	L12	L13
Mon810 xtract	0.959	1.958	1.903	1.036	1.295	1.290	1.717
BT11Xtract	1.362	2.151	2.237	1.403	1.633	1.723	2.111
Non GMO extract	0.081	0.178	0.353	0.123	0.094	0.101	0.066
Buffer	0.121	0.275	0.130	0.184	0.121	0.143	0.102

Fig. 2

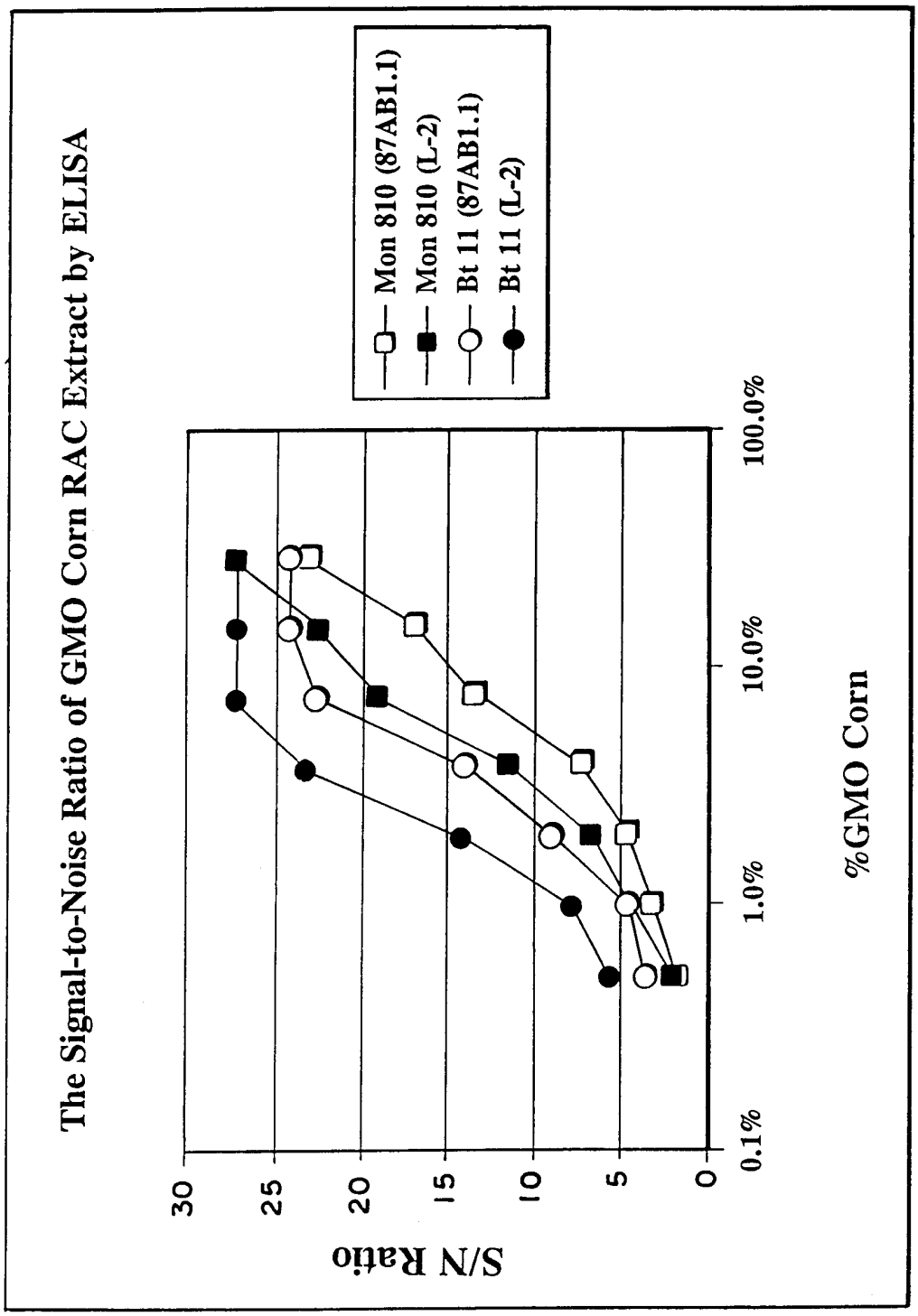

The Signal-to-Noise Ratio of GMO Corn RAC Extract by ELISA
Mon 810 (87AB1.1)
Mon 810 (L-2)
Bt 11 (87AB1.1)
Bt 11 (L-2)
S/N Ratio
30
25
20
15
10
5
0
0.1%
1.0%
10.0%
100.0%
%GMO Corn

Fig. 3

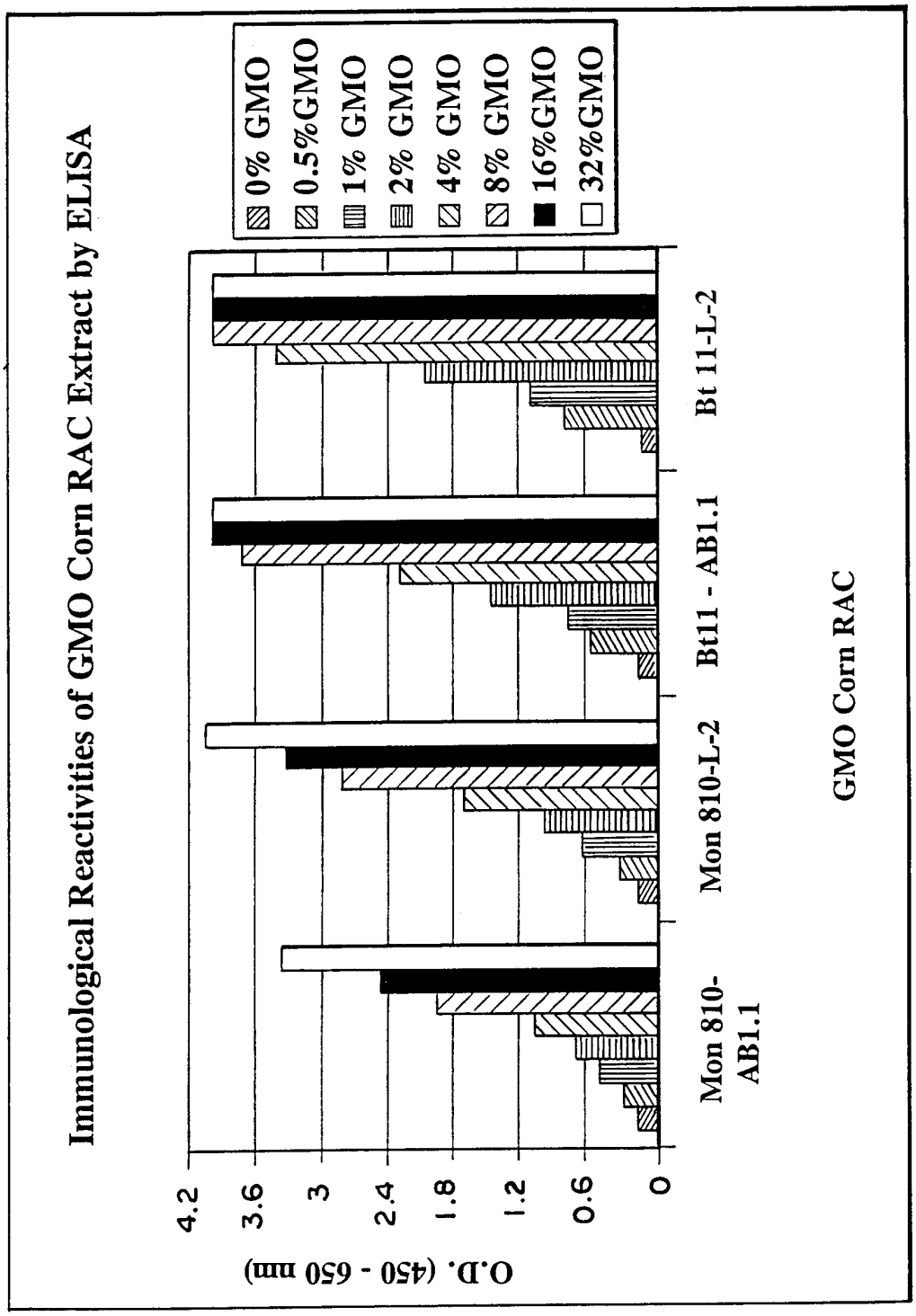

Immunological Reactivities of GMO Corn RAC Extract by ELISA
0% GMO
0.5%GMO
1% GMO
2% GMO
4% GMO
8% GMO
16%GMO
32%GMO
O.D. (450 - 650 nm)
4.2
3.6
3
2.4
1.8
1.2
0.6
0
Mon 810-AB1.1
Mon 810-L-2
Bt11 - AB1.1
Bt 11-L-2
GMO Corn RAC

Fig. 4

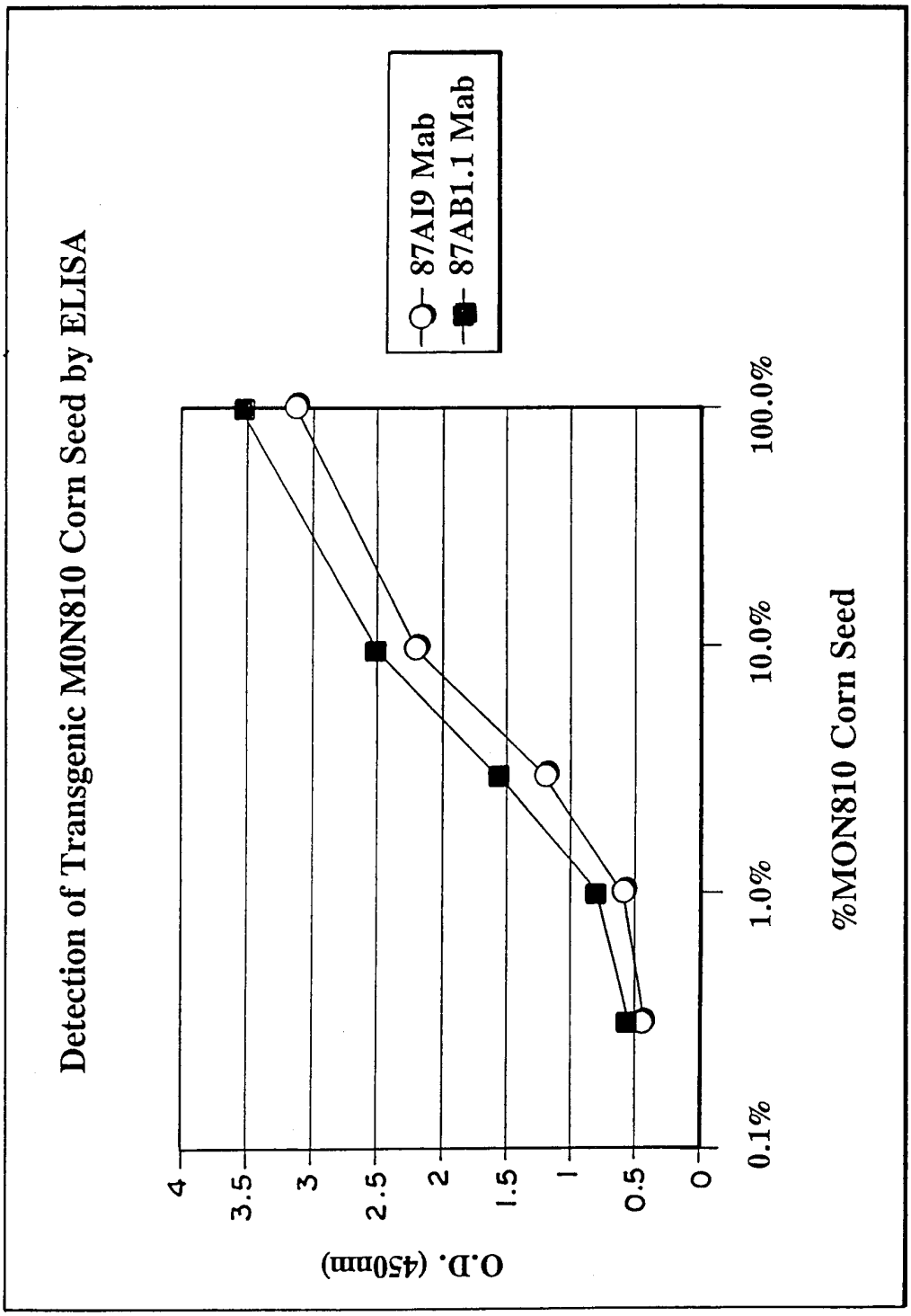

Detection of Transgenic M0N810 Corn Seed by ELISA
87AI9 Mab
87AB1.1 Mab
O.D. (450nm)
4
3.5
3
2.5
2
1.5
1
0.5
0
0.1%
1.0%
10.0%
100.0%
%MON810 Corn Seed

Fig. 5

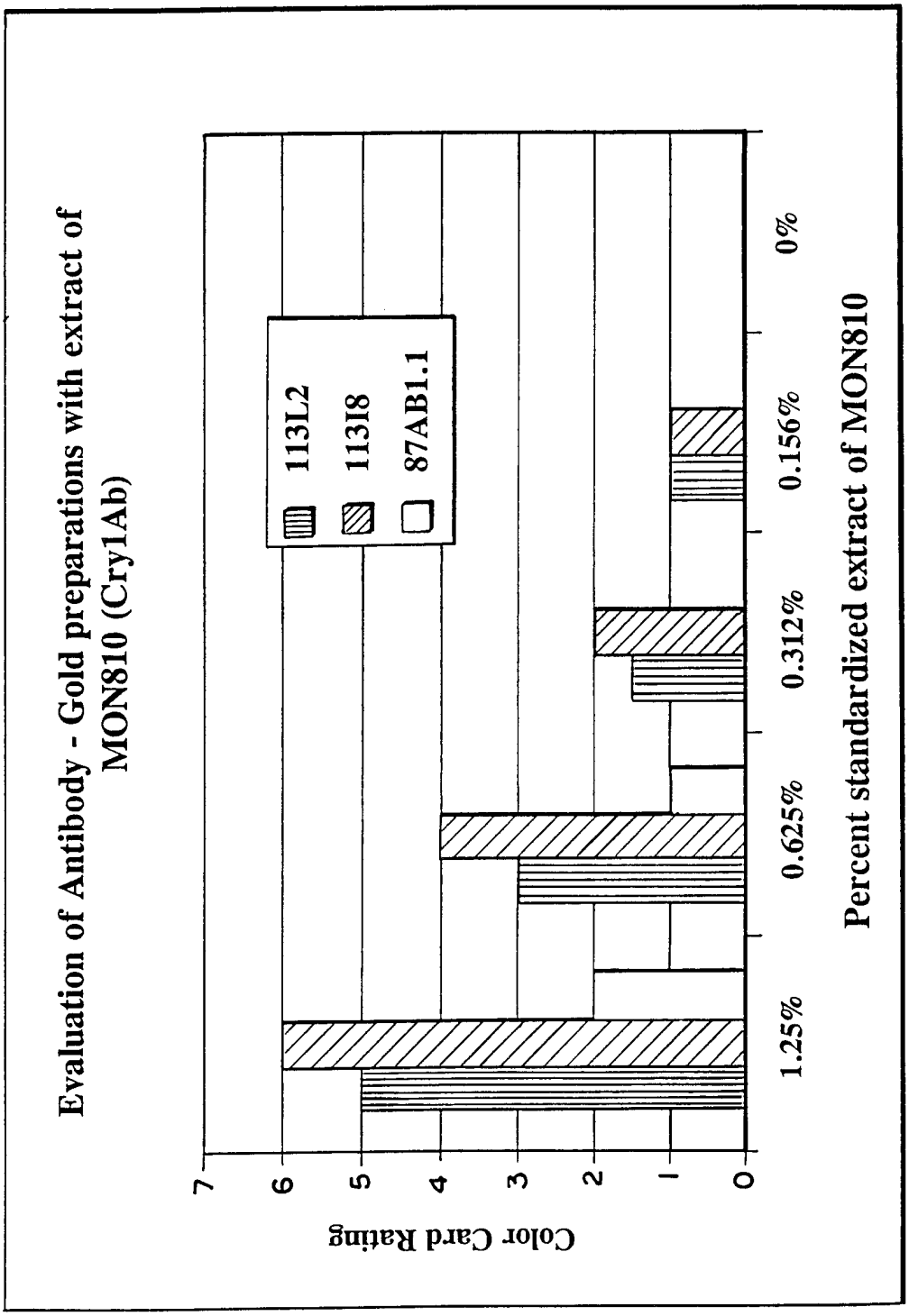

Evaluation of Antibody - Gold preparations with extract of MON810 (Cry1Ab)
113L2
113I8
87AB1.1
Color Card Rating
0
1
2
3
4
5
6
7
1.25%
0.625%
0.312%
0.156%
0%
Percent standardized extract of MON810

Fig. 6

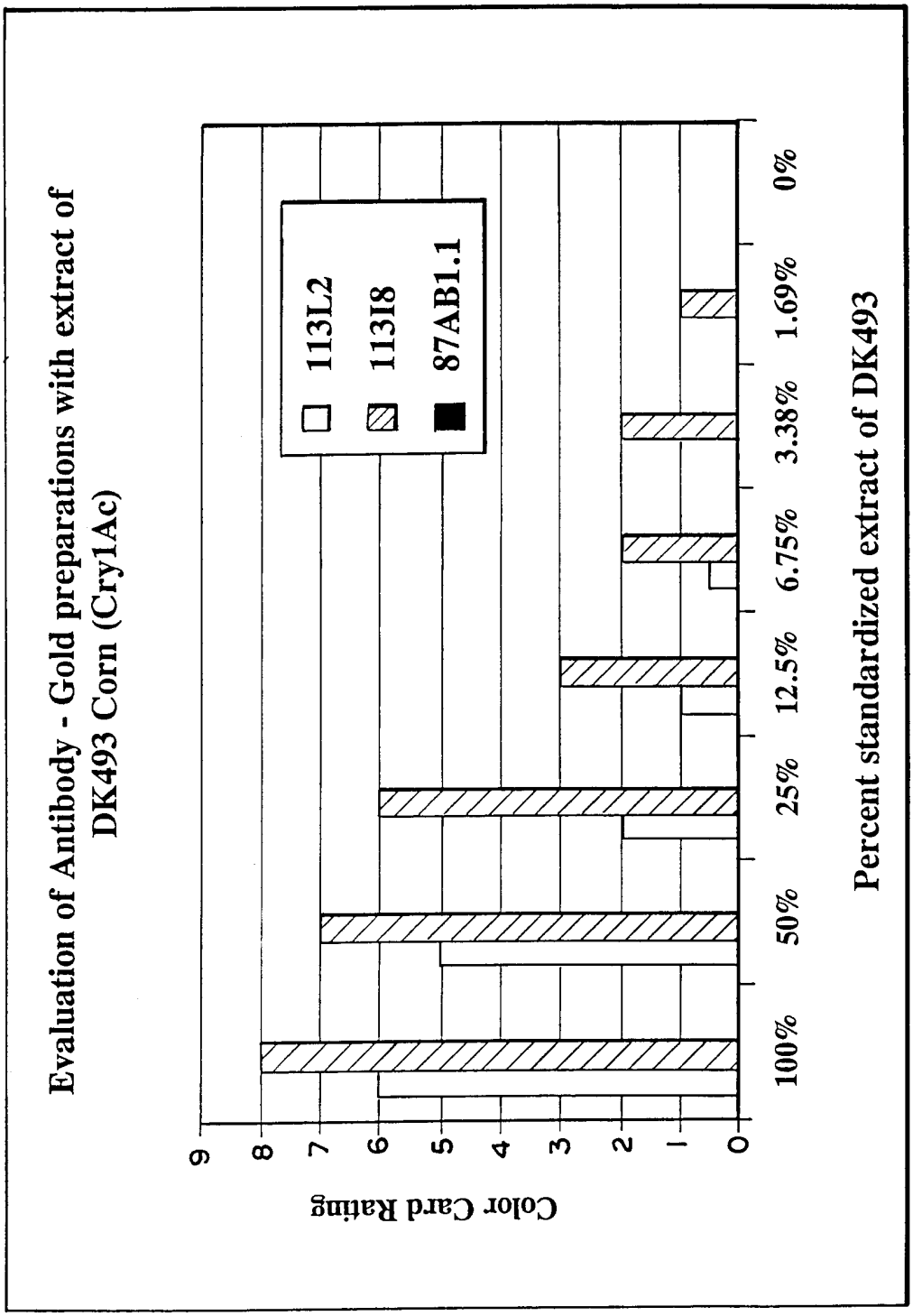

Evaluation of Antibody - Gold preparations with extract of DK493 Corn (Cry1Ac)
113L2
113I8
87AB1.1
Color Card Rating
0
1
2
3
4
5
6
7
8
9
100%
50%
25%
12.5%
6.75%
3.38%
1.69%
0%
Percent standardized extract of DK493

Fig. 7

Fig. 8

ug/mL	87AB1.1	87A19	113L2	113L10	113L11	113L12	113L13	11318	Neg. Control
20.000	0.063	0.051	2.116	0.403	0.052	0.056	1.807	2.168	0.047
6.667	0.048	0.046	2.13	0.146	0.048	0.05	1.264	2.129	0.05
2.222	0.048	0.049	1.974	0.081	0.049	0.049	0.719	2.09	0.051
0.741	0.053	0.055	2.028	0.066	0.053	0.052	0.36	1.889	0.06
0.247	0.053	0.056	1.817	0.056	0.05	0.053	0.164	1.69	0.057
0.082	0.053	0.052	1.789	0.051	0.049	0.052	0.09	1.418	0.058
0.027	0.056	0.055	1.55	0.061	0.058	0.06	0.08	0.883	0.059
0.000	0.056	0.063	0.057	0.058	0.059	0.061	0.057	0.056	0.053

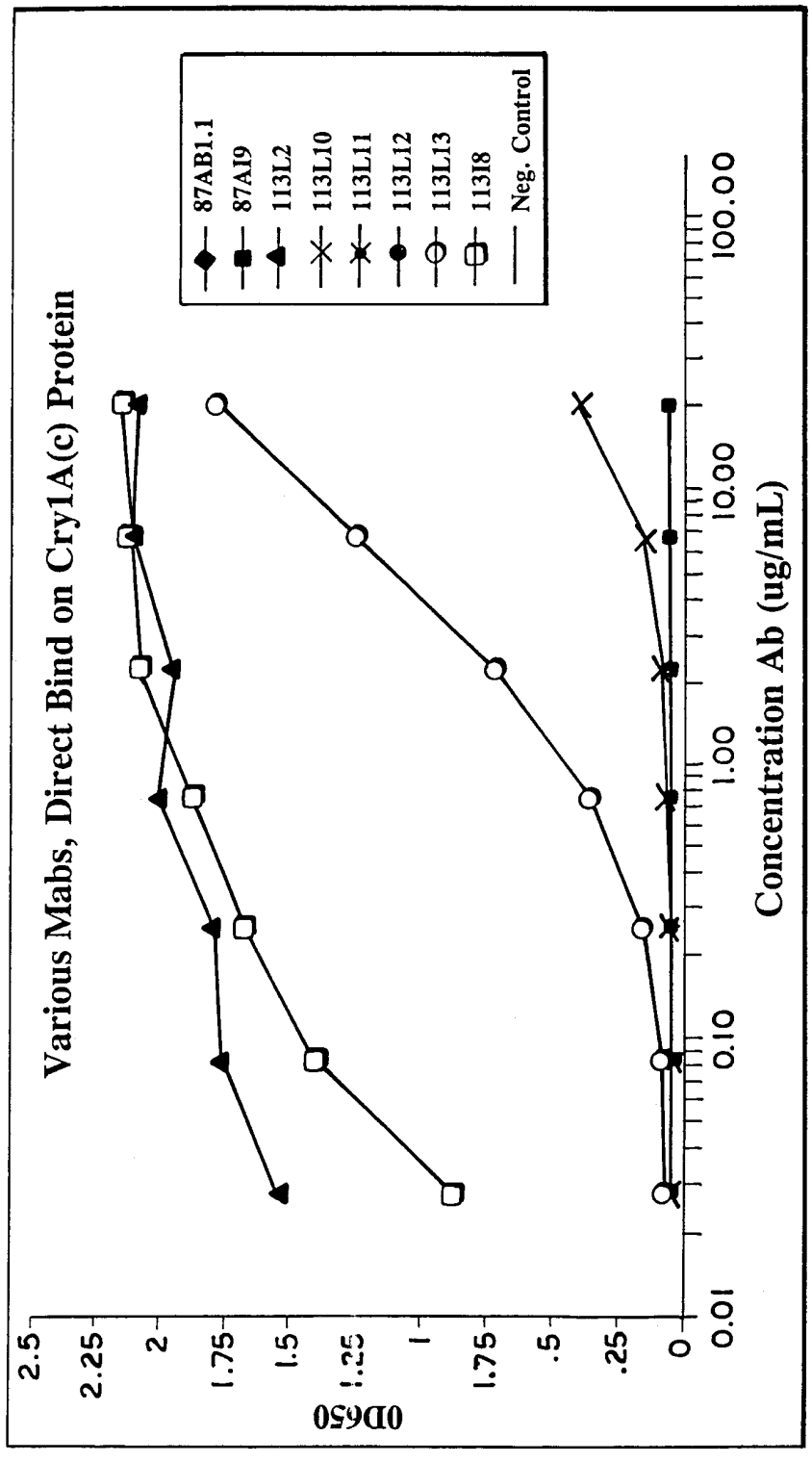


Fig_9

ug/mL	87AB1.1	87A19	113L2	113L10	113L11	113L12	113L13	11318	Neg. Control
20.000	0.726	1.892	1.342	0.955	0.83	0.889	0.961	1.538	0.05
6.667	0.848	1.827	1.307	0.953	0.977	0.841	0.99	1.349	0.047
2.222	0.841	1.835	1.263	0.82	0.908	0.834	0.9	1.211	0.057
0.741	0.746	1.634	1.21	0.858	0.839	0.778	0.909	0.997	0.049
0.247	0.742	1.384	1.008	0.772	0.798	0.719	0.763	0.887	0.04
0.082	0.605	0.955	0.853	0.506	0.565	0.528	0.618	0.586	0.023
0.027	0.35	0.607	0.872	0.326	0.374	0.366	0.291	0.274	0.025
0.000	0.021	0.022	0.019	0.025	0.02	0.023	0.025	0.023	0.021

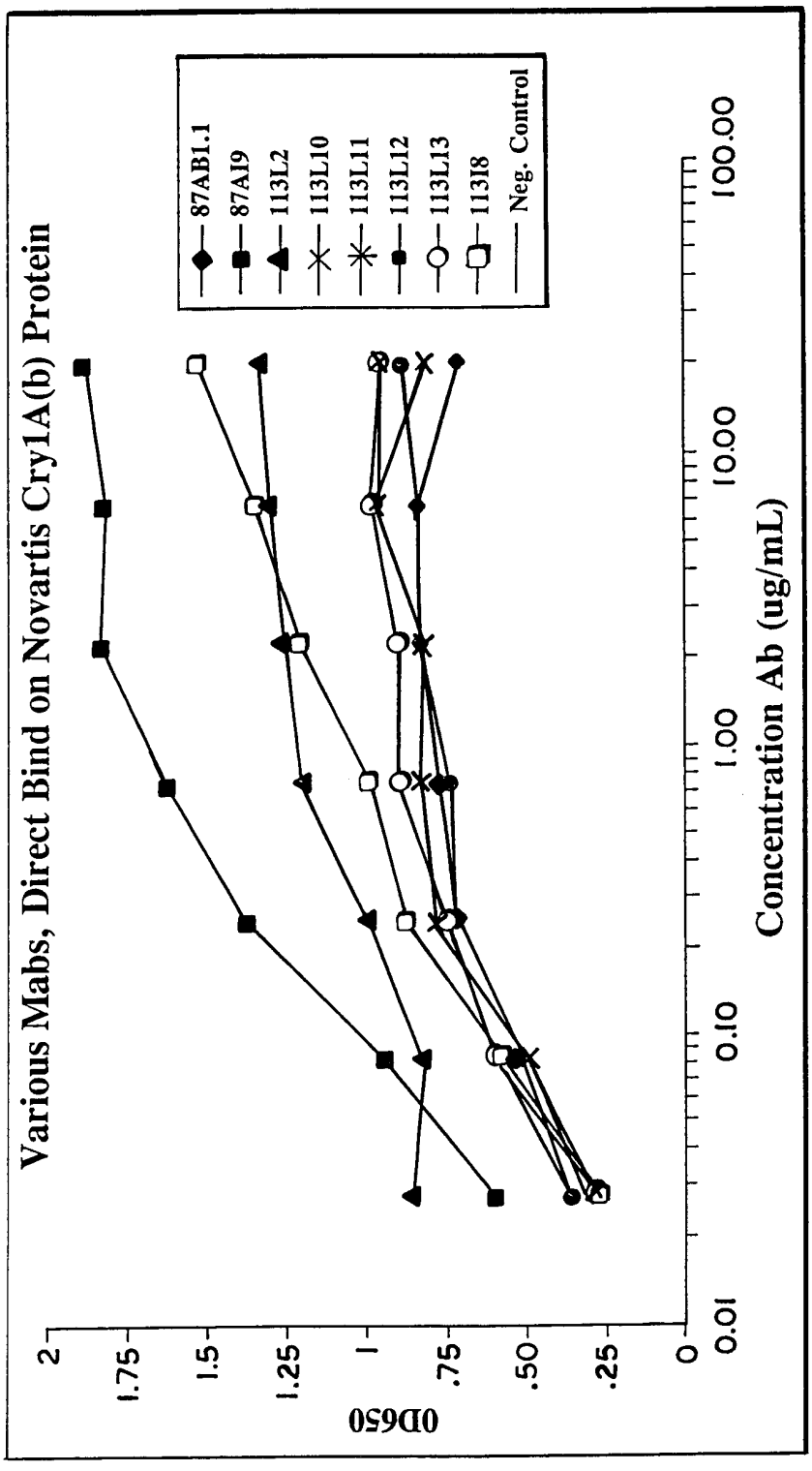
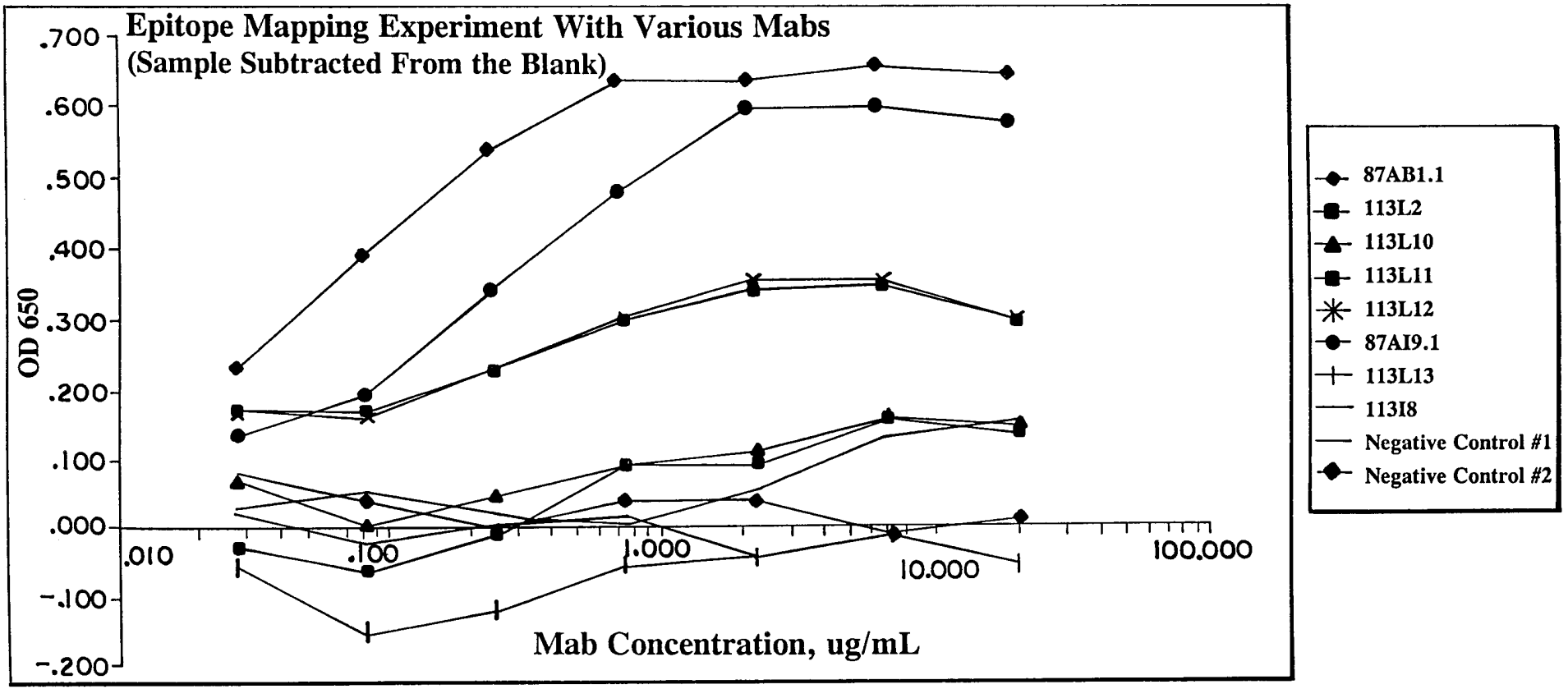


Fig_10

ug/mL	87AB1.1	113L2	113L10	113L11	113L12	87A19.1	113L13	11318	Neg. Cont. 1	Neg. Cont. 2
0.000	0.636	0.135	0.143	0.293	0.293	0.571	-0.059	0.150	0.011	0.009
6.667	0.652	0.154	0.157	0.344	0.351	0.593	-0.011	0.129	-0.012	-0.012
2.222	0.631	0.093	0.109	0.342	0.363	0.591	-0.044	0.056	-0.043	0.038
0.741	0.630	0.092	0.092	0.299	0.304	0.476	-0.054	0.008	0.017	0.039
0.247	0.637	-0.010	0.049	0.229	0.232	0.339	-0.118	0.021	0.010	0.004
0.082	0.390	-0.060	0.008	0.172	0.165	0.194	-0.150	0.057	-0.016	0.039
0.027	0.234	-0.023	0.075	0.176	0.173	0.138	-0.053	0.036	0.026	0.087
0.000	0.000	0.000	0.000	0.000	0.000	-0.008	0.000	0.000	0.000	0.000



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/34321

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : H01H 5/08, 3/48; 1/36
US CL : 435/7.2,7.32,7.92, 97.5; 536/503, 547,548

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.2, 7.32, 7.92, 975; 536/503, 547, 548

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, DIALOG, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOTA, M. et al. Overexpression of the Bacillus thuringiensis (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. PNAS Online. March 1999, Vol. 96. No. 5, pages 1840-1845, See entire document.	1, 3-5, 7-9, 11, 13
Y	US 5,804,393 A (GEISER et al.) 8 September 1998, See entire document.	1-20
Y	HUBER-LUKAC, M. et al. Characterization of Monoclonal Antibodies to a Crystal Protein of Bacillus thuringiensis subsp. kurstaki. Infection and Immunity October 1986, Vol. 54 No.1, pages 228-232, See entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2001 (23.03.2001)	14MAY 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Iesha Fields Telephone No. (703) 308-0196

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