BIOPESTICIDE REGISTRATION ACTION DOCUMENT

Bacillus thuringiensis Cry1A.105 and Cry2Ab2 Insecticidal Proteins and the Genetic Material Necessary for Their Production in Corn
[PC Codes 006515 (Cry2Ab2), 006514 (Cry1A.105)]

U.S. Environmental Protection Agency
Office of Pesticide Programs
Biopesticides and Pollution Prevention Division

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I. OVERVIEW

A. BACKGROUND

On June 10, 2008, EPA conditionally registered a plant-incorporated protectant product, event MON 89034 corn, containing two new active ingredients, *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 insecticidal proteins, and the genetic material necessary for their production. EPA also conditionally registered another product, MON 89034 x MON 88017 corn, which contains a previously registered *Bacillus thuringiensis* Cry3Bb1 protein in addition to the two new active ingredients. The MON 89034 corn registration will expire on midnight September 30, 2022 and the. MON 89034 x MON 88017 corn registration will expire on midnight September 30, 2015. The Agency determined that the use of these pesticide products is in the public interest and that their use will not cause any unreasonable adverse effects on the environment during the time the products are registered. The registrant for both products is Monsanto Company ("Monsanto").

Event MON 89034 corn produces its own insecticide derived from *Bacillus thuringiensis* (*Bt*), a naturally occurring soil bacterium. The *Bt* proteins produced in this product, called Cry1A.105, and Cry2Ab2, have been shown to effectively control highly destructive lepidopteran corn pests, including European corn borer (ECB), corn earworm (CEW), southwestern corn borer (SWCB), fall armyworm (FAW), and sugarcane borer (SCB), in field trials conducted during the 2003-2004 growing seasons in Puerto Rico and the United States. These pests feed on the base of seedlings and on the stalk, leaf, and ear tissue of corn plants, thereby destroying the entire plant, weakening the stalk, and/or damaging the ear. In areas where one or more of these pests is prevalent (*e.g.,* the corn belt), significant financial losses are realized from decreased corn yields and increased expenditures on chemical pest control agents, including organophosphate, carbamate and pyrethroid insecticides.

On June 10, 2008, when the conditional, time-limited registrations of MON 89034, and MON 89034 x MON 88017 were issued, the non-*Bt* corn borer refuge was required to be at least 20% for the corn belt. On December 15, 2008, EPA amended these product registrations to allow a reduction in the structured corn borer refuge requirement (5%) in the non-cotton-growing regions of the corn belt.

The data required to satisfy the conditions of these registrations are listed in Section III, "Regulatory Position for Cry1A.105, and Cry2Ab2."

On October 1, 2009, EPA announced a policy to provide a more meaningful opportunity for the public to participate on major registration decisions before they occur. According to this policy, EPA intends to provide a public comment period prior to making a registration decision for, at minimum, the following types of applications: new active ingredients; first food uses; first outdoor uses; first residential uses; and other actions for which the Agency anticipates that there will be significant public interest.

Consistent with the policy of making registration actions more transparent, the amendments to the expiring MON 89034 corn products were subject to a 30-day comment period because the Agency believed, given past experiences with PIPs in general, these actions would be of significant interest to the public. During this comment period, several comments were received from the following stakeholders: Mycogen Seeds c/o Dow AgroSciences LLC; Pioneer Hi-Bred International, Incorporated; Monsanto Company; National Corn Growers Association; Agricultural Biotechnology Stewardship Technical Committee; Center for Science in the Public Interest; and Association of American Seed Control Officials. After reviewing and considering all of the public comments received, the Agency still maintains that, based on all data submitted in support of the MON 89034 corn registrations (both for initial registrations and as responses to conditions of registration), it is in the best interest of the public and the environment to amend the currently existing MON 89034 registrations by extending their expiration dates (September 30, 2022 for MON 89034 corn; September 30, 2015 for MON 89034 x MON 88017 corn). The basis for this decision can be found in both the risk assessment for the MON89034 corn products, which is characterized throughout this Biopesticides Registration Action Document (BRAD), and the Agency's response to comments document.

All data and findings for the MON 89034 corn products are presented within the standard BRAD configuration for PIPs (i.e., information is placed into separate and distinct chapters according to scientific discipline or regulatory focus); this should be the most familiar format to outside stakeholders interested in reading further about these actions. In addition to the MON 89034 corn products, there are other *Bt* corn PIPs, expressing different proteins effective in controlling various lepidopteran pests or corn rootworm, that were due to expire in 2010, and for which the associated registrants formally requested an extension to expiration dates. Therefore, within the same docket (EPA-HQ-OPP-2010-0607) as this document, the following information[a] is also available for public examination:

- Cry1F and Cry1Ab BRAD (Draft - August 2010; Final - Sept. 2010)
- Cry3Bb1 BRAD (Draft - July 2010; Final - Sept. 2010)
- mCry3A BRAD (Draft - July 2010; Final - Sept. 2010)
- Cry1A.105 and Cry2Ab2 BRAD (Draft - August 2010; Final - September 2010)
- Optimum® AcreMax™ *B.t.* Seed Blends BRAD (Draft - August 2010; Final - Sept. 2010)
- Current Registration Terms and Conditions for *Bt* Corn Registrations Set to Expire in 2010
- Proposed Registration Terms and Conditions for *Bt* Corn Registrations Set to Expire in 2010
- Registration Terms and Conditions Established with the Finalized Amendments
- BPPD mCry3A, Cry3Bb1, and Cry34/35Ab1 Rootworm Monitoring Reviews (June 2010)
- Public Comments on EPA Docket Number EPA-HQ-OPP-2010-0607
- EPA's Response to Comments

[a] Each of the Biopesticides Registration Action Documents in this action are modified from previous versions to account for data/information submitted to fulfill terms and conditions of registration (see draft and final versions) and to respond, in part, to comments received on the information presented in Docket Number EPA-HQ-OPP-2010-0607 (see final versions only). All documents presented in the list can be retrieved from the following website: *http://www.regulations.gov*.

EPA made the decision to amend the registrations of eighteen (18) expiring *Bt* corn PIP registrations to extend the expiration dates. We conducted comprehensive assessments of each of these registrations, considering all toxicity and environmental effects data, data from insect resistance monitoring, and insect resistance refuge compliance reports, received and obtained since the last comprehensive evaluation of these products in 2001. Based upon our comprehensive assessment, we reached significant conclusions regarding the positive environmental impact of *Bt* corn PIPs, and we took several actions to strengthen the insect resistance management requirements to ensure continued success in the prevention of the evolution of resistance in target pests.

Since the commercialization of *Bt* crops, there have been a significant number of published field studies that, combined with the post-registration field studies required to be submitted to the Agency, have demonstrated that non-target invertebrates are generally more abundant in *Bt* cotton and *Bt* corn fields than in non-transgenic fields managed with chemical insecticides. Thus, these published and registrant-produced studies demonstrate that, not only are the *Bt* crops not causing any unreasonable adverse effects in the environment, but, arthropod prevalence and diversity is greater in *Bt* crop fields.

To strengthen insect resistance management of these corn PIPs and to address reports that compliance with the mandated refuge requirements has been decreasing, EPA is requiring enhanced compliance assurance programs (CAPs), and a phased requirement for seed bag labeling that clearly shows the refuge requirements. Also, given the increasing variety of PIP products and combinations, and the differing risk of resistance evolution that the various products represent, we are granting registrations for the corn PIP products for different timeframes, based on assessments of their likelihood of forestalling the evolution of insect resistance. We are registering differing categories of products for differing time periods to reflect the assessed level of risk of resistance posed by the various corn PIP products. The scheme that we are following includes registration periods generally of five, eight, and twelve years; with the possibility of a fifteen-year registration period for products that are demonstrated to meet specified criteria. We retain, however, the discretion to register products for time periods differing from these defaults where circumstances warrant.

B. EXECUTIVE SUMMARY

Product Characterization

MON 89034 was developed by *Agrobacterium*-mediated transformation of corn using the 2T-DNA plasmid vector PV-ZMIR245. The transformation produces two *Bacillus thuringiensis* proteins, Cry1A.105 and Cry2Ab2. Cry1A.105 is a chimeric protein composed of portions of Cry1Ab, Cry1Ac, and Cry1F proteins.

Mammalian Toxicity and Allergenicity Assessment

The acute oral toxicity data submitted by Monsanto demonstrated the lack of mammalian toxicity in rats and mice exposed to pure Cry1A.105 and Cry2Ab2 protein at doses well above the maximum levels anticipated in treated crops, based upon the demonstrated expression values of these two proteins.

Data demonstrating no mammalian toxicity at high levels of exposure confirm the safety of the product at levels well above any possible maximum exposure levels anticipated for a plant-incorporated protectant. This is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which these plant-incorporated protectants were derived. [See 40 CFR Sec. 158.2130 and 158.2140.] For microbial products, further toxicity testing to verify the observed effects and clarify the source of the effects (Tiers II & III) and residue data are triggered by significant acute effects in studies such as the mouse oral toxicity study.

Since no acute effects were observed in the submitted studies, even at relatively high dose levels, the Cry1A.105 and Cry2Ab2 proteins are not considered to be toxic. This conclusion was supported by amino acid sequence comparisons of the Cry1A.105 and Cry2Ab2 proteins with databases of known toxic proteins, which showed no similarities that would raise a safety concern. In addition, the data submitted by Monsanto demonstrated that the Cry1A.105 and Cry2Ab2 proteins were substantially degraded by heat when examined by immunoassay. This instability to heat would decrease the potential for dietary exposure to intact Cry1A.105 and Cry2Ab2 proteins in cooked or processed foods. These biochemical features, along with the lack of adverse results in the acute oral toxicity tests, support the Agency's conclusion that there is a reasonable certainty of no harm from dietary exposure to Cry1A.105 and Cry2Ab2 containing crops.

Since Cry1A.105 and Cry2Ab2 are proteins, their potential for food allergenicity was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a "weight-of-evidence" approach when considering the allergenic potential for a PIP protein, and bases its conclusions upon the following factors: the source of the trait, the amino acid sequence compared with known allergens, and the biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The Agency's allergenicity assessment for the Cry1A.105 and Cry2Ab2 proteins follows:

1. Source of the traits. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequences of Cry1A.105 and Cry2Ab2 with known allergens showed no sequence similarity or identity at the level of

eight contiguous amino acid residues, which is considered to be the smallest amino acid sequence necessary to induce an immune response.

3. Digestibility. The Cry1A.105 and Cry2Ab2 proteins were digested rapidly in simulated gastric fluid containing pepsin, the enzyme produced by the stomach that digests proteins so they can be absorbed as nutrients into the body via the small intestine. The rapid degradation of Cry1A.105 and Cry2Ab2 in the simulated gastric environment indicated that the intact protein will not pass from the stomach into the intestinal lumen, where sensitization of the immune system to food allergens occurs.
4. Glycosylation. Cry1A.105 and Cry2Ab2 proteins expressed in corn are not glycosylated.[1]
5. Conclusion. EPA concluded that the potential for Cry1A.105 and Cry2Ab2 to be a food allergen is minimal.

The information on the safety of pure Cry1A.105 and Cry2Ab2 proteins provides adequate justification to address possible exposures in all corn crops.

Environmental Hazard Assessment

Maximum hazard dose toxicity testing on representative beneficial organisms from several taxa was performed in support of the registrations of Cry1A.105 and Cry2Ab2 proteins expressed in corn. The toxicity of the Cry1A.105 and Cry2Ab2 proteins was evaluated on several species of invertebrates, including the lady beetle, minute pirate bug, parasitic hymenoptera, Collembola, *Daphnia*, honey bee, and earthworm. Developmental observations were also made in the lady beetle, minute pirate bug, and honeybee studies. Observations of possible reproductive effects were also made in the Collembolan studies. In addition, earthworm studies were voluntarily submitted to the Agency to ascertain the potential effects of the Cry1A.105 and Cry2Ab2 proteins on beneficial decomposer species. Avian dietary studies and soil fate data were also submitted.

The test substances used for the studies submitted in support of the MON 89034 registrations included bacterially produced, purified Cry1A.105 and Cry2Ab2 proteins, and MON 89034 corn leaf tissue, pollen, and grain. The October 2000 FIFRA Science Advisory Panel (SAP) recommended that while actual plant material is the preferred test material, bacterially derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume corn plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. An insect feeding study, which compared the relative potency of plant produced Cry1A.105 and Cry2Ab2 proteins to the microbe produced proteins, indicated that plant produced protein was similar in toxicity to the bacterially produced protein (Edelstein Memo, November 7, 2007).

[1] Although this was only demonstrated in corn, these expressed proteins are unlikely to be glycosylated if produced in any other crops since the mechanisms of protein glycosylation are similar in different plants (Lerouge, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., "N-Glycoprotein biosynthesis in plants: recent developments and future trends," *Plant Molecular Biology* 38: 31-48, 1998

The potential interactions between the Cry1A.105 and Cry2Ab2 proteins was addressed in a memorandum for the MON 89034 Experimental Use Permit accompanying the Agency's review, "Evaluation of the Potential for Interactions Between the *Bacillus thuringiensis* Proteins Cry1A.105 and Cry2Ab2," (Hunter, M., July 6, 2006). The study provided evidence that the proteins do not interact in either an antagonistic or synergistic manner, and that there will not be any unexpected interactions with regard to target and non-target insects. New data on the potential interaction between the combined Cry1A.105, Cry2Ab2 and Cry3Bb1 proteins were submitted. The results from that study demonstrated that the combined Cry1A.105 and Cry2Ab2 activity was not affected by the Cry3Bb1 protein, and that the Cry3Bb1 activity was unaffected by combined Cry1A.105 and Cry2Ab2 activity (MRIDs 469513-05 & 469513-06).

Insect Resistance Management

Monsanto has demonstrated that the Cry1A.105 and Cry2Ab2 toxins have different modes of action, and consequently, a low likelihood of cross-resistance. Therefore, Cry1A.105 and Cry2Ab2 are suitable partners in a pyramided product. Monsanto has also shown that there is a low likelihood of cross-resistance between Cry1A.105 and Cry1Ab. Monsanto has previously demonstrated that there is a low likelihood of cross-resistance between Cry2Ab2 and Cry1Ac. Both Cry1Ab and Cry1Ac are expressed in other registered *Bt* corn and *Bt* cotton PIPs. Monsanto did not, however, address the likelihood of cross-resistance between Cry1A.105 and Cry1Ac, and Cry1Fa (*Bt* proteins already in existing *Bt* corn and *Bt* cotton products), and what impact such cross-resistance would have on the durability of MON 89034. As a result, Monsanto was required to provide additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance could impact the durability of MON 89034.

Monsanto originally proposed that a 5% structured refuge, rather than the 20% structured refuge required for other *Bt* corn registrations, be applied to field corn uses of MON 89034 in the U.S. Corn Belt. But, the data and simulation modeling in Monsanto's initial application did not support the 5% proposed refuge for MON 89034 in the Corn Belt. There were uncertainties regarding the dose determination for susceptible and heterozygote (i.e., partially resistant) insects (ECB, SWCB, CEW, and FAW), the cross-resistance potential of Cry1A.105, Cry1Ac and Cry1Fa and any impacts on the durability of MON 89034, and limitations in the simulation modeling. Therefore, the field corn uses of MON 89034 in the Corn Belt were registered with a 20% refuge requirement until such time as Monsanto could address the uncertainties. EPA determined, however, that the data did support reduction of the refuge from 50% to 20% in cotton-growing regions in the southeastern U.S., where a 50% non-*Bt* corn refuge has been required for other *Bt* corn registrations.

Subsequent to the registrations of the event MON 89034 corn and MON 89034 x MON 88017 corn products, Monsanto submitted additional data and an analysis of potential resistance risks to support an amendment to reduce the required non-*Bt* corn refuge for MON 89034 corn from 20%

to 5% in the U.S. Corn Belt. After reviewing these data, EPA determined that a 5% refuge in the U.S. Corn Belt should not significantly increase the risk of resistance for ECB, CEW, and SWCB. Monsanto sufficiently addressed the requirement to analyze potential cross resistance in existing *Bt* corn and *Bt* cotton products for Cry1A.105 and Cry1Fa, but additional analysis and information is still needed to fully assess the cross resistance potential for Cry1Ac and Cry1A.105.

C. USE PROFILE

Active Ingredient Name: *Bacillus thuringiensis* Cry1A.105, and Cry2Ab2 insecticidal protein and the genetic material necessary for their production in corn

Trade and Other Name(s): MON 89034

OPP Chemical Codes: 006515 (Cry2Ab2) and 006514 (Cry1A.105)

Basic Manufacturer: Monsanto Company
800 North Lindbergh Blvd.
St. Louis, MO 63167

Type of Pesticide: Plant-incorporated Protectant

Uses: Field Corn and Sweet Corn

Target Pests for Active Ingredient: European corn borer (*Ostrinia nubilalis*), Southwestern corn borer (*Diatraea grandiosella*), Southern cornstalk borer (*Diatraea crambidoides*), Corn earworm (*Helicoverpa zea*), Fall armyworm (*Spodoptera frugiperda*), Corn stalk borer (*Papaipema nebris)*, and Sugarcane borer (*Diatreae saccharalis)*

D. REGULATORY HISTORY

Monsanto previously submitted an Experimental Use Permit (EUP) application for events MON 89034, MON 88017, and MON 89034 x MON 88017. MON 89034 was developed by *Agrobacterium*-mediated transformation of corn using the 2T-DNA plasmid vector PV-ZMIR245, and produces two *Bacillus thuringiensis* (*Bt*) proteins, Cry1A.105 and Cry2Ab2. These proteins are intended to provide protection from feeding damage caused by a number of lepidopteran pests. Cry1A.105 is a chimeric protein composed of portions of Cry1Ab, Cry1Ac, and Cry1F proteins. On July 17, 2006, EPA established temporary exemptions from the requirement of a tolerance for both Cry1A.105 (71 FR 40427 and 72 FR 20434; 40 CFR

174.502) and Cry2Ab2 (71 FR 40431 and 72 FR 20434; 40 CFR 174.503) in the food and feed commodities of corn; these exemptions were set to expire on June 30, 2009.

On November 15, 2006, Monsanto submitted petitions to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act of 1996 (FQPA), requesting amendment of the existing temporary tolerances in 40 CFR 174.503 for the *Bacillus thuringiensis* Cry2Ab2 insecticidal protein to establish a permanent exemption from the requirement of a tolerance for the *Bacillus thuringiensis* Cry2Ab2 insecticidal protein and in 40 CFR 174.502 for the *Bacillus thuringiensis* Cry 1A.105 insecticidal protein to establish a permanent exemption from the requirement of a tolerance for the *Bacillus thuringiensis* Cry 1A.105 insecticidal protein in field corn, sweet corn, and popcorn.

On September 29, 2006, Monsanto submitted an application to register MON 89034 and MON 89034 x MON 88017 under Section 3 of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

On March 9, 2007, Monsanto resubmitted petitions to EPA under the Federal Food, Drug, and Cosmetic Act (FFDCA), requesting amendment of the existing temporary tolerances in 40 CFR 174.503 for the *Bt* Cry2Ab2 insecticidal protein to establish a permanent exemption from the requirement of a tolerance for the *Bt* Cry2Ab2 insecticidal protein, and in 40 CFR 174.502 for the *Bt* Cry 1A.105 insecticidal protein to establish a permanent exemption from the requirement of a tolerance for the *Bt* Cry 1A.105 insecticidal protein in all crops and agricultural commodities

On June 10, 2008, conditional registrations were issued for MON 89034 and MON 89034 x MON 88017 products.

On July 2, 2008 (73 FR No. 128), the existing permanent exemption from the requirement of a tolerance for residues of the *Bacillus thuringiensis* Cry2Ab2 protein under 174.519 was amended to include corn or cotton when used as a plant-incorporated protectant in the food and feed commodities: field corn, sweet corn, popcorn, cotton seed, cotton oil, cotton meal, cotton hay, cotton hulls, cotton forage, and cotton gin byproducts in accordance with good agricultural practices.

On July 16, 2008 (73 FR No. 137), the Agency established permanent exemptions from the requirement of a tolerance for residues of the *Bacillus thuringiensis* Cry1A.105 protein in or on the food and feed commodities: field corn, sweet corn, and popcorn when used as plant incorporated protectant in all food commodities in accordance with good agricultural practices.

On December 15, 2008, the conditional registrations were amended for MON 89034 and MON 89034 x MON 88017, to allow for a 5% structured refuge in the corn belt (in non-cotton growing regions) for corn borers.

II. SCIENCE ASSESSMENT

A. PRODUCT CHARACTERIZATION

MON 89034 was developed by *Agrobacterium*-mediated transformation of corn using the 2T-DNA plasmid vector PV-ZMIR245 and produces two *Bacillus thuringiensis* proteins, Cry1A.105 and Cry2Ab2. These proteins are intended to provide protection from feeding damage caused by a number of lepidopteran pests. Cry1A.105 is a chimeric protein composed of portions of Cry1Ab, Cry1Ac, and Cry1F proteins.

Transformation System:
PV-ZMIR245 is a binary vector containing two separate transfer DNAs (2T-DNA). The first T-DNA contains the *cry1A.105* and the *cry2Ab2* expression cassettes. The second T-DNA contains the *nptII* (neomycin phosphotransferase II) expression cassette. The *cry1A.105* expression cassette contains the *cry1A.105* coding sequence under the regulation of the *e35S* promoter, *Ract1* intron, and the *Hsp17* 3' end sequence. The *cry2Ab2* expression cassette contains the *cry2Ab2* coding sequence under the regulation of the *FMV* promoter, the *Hsp70* intron, a chloroplast transit peptide (TS-*SSU-CTP*), and the *nos* 3' end sequence. The *nptII* expression cassette contains the *nptII* coding sequence under the regulation of the CaMV *35S* promoter and the *nos* 3' end sequence. During transformation, both T-DNAs were inserted into the genome. The *nptII* selectable marker gene was used to select for transformed cells. Traditional breeding was then used to isolate plants that only contain the *cry1A.105* and *cry2Ab2* expression cassettes and not the *nptII* expression cassette.

Characterization of the DNA Inserted in the Plant and Inheritance and Stability:
Characterization of the DNA isolated from event MON 89034 corn using restriction enzyme digests and Southern blot analysis as well as DNA sequencing indicates that the DNA was inserted in the corn genome at a single locus, and the insert contains one copy each of the *cry1A.105* and *cry2Ab2* expression cassettes. There were no other detectable elements other than those associated with the respective cassettes. No backbone sequences from plasmid PV-ZMIR245 or *nptII* coding sequences were detected in the corn genome. Southern blot analysis also demonstrated the stability of the insert over multiple generations. DNA sequencing indicated that the genetic elements were present in the inserted DNA as expected except that the *e35S* promoter was modified, and the right border sequence present in PV-ZMIR245 was replaced by a left border sequence in MON 89034.

Protein Characterization:
Protein characterization data demonstrate that the plant-produced Cry1A.105 and Cry2Ab2 proteins have biochemical and functional activities that are similar to those of the *E. coli*-produced proteins that were used in several toxicity studies. The following techniques were used to characterize and compare the plant-produced and the *E. coli*-produced proteins: sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass

spectrometry, glycosylation analysis, N-terminal amino acid sequencing, and insecticidal activity assays. Glycoslyation analysis indicated that the proteins are not glycoslyated. These analyses demonstrated the structural and functional similarity between the plant-produced and the *E. coli*-produced Cry1A.105 and Cry2Ab2 proteins and justified the use of *E. coli*-produced proteins in toxicity studies. Monsanto also provided information showing the similarity between Cry1A.105, Cry1Ab, and Cry1Ac.

In addition, Monsanto provided information comparing the expected (deduced) amino acid sequence of Cry2Ab2 expressed in MON 89034 corn, the native form of the protein in *B. thuringiensis*, and in Bollgard II cotton. Monsanto stated that Cry2Ab2 in MON 89034 and Bollgard II are identical. Different chloroplast transit peptide sequences were used in the different products; however, these are expected to be cleaved and degraded in the plants upon uptake into the chloroplasts. When Monsanto attempted to determine the N-terminal sequence of Cry2Ab2 from MON 89034 or Bollgard II, the results indicated that the N-terminus is blocked in both. Therefore, Monsanto was unable to determine the cleavage site of the chloroplast transit peptide. Because the chloroplast transit peptides used in MON 89034 and Bollgard II have potential cleavage sites (methionine) three amino acids upstream from the start of the Cry2Ab2 protein sequence, the Cry2Ab2 produced in Bollgard II and in MON 89034 may differ by one amino acid (leucine vs. glutamine) if the cleavage site is within the transit peptide. The *E. coli*-produced Cry2Ab2 protein used in the toxicity studies for MON 89034 includes the three additional amino acids from the chloroplast transit peptide at the N-terminus. Monsanto stated that the Cry2Ab2 proteins produced in MON 89034 and Bollgard II are variants of the wild type Cry2Ab2 protein produced in *B. thuringiensis*. The *Bt*-produced protein was used in some of the previously submitted studies that are cited to support the ecological risk assessment for MON 89034. Monsanto therefore submitted a study demonstrating that the *E. coli*-produced Cry2Ab2 and the *Bt*-produced Cry2Ab2 have equivalent biological activity (EC50 values and rates of growth inhibition) in a larval corn earworm diet-incorporation bioassay.

Analytical Detection Methods:
Short descriptions of enzyme-linked immunosorbent assay (ELISA) methods for detecting and quantifying Cry1A.105 and Cry2Ab2 as well as standard operating procedures for the methods were provided with the registration application. Monsanto stated that these methods have been validated and provided validation results in an appendix to MRID 46951403; but an independent lab validation study was not provided for either method. In addition, Monsanto did not indicate whether the ELISA method for Cry1A.105 will distinguish between Cry1A.105, Cry1Ab, Cry1Ac, and Cry1F. Since Cry1A.105 contains portions of all three proteins, there may be cross-reactivity in the assay. Monsanto also provided a study demonstrating that a commercially available qualitative immunochromatographic test strip can detect Cry2Ab2 in MON 89034 corn. Since event MON 89034 is the only product that expresses Cry1A.105 and the only corn product that expresses Cry2Ab2, the detection method for Cry2Ab2 can be used for detecting both Cry2Ab2 and Cry1A.105. The presence of Cry2Ab2 in corn should also indicate the presence of Cry1A.105.

When *Bt* Cry1A.105, and Cry2Ab2 Protein in corn (EPA Reg. No. 524-575, and 524-576) were initially registered, the Agency issued registration notices to Monsanto that contained the following requirement for further product characterization information:

> "For event MON 89034 corn, an independent lab validation of the analytical method for the detection of Cry2Ab2 and/or Cry1A.105 [is required]. You must also agree to provide to the EPA laboratory (Ft. Meade, MD) methodology and/or reagents necessary for validation of such analytical method within 6 months from the date that the Agency requests them."

Monsanto has provided an independent lab validation of this method (MRID 47731601) as required in the conditions of registration. When the evaluation of the independent lab validation of an analytical detection method for Cry2Ab2 protein in corn was conducted, it was determined that the Cry2Ab2 protein can be detected at a level of detection (LOD) of 1.0%. The study effectively demonstrated that performance based on the number of blinded samples tested and also confirmed that at the 1.0% LOD the dipstick reagents show zero false positive and false negative results. This indicates acceptable performance standards for a rapid analytical method but significantly does not address several performance criteria required by GIPSA for its dipstick test kit validation. The use of the kit manufacturer for independent validation is also questionable.

Conclusion: The test verified the claim that the EnviroLogix QuickStix™ Kit for Cry2Ab2 Bulk Grain can consistently detect Cry2Ab2 present in corn at a concentration of ≥1%. This study was deemed acceptable.

Protein Expression:
Expression level data were provided for Cry1A.105 and Cry2Ab2 in different plant tissues and at different growth stages. Both proteins are expressed at relatively low levels in event MON 89034 corn. The data were produced using ELISA methods for each protein. Summary results are provided below in Table 1. Table 2 provides summaries of the product characterization studies and data provided.

Table 1. Mean Expression Levels of Cry1A.105 and Cry2Ab2 from MON 89034 Plant Tissues

Tissue Type	Cry1A.105 (µg/g dry weight ± standard deviation)*	Cry2Ab2 (µg/g dry weight ± standard deviation)*
Leaf	72 ± 14 – 520 ± 130	130 ± 34 – 180 ± 59
Root	11 ± 1.4 -79 ± 17	21 ± 5.9 -58 ± 18
Whole Plant	100 ± 26 – 380 ± 90	39 ± 16 – 130 ± 51
Pollen	12 ± 1.7	0.64 ± 0.091

Silk	26 ± 3.9	71 ± 35
Forage	42 ± 9.4	38 ± 14
Grain	5.9 ± 0.77	1.3 ± 0.36

*Ranges reflect means at different growth stages for the first three tissue types

Table 2. Product Characterization Data Submitted

Study Type/Title	Summary	MRID #
Characterization of the inserted DNA/ Summary of Southern Blot Analyses of MON 89034 and MON 89697 Corn[2]	Southern blot analyses indicate that MON 89034 and MON 89597 have the introduced DNA inserted in the corn genome at a single locus and contain one copy each of the cry1A.105 and cry1A.105 expression cassettes. All expression elements are shown to be present in each of the inserts, and there are no other elements detectable other than those associated with the respective cassettes. No backbone sequences from plasmid PV-ZMIR245 or *nptII* coding sequences were detected in the corn genome. **Classification: ACCEPTABLE**	46694501
Analytical detection method/Qualitative Detection Method for the Cry2Ab2 Protein in Corn Leaf and Seed of MON 89034 and MON 89597[2]	A commercially available qualitative immunochromatographic test strip (QuickStix™ kit AS 005 LS) was obtained from EnviroLogix Inc. to determine if the strips can detect the Cry2Ab2 protein produced in MON 89034 and MON 89597. The QuickStix™ kit AS 005 LS detected the presence of Cry2Ab2 in MON 89034 and 89597. It was demonstrated that extracts of leaves or seed from MON 89034 or MON 89597 (both expressing Cry2Ab2) can be distinguished from corn plants that do not express the Cry2Ab2 protein. The study (MRID #477316-01) verified the claim that the EnviroLogix QuickStix™ Kit for Cry2Ab2 Bulk Grain can consistently detect Cry2Ab2 present in corn at a concentration of ≥1%. **Classification: ACCEPTABLE**	46694503 47731601
Characterization of the active ingredient/ Structural and Functional Similarity of the Cry1A.105 Protein to Cry1A Class of *Bacillus thuringiensis* Proteins: Final Report[2]	A summary of current information about the structural and functional similarities of the Cry1A.105 protein to other *Bt* Cry1 proteins is presented in this submission. The Cry1A.105 protein is chimeric, with overall amino acid sequence identity to the Cry1Ac, Cry1Ab and Cry1F proteins of 93.6, 90.0 and 76.7%, respectively. A structural model of the Cry1A.105 protein was developed using the X-ray crystal structure of the Cry1Aa protein. The model demonstrated high overall main chain structural similarity with Cry1Aa. Models of Cry1Ab and Cry1Ac were also prepared using the Cry1A.105 model. Comparison of the aligned folds of all three proteins showed that Cry1Ab and Cry1A.105 have essentially the same main chain structure, and that Cry1Ac differs slightly in its main chain structure from the other two in domain III. Thus, comparison of the modeled crystal structures of the Cry1A.105, Cry1Ab, and Cry1Ac with that of the experimental Cry1Aa X-ray crystal structure demonstrated high structure similarity between the four proteins. Monsanto also summarized results from bioactivity assays using	46694601

[2] Study submitted with EUP request and reviewed in memorandum from R. Edelstein and I. Barsoum to M. Mendelsohn dated June 16, 2006.

Study Type/Title	Summary	MRID #
	Cry1A.105, Cry1Ab, and Cry1Ac in this submission and indicates that complete study reports will be submitted for registration. Monsanto states that purified *E.coli*-produced Cry1A.105 protein had significant activity against each representative lepidopteran insect larvae in laboratory diet bioassays. Tests species included; black cutworm (*Agrotis ipsilon*), corn earworm (*Helicoverpa zea*), fall armyworm (*Spodoptera frugiperda*) and European corn borer (*Ostrinia nubilalis*). Cry1A.105 insecticidal activity was similar to other Cry1 proteins (i.e., Cry1Ac, Cry1F, Cry1Ab). Coleopteran and heteropteran larvae showed no indication of sensitivity to the Cry1A.105 protein. The results of tests with purified Cry1A.105 protein against non-target invertebrates from different orders, such as honey bee, minute pirate bug, earthworms, parasitic hymenoptera and ladybird beetle, demonstrated no meaningful activity. Corn tissues from MON 89034 were tested in a bioassay for potential activity of the Cry1A.105 and Cry2Ab2 proteins against Collembola (*Folsomia candida*), *Daphnia magna* and bobwhite quail with results indicating no effect on the tested non-target organisms. **Classification: ACCEPTABLE**	
Characterization of the active ingredient/Characterization of the Cry1A.105 Protein Purified from the Corn Grain of MON 89034 and Comparison of the Physiochemical and Functional Properties of the Plant-Produced and *E. coli*-Produced Cry1A.105 Proteins[2]	The physicochemical properties and functional properties of the plant-produced Cry1A.105 were analyzed and compared with the properties of the *E. coli* produced Cry1A.105 using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, glycosylation analysis, and a Cry1A.105 insecticidal activity assay. Similar immunoreactive bands migrating between approximately 85 and 130 kDa were observed in the plant-produced Cry1A.105 and *E. coli*-produced reference samples, and the full-length Cry1A.105 protein (~130 kDa) was observed in both the plant-produced and *E. coli*-produced protein samples. MALDI-TOF mass spectrometry analysis of the ~130 kDa band after trypsin digestion yielded peptide masses consistent with peptide masses of the predicted sequence of the Cry1A.105 protein. The identified peptide masses yielded 43.8% overall coverage of the expected peptide sequence (516 of the 1177 amino acids). Immunoreactivity with the N-terminal peptide antibody demonstrated that the N-terminus in the plant-produced full-length Cry1A.105 protein was intact. Glycosylation analysis demonstrated that neither the plant-produced nor the *E. coli*-produced Cry1A.105 protein is glycosylated. The plant-produced and *E. coli*-produced proteins gave similar results in the corn earworm diet-incorporation bioactivity assay: the mean EC_{50} values for the plant-produced Cry1A.105 protein and *E. coli*-produced reference standard were determined to be 0.0074 and 0.012 µg Cry1A.105 per mL diet, respectively. The results of this study demonstrate the structural and functional similarity between the plant-produced and the *E. coli*-produced Cry1A.105 proteins. **Classification: ACCEPTABLE**	46694604

Study Type/Title	Summary	MRID #
Characterization of the inserted DNA/ Amended Report for MSL-20072: Molecular Analysis of Corn MON 89034	The DNA inserted in event MON 89034 was characterized by Southern blot analysis and DNA sequencing. Southern blot analysis indicates that MON 89034 contains a single copy of the *cry1A.105* and *cry2Ab2* expression cassettes at a single locus. No backbone sequences from plasmid PV-ZMIR245 or *nptII* coding sequences were detected in the corn genome. Southern blot analysis of DNA from several generations of MON 89034 demonstrated the stability of the insert over seven generations. In addition, the DNA sequence of the insert and surrounding genomic sequences was determined using PCR and DNA sequencing; this analysis confirmed the organization of the elements within the insert and identified the 5' and 3' insert-to-genomic DNA junctions. **Classification: ACCEPTABLE**	46951402
Expression levels/Assessment of the Cry1A.105 and Cry2Ab2 Protein Levels in Tissues of Insect-protected corn MON 89034 Produced in 2005 U.S. Field Trials	The levels of Cry1A.105 and Cry2Ab2 in corn tissues collected from MON 89034 plants grown at five field sites in the U.S. were determined using enzyme-linked immunosorbent assays (ELISA). The means for Cry1A.105 protein levels across all sites were 5.9 µg/g dry weight (dwt) in grain, 42 µg/g dwt in forage, 12 µg/g dwt in pollen, 520 µg/g dwt in over season leaf collected at growth stage V2-V4 (OSL-1), 120 µg/g dwt in leaves OSL-4 (collected at growth stage pre-VT), 12 µg/g dwt in forage root, and 50 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry1A.105 protein levels across all sites ranged from 72-520 µg/g dwt in leaf, 42-79 µg/g dwt in root, and 100-380 µg/g dwt in whole plant. The means for Cry2Ab2 protein levels across all sites were 1.3 µg/g dwt in grain, 38 µg/g dwt in forage, 0.64 µg/g dwt in pollen, 180 µg/g dwt in OSL-1, 160 µg/g dwt in OSL-4, 21 µg/g dwt in forage root, and 62 µg/g dwt in stover. In tissues harvested throughout the growing season, mean Cry2Ab2 protein levels across all sites ranged from 130-180 µg/g dwt in leaf, 26-58 µg/g dwt in root, and 39-130 µg/g dwt in whole plant. **Classification: ACCEPTABLE**	46951403

Study Type/Title	Summary	MRID #
Characterization of the active ingredient/ Characterization of the Cry2Ab2 Protein Purified from the Corn Grain of MON 89034 and Comparison of the Physicochemical and Functional Properties of the Plant-Produced and *E. coli*-produced Cry2Ab2 Proteins	The physicochemical and functional properties of the plant-produced Cry2Ab2 were analyzed and compared with the properties of the *E. coli* produced Cry2Ab2 using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, densitometry, matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry, glycosylation analysis, and a diet-incorporation corn earworm (CEW) bioactivity assay. Similar immunoreactive bands migrating at approximately 61 kDa were observed in the plant-produced Cry2Ab2 and *E. coli*-produced reference samples. The plant-produced protein sample had an additional immunoreactive band migrating at approximately 50 kDa; N-terminal amino acid analysis of this protein indicated that it is a truncated Cry2Ab2 protein with its N-terminus starting at amino acid 145. MALDI-TOF mass spectrometry analysis of the ~61 and 50 kDa bands after trypsin digestion yielded peptide masses consistent with peptide masses of the predicted sequence of the Cry2Ab2 protein. Glycosylation analysis indicated that the Cry2Ab2 protein is not glycosylated. The plant-produced and *E. coli*-produced proteins gave similar results in the bioactivity assay: the mean EC_{50} values for the plant-produced Cry2Ab2 protein and *E. coli*-produced reference standard were both determined to be 0.16 µg Cry2Ab2 per mL diet, with standard deviations of 0.04 and 0.01 µg Cry2Ab2 per mL diet, respectively. The results of this study demonstrate the structural and functional similarity between the plant-produced and the *E. coli*-produced Cry2Ab2 proteins. **Classification: ACCEPTABLE**	46951404
Characterization of active ingredient/Evaluation of the Functional Equivalence of the Cry2Ab2 Protein Produced in E. coli and *Bt* Against a Sensitive Lepidopteran Species	The functional activity of purified Cry2Ab2 produced from *E. coli* and Cry2Ab2 produced from *Bt* was evaluated using a corn earworm larvae diet incorporation bioassay. There was no significant difference between the EC50 values (the effective concentration to inhibit growth of the target insect by 50%) for the two proteins, as shown by the large overlap in the 95% confidence intervals and the nearly identical dose response curves. In addition, the two proteins showed the same rates of concentration-dependent growth inhibition, indicating that the proteins have the same mechanism of insecticidal action. **Classification: ACCEPTABLE**	46951405
Response to EPA Questions/ Responses to EPA Questions Regarding Applications 524-LTL and 524-LTA to Register Insect-protected Corn MON 89034 and MON 89034 x MON 88017	In an email message from S. Cerrelli to N. Bogdanova dated April 23, 2007, EPA identified some deficiencies in the applications to register MON 89034 and MON 89034 x MON 88017 and requested some additional information from Monsanto. In MRIDs 47127501-47127505, Monsanto responds to the questions and supplies the requested additional information. Monsanto's responses are adequate. **Classification: ACCEPTABLE**	47127501-47127505

B. HUMAN HEALTH ASSESSMENT OF Cry1A.105

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue... ."

Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

1. Toxicological Profile

Consistent with section 408(b)(2)(D) of the FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness and reliability, and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

Mammalian Toxicity and Allergenicity Assessment

Monsanto submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry1A.105 protein. These data demonstrate the safety of the product at a level well above maximum possible exposure levels that are reasonably anticipated in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity testing and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant incorporated protectant was derived (See 40 CFR Sec. 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered by significant adverse acute effects in studies such as the mouse oral toxicity study, to verify the observed adverse effects and clarify the source of these effects (Tiers II & III).

An acute oral toxicity study in mice (MRID 46694603) indicated that Cry1A.105 is non-toxic to humans. Cry1A.105 produced from microbial culture was dosed by gavage as two doses separated by 4 hours (±20 minutes) to 10 females and 10 males (2072 mg/kg body weight). Two control groups were also included in the study: a bovine serum albumin protein control, and a vehicle control. One male in the test protein group was moribund and sacrificed on day 1 due to a mechanical dosing error; this death was not attributed to the test material. All other mice survived the study. There were no significant differences in body weight or body weight change among the three groups during the study, and no treatment-related gross pathological findings were observed. The oral LD_{50} for males, females, and combined mice was greater than 2072 mg/kg.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjoblad, Roy D., et al., "Toxicological Considerations for Protein Components of Biological Pesticide Products," Regulatory Toxicology and Pharmacology 15, 3-9 (1992)). Therefore, since no acute effects were shown to be caused by Cry1A.105, even at relatively high dose levels, the Cry1A.105 protein is not considered toxic. Further, amino acid sequence comparisons showed no similarities between the Cry1A.105 and known toxic proteins in protein databases that would raise a safety concern.

Since Cry1A.105 is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of-evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in-vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Cry1A.105 follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Cry1A.105 with known allergens showed no overall sequence similarity or identity at the level of eight contiguous amino acid residues.
3. Digestibility. The Cry1A.105 protein was digested within 30 seconds in simulated gastric fluid containing pepsin.
4. Glycosylation. Cry1A.105 expressed in corn was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Cry1A.105 to be a food allergen is minimal.

Although Cry1A.105 was only shown not to be glycosylated in corn, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it needs to contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms

of protein glycosylation are similar in different plants (Lerouge, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., "N-Glycoprotein biosynthesis in plants: recent developments and future trends," *Plant Molecular Biology* **38**: 31-48, 1998).

2. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses).

The Agency has considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for residues of the plant-incorporated protectants, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely, since the plant incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. In addition, even if exposure can occur through inhalation, the potential for Cry1A.105 to be an allergen is low, as discussed previously. Although the allergenicity assessment focused on the Cry1A.105 protein's potential to be a food allergen, the data also indicated a low potential for Cry1A.105 to be an inhalation allergen. Exposure to infants and children via residential or lawn use is not expected, because the use sites for the Cry1A.105 protein is agricultural. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, theoretically, drinking water. However oral toxicity testing in mammals showed no adverse effects.

3. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from the plant-incorporated protectant, EPA concluded that there are no cumulative effects for the Cry1A.105 protein.

4. Determination of Safety for U.S. Population, Infants and Children

a) Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry1A.105 protein included the characterization of the expressed Cry1A.105 protein in corn, as well as the acute oral toxicity study, amino acid sequence comparisons to known allergens and toxins, and in vitro

digestibility of the protein. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the Cry1A.105 test material derived from microbial culture was biochemically and functionally equivalent to the protein produced by the plant-incorporated protectant ingredient in the plant. Microbially produced protein was used in the studies so that sufficient material for testing was available.

The acute oral toxicity data submitted support the prediction that the Cry1A.105 protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels. Given that no treatment-related adverse effects were shown to be caused by the Cry1A.105 protein, even at relatively high dose levels, the Cry1A.105 protein is not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing or residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects (Tiers II and III).

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. Data submitted by the applicant, however, demonstrated low levels of Cry1A.105 in corn tissues.

Since Cry1A.105 is a protein, potential allergenicity is also considered as part of the toxicity assessment. Considering all of the available information (1) Cry1A.105 originates from a non-allergenic source; (2) Cry1A.105 has no sequence similarities with known allergens; (3) Cry1A.105 is not glycosylated; and (4) Cry1A.105 is rapidly digested in simulated gastric fluid; EPA has concluded that the potential for Cry1A.105 to be a food allergen is minimal.

The Agency did not evaluate information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) or apply safety factors that are generally recognized as appropriate when animal experimentation data are used to assess risks to humans. The lack of mammalian toxicity at high levels of exposure to the Cry1A.105 protein, as well as the minimal potential to be a food allergen, satisfactorily demonstrated the safety of the products at levels well above the anticipated maximum exposure levels.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA), necessary for the production of the Cry1A.105 protein has been exempted from the requirement of a tolerance under 40 CFR 174.507 "Nucleic acids that are part of a plant-incorporated protectant."

b) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues, and the cumulative effects on infants and children of the residues and other substances with a common mechanism of toxicity. In addition, FFDCA section 408(b)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concluded that there is a finding of no toxicity for the Cry1A.105 protein. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

c) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry1A.105 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as previously discussed, no toxicity to mammals has been observed, nor any indication of allergenicity potential for this plant-incorporated protectant.

5. Other Considerations

a) Endocrine Disruptors

As required under FFDCA section 408(p), EPA has developed the Endocrine Disruptor Screening Program (EDSP) to determine whether certain substances (including pesticide active and other ingredients) may have an effect in humans or wildlife similar to an effect produced by a "naturally occurring estrogen, or other such endocrine effects as the Administrator may designate." The EDSP employs a two-tiered approach to making the statutorily required determinations. Tier 1 consists of a battery of 11 screening assays to identify the potential of a chemical substance to interact with the estrogen, androgen, or thyroid (E, A, or T) hormonal

systems. Chemicals that go through Tier 1 screening and are found to have the potential to interact with E, A, or T hormonal systems will proceed to the next stage of the EDSP where EPA will determine which, if any, of the Tier 2 tests are necessary based on the available data. Tier 2 testing is designed to identify any adverse endocrine related effects caused by the substance, and establish a quantitative relationship between the dose and the E, A, or T effect.

Between October 2009 and February 2010, EPA issued test orders/data call-ins for the first group of 67 chemicals, which contains 58 pesticide active ingredients and 9 inert ingredients. This list of chemicals was selected based on the potential for human exposure through pathways such as food and water, residential activity, and certain post-application agricultural scenarios. This list should not be construed as a list of known or likely endocrine disruptors.

Bt Cry1A.105, and Cry2Ab2 Protein in corn is not among the group of 58 pesticide active ingredients on the initial list to be screened under the EDSP. Under FFDCA § 408(p) the Agency must screen all pesticide chemicals. Accordingly, EPA anticipates issuing future EDSP orders/data call-ins for all Registration Review cases, including those for which EPA has already opened a Registration Review docket for a pesticide active ingredient.

For further information on the status of the EDSP, the policies and procedures, the list of 67 chemicals, the test guidelines and the Tier 1 screening battery, please visit our website: http://www.epa.gov/endo/.

b) Analytical Method(s)

A standard operating procedure for an enzyme-linked immunosorbent assay for the detection and quantification of Cry1A.105 in corn tissue has been submitted.

c) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* Cry1A.105 protein.

The human health studies submitted for Cry1A.105 are summarized in Table 3 below.

Table 3. Summary of Cry1A.105 Human Health Data

Study Type/Title	Summary	MRID #
Acute oral toxicity (OPPTS 870.1100)/ Acute Oral Toxicity Study in Mice with Cry1A.105 Protein[2]	The Cry1A.105 test protein (2072 mg/kg body weight) was dosed by gavage as two doses separated by 4 hours (±20 minutes). The BSA protein control (1998 mg/kg body weight) was dosed using the same procedure as for the test protein group. The vehicle control group was dosed with carbonate-bicarbonate with reduced glutathione. Body weight was recorded prior to fasting, prior to dosing, and on days 7 and 14. The test animals were observed for clinical signs of toxicity two times post-dosing and for 14 days.	46694603

Study Type/Title	Summary	MRID #
	A general health/mortality check was done twice daily. All animals were necropsied. One male in the test protein group was moribund and sacrificed on day 1 due to a mechanical dosing error, which resulted in a perforated esophagus. All other mice survived the study. There were no significant differences in body weight or body weight change among the three groups during the study. The oral LD_{50} for males, females, and combined mice was greater than 2072 mg/kg. This places Cry1A.105 Protein in TOXICITY CATEGORY III due to dose amounts only; no signs of toxicity were observed. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Bioinformatics Analysis of the Cry1A.105 Protein Utilizing the AD6, Toxin5, and Allpeptides Databases [2]	Bioinformatic analyses were used to search for sequence similarities between the Cry1A.105 protein and toxins and allergens. The FASTA alignment tool and the allergen (AD5), toxin (TOXIN5), and public domain (ALLPEPTIDES) database sequences were used to assess structural similarity. No significant similarities were found, other than with the Cry1Ac protein; this alignment is not surprising, since the Cry1A.105 protein contains a significant portion of the Cry1Ac protein. The Cry1A.105 protein sequence was also screened against the AD5 sequence database using a pair-wise comparison algorithm. No matches of 8 amino acids or more were found for the Cry1A.105 protein in the AD5 database. No similarities between Cry1A.105 protein and known allergens, human or animal toxins, or pharmacologically active proteins were found in the study. **Classification: ACCEPTABLE**	46694605
In vitro digestibility/ Assessment of the In Vitro Digestibility of the Cry1A.105 Protein in Simulated Gastric Fluid[2]	No bands representative of intact Cry1A.105 protein were identified by SDS-PAGE after ≥30 seconds incubation with simulated gastric fluid containing pepsin. A very faint band of 4.5 kDaltons was observed between the 30 second and 20 minute digestions but was not observed after 20 minutes. The limit of detection for the SDS-PAGE method was determined to be 5 ng for the full-length Cry1A.105 protein. Both the pepsin stability and test material stability controls gave appropriate responses. In the Western Blot assay, Cry1A.105 was not immunologically identifiable within 30 seconds of incubation. The limit of detection for the method was determined to be 1 ng for the full-length Cry1A.105 protein. The pepsin stability and test material stability controls gave appropriate responses. **Classification: ACCEPTABLE**	46694606
Heat stability/ Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in Corn Grain from MON 89034 Following Heat Treatment [2]	The immunodetectability of the Cry1A.105 and Cry2Ab2 proteins in corn grain from MON 89034 following heat treatment was assessed. MON 89034 and conventional grain were ground, mixed with water, and then heated in an oven at 204 ºC for 20 minutes to simulate the heating process used commercially to process grain. Heated and unheated grain was extracted with two buffers: 50 mM CAPS and 50 mM NLS (CAPS containing 2% N-Lauroyl sarcosine). The extracts were analyzed using western blot to detect the presence of the Cry1A.105 and Cry2Ab2 proteins. The amount of immunodetectable Cry2Ab2 protein in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD. Based on the LOD and the estimated amount of protein in the unheated	46694607

Study Type/Title	Summary	MRID #
	extract, it was determined that the Cry2Ab2 protein decreased at least 77% and 70%, respectively following heat treatment, relative to their original values. Likewise, the amount of immunodetectable Cry1A.105 protein in either CAPS or NLS buffer extracts of MON 89034 after heating was below the limit of detection (LOD) and had decreased by at least 94% and 78%, respectively, relative to their original values. This loss is likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment. **Classification: ACCEPTABLE**	
Amino acid sequence comparison/ Bioinformatics Analysis of the Cry1A.105 Protein Utilizing the AD6, Toxin5, and Allpeptides Databases	The amino acid sequence of the Cry1A.105 protein was compared to the sequences of known allergens and toxins using allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) databases and the FASTA algorithm. In addition to the FASTA comparisons, the Cry1A.105 protein sequence was compared to the AD6 database using 8 amino acid sliding blocks and the ALLERGENSEARCH algorithm. No proteins were identified with an *E* score of less than $1x10^{-5}$ from the search using the AD6 database, indicating that Cry1A.105 has no structural similarity to any known allergens, gliadins, or glutenins. In addition, no matches of 8 or more amino acids were found between the Cry1A.105 protein and sequences in the AD6 database. In the comparisons using the TOXIN5 and ALLPEPTIDES databases, the highest similarity identified was with *Bacillus thuringiensis* pesticidal protein Cry1Ac (92% identity over an 1,182 aa window and an *E* score of 0). This result is not surprising, given that the Cry1A.105 protein contains a significant portion of the Cry1Ac protein. No significant similarities between Cry1A.105 protein and known allergens, human or animal toxins, or pharmacologically active proteins were found in this study. **Classification: ACCEPTABLE**	46951410
In vitro digestibility/ Assessment of the *in vitro* Digestibility of the Cry1A.105 Protein in Simulated Intestinal Fluid	The in vitro digestibility of Cry1A.105 in simulated intestinal fluid containing pancreatin was investigated using western blot analysis. The band for the full-length Cry1A.105 was below the LOD in the 5 minute time-point sample and in the later time-point samples. The LOD for the full-length Cry1A.105 protein by western blot analysis was estimated to be 0.1 ng, which was 0.5 % of the total protein loaded. Therefore, at least 99.5% of the full-length protein was digested within 5 minutes. Bands from proteolytic fragments with approximate molecular weights of 60, 32, and 30 kDa were visible in the five-minute time-point sample. The ~32 kDa fragment was digested and undetectable at the 2-hour time-point and after. The ~30 kDa fragment, which appears as a doublet, was still visible in the 24-hour time-point sample, but its intensity decreased substantially. The ~60 kDa fragment, which also appears as a doublet, represents the trypsin-resistant core and appears to be fairly stable throughout the 24-hour digestion experiment. **Classification: ACCEPTABLE**	46951408

C. HUMAN HEALTH ASSESSMENT Cry2Ab2

Section 408(c)(2)(A)(i) of the FFDCA allows EPA to establish an exemption from the requirement for a tolerance (the legal limit for a pesticide chemical residue in or on a food) only if EPA determines that the exemption is "safe." Section 408(c)(2)(A)(ii) of the FFDCA defines "safe" to mean that "there is a reasonable certainty that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information." This includes exposure through drinking water and in residential settings, but does not include occupational exposure. Pursuant to section 408(c)(2)(B), in establishing or maintaining in effect an exemption from the requirement of a tolerance, EPA must take into account the factors set forth in section 408(b)(2)(C), which require EPA to give special consideration to exposure of infants and children to the pesticide chemical residue in establishing a tolerance and to "ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue... ."

Additionally, section 408(b)(2)(D) of the FFDCA requires that the Agency consider "available information concerning the cumulative effects of a particular pesticide's residues" and "other substances that have a common mechanism of toxicity." EPA performs a number of analyses to determine the risks from aggregate exposure to pesticide residues. First, EPA determines the toxicity of pesticides. Second, EPA examines exposure to the pesticide through food, drinking water, and through other exposures that occur as a result of pesticide use in residential settings.

1. Toxicological Profile

Consistent with section 408(b)(2)(D) of the FFDCA, EPA has reviewed the available scientific data and other relevant information in support of this action and considered its validity, completeness and reliability and the relationship of this information to human risk. EPA has also considered available information concerning the variability of the sensitivities of major identifiable subgroups of consumers, including infants and children.

Mammalian Toxicity and Allergenicity Assessment

Monsanto has submitted acute oral toxicity data demonstrating the lack of mammalian toxicity at high levels of exposure to the pure Cry2Ab2 protein. These data demonstrate the safety of the product at a level well above maximum possible exposure levels that are reasonably anticipated in the crop. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the position regarding toxicity testing and the requirement of residue data for the Agency's microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR Sec. 158.740(b)(2)(i)). For microbial products, further toxicity testing (Tiers II & III) and residue data are triggered by significant adverse acute effects in studies such as the acute oral toxicity study, to verify the observed adverse effects and clarify the source of these effects.

An acute oral toxicity study in mice (MRID 44966602) indicated that Cry2Ab2 is non-toxic to humans. Three groups of ten male and ten female mice were dosed by oral gavage with 30, 300, or 1000 mg/kg bodyweight of microbially produced Cry2Ab2 protein. Two negative control groups were also included in the study: bovine serum albumin protein control, and a vehicle control (purified water). There were no significant differences between the test and control groups; therefore, the Cry2Ab2 protein does not appear to cause any significant adverse effects at an exposure level of up to 1000 mg/kg bodyweight.

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels. Therefore, given that no acute effects were shown to be caused by Cry2Ab2, even at relatively high dose levels, the Cry2Ab2 protein is not considered toxic. Further, amino acid sequence comparisons showed no similarities between the Cry2Ab2 protein and known toxic proteins in protein databases that would raise a safety concern.

Since Cry2Ab2 is a protein, allergenic potential was also considered. Currently, no definitive tests for determining the allergenic potential of novel proteins exist. Therefore, EPA uses a weight-of- evidence approach where the following factors are considered: source of the trait; amino acid sequence comparison with known allergens; and biochemical properties of the protein, including in vitro digestibility in simulated gastric fluid (SGF) and glycosylation. This approach is consistent with the approach outlined in the Annex to the Codex Alimentarius "Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Plants." The allergenicity assessment for Cry2Ab2 follows:

1. Source of the trait. *Bacillus thuringiensis* is not considered to be a source of allergenic proteins.
2. Amino acid sequence. A comparison of the amino acid sequence of Cry2Ab2 with known allergens showed no significant overall sequence similarity or identity at the level of eight contiguous amino acid residues.
3. Digestibility. The Cry2Ab2 protein was digested within 15 seconds in simulated gastric fluid containing pepsin.
4. Glycosylation. Cry2Ab2 expressed in corn was shown not to be glycosylated.
5. Conclusion. Considering all of the available information, EPA has concluded that the potential for Cry2Ab2 to be a food allergen is minimal.

Although Cry2Ab2 was only shown not to be glycosylated in corn, it is unlikely to be glycosylated in any other crops because in order for a protein to be glycoslyated, it must contain specific recognition sites for the enzymes involved in glycosylation, and the mechanisms of protein glycosylation are similar in different plants (Lerouge, P. Cabanes-Macheteau, M., Rayon, C., Fichette-Lainé, A-C., Gomord, V., and Faye, L., "N-Glycoprotein biosynthesis in plants: recent developments and future trends," *Plant Molecular Biology* **38**: 31-48, 1998).

2. Aggregate Exposures

Pursuant to FFDCA section 408(b)(2)(D)(vi), EPA considers available information concerning aggregate exposures from the pesticide residue in food and all other non-occupational exposures, including drinking water from ground water or surface water and exposure through pesticide use in gardens, lawns, or buildings (residential and other indoor uses). The Agency considered available information on the aggregate exposure levels of consumers (and major identifiable subgroups of consumers) to the pesticide chemical residue and to other related substances. These considerations include dietary exposure under the tolerance exemption and all other tolerances or exemptions in effect for the Plant Incorporated Protectant's chemical residue, and exposure from non-occupational sources. Exposure via the skin or inhalation is not likely since the plant incorporated protectant is contained within plant cells, which essentially eliminates these exposure routes or reduces exposure by these routes to negligible. In addition, even if exposure can occur through inhalation, the potential for Cry2Ab2 protein to be an allergen is low, as previously discussed. Although the allergenicity assessment focused on Cry2Ab2 protein's potential to be a food allergen, the data also indicated a low potential for Cry2Ab2 to be an inhalation allergen. Exposure to infants and children via residential or lawn use is also not expected because the use sites for the Cry2Ab2 protein is agricultural. Oral exposure, at very low levels, may occur from ingestion of processed corn products and, theoretically, drinking water. However, oral toxicity testing in laboratory mammals showed no adverse effects.

3. Cumulative Effects

Pursuant to FFDCA section 408(b)(2)(D)(v), EPA has considered available information on the cumulative effects of such residues and other substances that have a common mechanism of toxicity. These considerations included the cumulative effects on infants and children of such residues and other substances with a common mechanism of toxicity. Because there is no indication of mammalian toxicity from the plant incorporated protectant, we conclude that there are no cumulative effects for the Cry2Ab2 protein.

4. Determination of Safety for U.S. Population, Infants and Children
Toxicity and Allergenicity Conclusions

The data submitted and cited regarding potential health effects for the Cry2Ab2 protein included the characterization of the expressed Cry2Ab2 protein in corn, as well as the acute oral toxicity study, amino acid sequence comparisons to known allergens and toxins, and in vitro digestibility of the protein. The results of these studies were used to evaluate human risk, and the validity, completeness, and reliability of the available data from the studies were also considered.

Adequate information was submitted to show that the Cry2Ab2 test material derived from microbial culture was biochemically and functionally equivalent to the protein in the plant.

Microbially produced protein was used in the safety studies so that sufficient material for testing was available.

The acute oral toxicity data submitted by Monsanto support the prediction that the Cry2Ab2 protein would be non-toxic to humans. As mentioned above, when proteins are toxic, they are known to act via acute mechanisms and at very low dose levels. Given that no treatment-related adverse effects were shown to be caused by the Cry2Ab2 protein, even at relatively high dose levels, the Cry2Ab2 protein is not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing and residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bacillus thuringiensis* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)). For microbial products, further toxicity testing and residue data are triggered when significant adverse effects are seen in studies such as the acute oral toxicity study. Further studies verify the observed adverse effects and clarify the source of these effects.

Residue chemistry data were not required for a human health effects assessment of the subject plant-incorporated protectant ingredients because of the lack of mammalian toxicity. Data submitted by the applicant however, demonstrated low levels of the Cry2Ab2 in corn tissues.

Since Cry2Ab2 is a protein, potential allergenicity is also considered as part of the toxicity assessment. Considering that (1) Cry2Ab2 originates from a non-allergenic source, (2) Cry2Ab2 has no sequence similarities with known allergens, (3) Cry2Ab2 is not glycosylated, and (4) Cry2Ab2 is rapidly digested in simulated gastric fluid, EPA concluded that the potential for Cry2Ab2 to be a food allergen is minimal.

The Agency did not consider information concerning the dietary consumption patterns of consumers (and major identifiable subgroups of consumers including infants and children) or safety factors that are generally recognized as appropriate when animal experimentation data are used to assess risks to humans. The lack of mammalian toxicity at high levels of exposure to the Cry2Ab2 protein, as well as the minimal potential to be a food allergen, demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated in the crop.

The genetic material necessary for the production of the plant-incorporated protectant active ingredient include the nucleic acids (DNA, RNA) that encode these proteins and regulatory regions. The genetic material (DNA, RNA) necessary for the production of the Cry2Ab2 protein has been exempted from the requirement of a tolerance under 40 CFR 174.507 “Nucleic acids that are part of a plant-incorporated protectant.”

a) Infants and Children Risk Conclusions

FFDCA section 408(b)(2)(C) provides that EPA shall assess the available information about consumption patterns among infants and children, special susceptibility of infants and children to pesticide chemical residues and the cumulative effects on infants and children of the residues and

other substances with a common mechanism of toxicity. In addition, FFDCA section 408(b)(2)(C) also provides that EPA shall apply an additional tenfold margin of safety for infants and children in the case of threshold effects to account for prenatal and postnatal toxicity and the completeness of the database unless EPA determines that a different margin of safety will be safe for infants and children.

In this instance, based on all the available information, the Agency concluded that there is a finding of no toxicity for the Cry2Ab2 protein. Thus, there are no threshold effects of concern and, as a result, the provision requiring an additional margin of safety does not apply. Further, the considerations of consumption patterns, special susceptibility, and cumulative effects do not apply.

b) Overall Safety Conclusion

There is a reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children, to the Cry2Ab2 protein and the genetic material necessary for its production. This includes all anticipated dietary exposures and all other exposures for which there is reliable information. The Agency has arrived at this conclusion because, as discussed above, no toxicity to mammals has been observed, nor any indication of allergenicity potential for the plant-incorporated protectant.

5. Other Considerations

a) Endocrine Disruptors

The pesticidal active ingredient is a protein, derived from a source that is not known to exert an influence on the endocrine system. Therefore, the Agency is not requiring information on the endocrine effects of this plant-incorporated protectant at this time.

b) Analytical Method(s)

A protocol for an enzyme-linked immunosorbent assay for the detection and quantification of Cry2Ab2 in corn tissue has been submitted, and a commercially available qualitative immunochromatographic test strip was shown to detect the Cry2Ab2 protein in corn tissues.

c) Codex Maximum Residue Level

No Codex maximum residue level exists for the plant-incorporated protectant *Bacillus thuringiensis* Cry2Ab2 protein and the genetic material necessary for its production in corn.

Table 4. Summary of Cry2Ab2 Human Health Data

Study Type/Title	Summary	MRID #
Acute oral toxicity (OPPTS 870.1100)/ An Acute Oral Toxicity Study in Mice with Cry2Ab2 Protein	The acute oral toxicity of the Cry2Ab2 protein was assessed in CD-1 mice. Ten male and 10 female mice received *E. coli*-produced Cry2Ab2 protein at a dose of 2198 mg/kg by oral gavage in two doses (test protein group); ten male and 10 female mice were treated with 2 mM carbonate-bicarbonate, 2mM reduced glutathione buffer (vehicle control group); and ten male and 10 female mice received bovine serum albumin at a dose of 2424 mg/kg (protein control group). Body weight was recorded prior to fasting, prior to dosing, and on days 7 and 14. The test animals were observed for clinical signs of toxicity two times post-dosing and daily for 14 days. A general health/mortality check was done twice daily. All animals were euthanized and necropsied on day 14. All mice survived the study. There were no significant differences in body weight or body weight change among the three groups during the study. The oral LD_{50} for males, females, and combined mice was greater than 2198 mg/kg. This places Cry2Ab2 protein in TOXICITY CATEGORY III because of dose amounts only; no signs of toxicity were observed. **Classification: ACCEPTABLE**	46951406
In vitro digestibility/Assessment of the *in vitro* digestibility of the Cry2Ab2 protein in simulated gastric fluid	The in vitro digestibility of Cry2Ab2 in simulated gastric fluid was investigated. No bands representative of intact Cry2Ab2 protein were identified by SDS-PAGE or western blot analysis after ≥ 30 seconds incubation with simulated gastric fluid containing pepsin. In the stained gel, a faint band with molecular weight ~5 kDa was visible in the 30-second time-point, but not in any other samples. No proteolytic fragments were observed in the western blot. The limit of detection (LOD) for the full-length Cry2Ab2 protein by SDS-PAGE with staining was determined to be 5 ng or approximately 0.6% of the total protein loaded. Therefore, at least 99.4% of the full-length Cry2Ab2 protein was digested within 30 seconds. The LOD for the full-length Cry2Ab2 protein by western blot analysis was determined to be 0.2 ng or 1% of the total protein loaded. Based on the fact that no band was observed for the Cry2Ab2 protein in the 30 second time-point sample in the western blot and the LOD for the protein using this method, it was concluded that at least 99% of the Cry2Ab2 protein was digested within 30 seconds. **Classification: ACCEPTABLE**	46951407
In vitro digestibility/ Assessment of the *in vitro* Digestibility of the Cry2Ab2 Protein in Simulated Intestinal Fluid	The in vitro digestibility of Cry2Ab2 in simulated intestinal fluid containing pancreatin was investigated using western blot analysis. The band for the full-length Cry2Ab2 was below the LOD in the 15 minute time-point sample and in the later time-point samples. The LOD for the full-length Cry2Ab2 protein by western blot analysis was estimated to be 0.5 ng, which was 2.5% of the total protein loaded. Therefore, at least 97.5% of the full-length protein was digested within 15 minutes. Bands from proteolytic fragments with approximate molecular weights of 60, 55, 50, 40, 12, and 10 kDa were visible in the five-minute time-point sample. The bands for all of these proteolytic fragments except for the 50 kDa fragment were undetectable at	46951409

Study Type/Title	Summary	MRID #
	the 24-hour incubation time-point. **Classification: ACCEPTABLE**	
Heat stability/ Immunodetection of Cry2Ab2 and Cry1A.105 Proteins in Corn Grain from MON 89034 Following Heat Treatment [2]	The immunodetectability of the Cry1A.105 and Cry2Ab2 proteins in corn grain from MON 89034 following heat treatment was assessed. MON 89034 and conventional grain were ground, mixed with water, and then heated in an oven at 204 ºC for 20 minutes to simulate the heating process used commercially to process grain. Heated and unheated grain was extracted with two buffers: 50 mM CAPS and 50 mM NLS (CAPS containing 2% N-Lauroyl sarcosine). The extracts were analyzed using western blot to detect the presence of the Cry1A.105 and Cry2Ab2 proteins. The amount of immunodetectable Cry2Ab2 protein in either CAPS or NLS buffer extracts of MON 89034 after heating was below the lower LOD. Based on the LOD and the estimated amount of protein in the unheated extract, it was determined that the Cry2Ab2 protein decreased at least 77% and 70%, respectively following heat treatment, relative to their original values. Likewise, the amount of immunodetectable Cry1A.105 protein in either CAPS or NLS buffer extracts of MON 89034 after heating was below the limit of detection (LOD) and had decreased by at least 94% and 78%, respectively, relative to their original values. This loss is likely due to protein degradation or aggregation into an insoluble complex as a result of heat treatment. **Classification: ACCEPTABLE**	46694607
Amino acid sequence comparison/ Bioinformatic Evaluation of the Cry2Ab2 Protein Utilizing the AD6, TOXIN5, and ALLPEPTIDES Databases	The amino acid sequence of the Cry2Ab2 protein was compared to the sequences of known allergens and toxins using allergen (AD6), toxin (TOXIN5), and public domain (ALLPEPTIDES) databases and the FASTA algorithm. In addition to the FASTA comparisons, the Cry2Ab2 protein sequence was compared to the AD6 database using 8 amino acid sliding blocks and the ALLERGENSEARCH algorithm. No proteins were identified with an *E* score of less than $1x10^{-5}$ from the search using the AD6 database, indicating that Cry2Ab2 has no structural similarity to any known allergens, gliadins, or glutenins. In addition, no matches of 8 amino acids were found between the Cry2Ab2 protein and sequences in the AD6 database. In the comparisons using the TOXIN5 and ALLPEPTIDES databases, the highest similarities identified were with Cry protein homologues derived from *Bacillus thuringiensis*, *Clostridium bifermentans, Paenibacillus popilliae, and Paenibacillus lentimorbus*. The results indicate that the Cry2Ab2 protein does not share sequence homology with any proteins that may have adverse effects in humans or animals.	46951411

D. ENVIRONMENTAL ASSESSMENT for MON 89034

Background

Monsanto has requested a registration for *Bacillus thuringiensis* Cry1A.105 and Cry2Ab2 proteins and the genetic material necessary for their production in all corn lines and varieties. The nptII selectable marker gene was used in the transformation process, but was isolated and removed from transformed plants via traditional breeding. The result is marker-free MON 89034 corn. The Cry proteins expressed in this event are intended to control the lepidopteran pests European corn borer (ECB, *Ostrinia nubilalis*), corn ear worm (CEW, *Helicoverpa zea*), fall army worm (FAW, *Spodoptera frugiperda*), and black cutworm (BCW, *Agrotis ipsilon*) which are primary pests of corn in the United States. These pests feed on the base of seedlings and on the stalk, leaf, and ear tissue of corn plants, thereby destroying the entire plant, weakening the stalk, and/or damaging the ear. In areas where one or more of these pests is prevalent, significant financial losses are realized from decreased corn yields and increased expenditures on chemical pest control agents, including organophosphate, carbamate and pyrethroid insecticides.

EPA has conducted an environmental risk assessment of Cry1A.105, Cry2Ab2 and MON 89034 when expressed in corn. General topics covered in this assessment include effects on wildlife, gene flow to related wild plants and its potential effects, and fate of these Cry proteins in the environment. This assessment is based on data submitted to EPA during the development of Event MON 89034 corn lines, additional data submitted for registration, Federal Insecticide Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) recommendations, consultations with scientific experts, and public comments on Plant-incorporated Protectant (PIP) regulation.

1. Tiered Testing Hazard and Risk Assessment Process

To minimize data requirements and avoid unnecessary tests, risk assessments are structured such that risk is determined first from estimates of hazard under "worst-case" exposure conditions. A lack of adverse effects under these conditions would provide enough confidence that there is no risk and no further data would be needed. Hence, such screening tests conducted early in an investigation tend to be broad in scope but relatively simple in design, and can be used to demonstrate acceptable risk under most conceivable conditions. When screening studies suggest potentially unacceptable risk, additional studies are designed to assess risk under more realistic field exposure conditions. These later tests are more complex than earlier screening studies. Use of this "tiered" testing framework saves valuable time and resources by organizing the studies in a cohesive and coherent manner and eliminating unnecessary lines of investigation. Lower tier, high dose screening studies also allow tighter control over experimental variables and exposure conditions, resulting in a greater ability to produce statistically reliable results at relatively low cost[3].

[3] Non-target invertebrate hazard tests often are conducted at exposure concentrations several times higher than the

Tiered tests are designed to first represent unrealistic worst case scenarios and ONLY progress to real world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk. Screening (Tier I) non-target organism hazard tests are conducted at exposure concentrations several times higher than the highest concentrations expected to occur under realistic field exposure scenarios. This has allowed an endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Less than 50% mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic field exposure scenarios.

EPA uses a tiered (Tiers I-IV) testing system to assess the toxicity of a PIP to representative non-target organisms that could be exposed to the toxin in the field environment. Tier I high dose studies reflect a screening approach to testing designed to maximize any toxic effects of the test substance on the test (non-target) organism. The screening tests evaluate single species in a laboratory setting with mortality as the end point. Tiers II – IV generally encompass definitive hazard level determinations, longer term greenhouse or field testing, and are implemented when unacceptable effects are seen at the Tier I screening level.

Testing methods that utilize the tiered approach were last published by EPA as Harmonized OPPTS Testing Guidelines, Series 850 and 885 (EPA 712-C-96-280, February 1996)[4]. These guidelines, as defined in 40 CFR 152.20, apply to microbes and microbial toxins when used as pesticides, including those that are naturally occurring, and those that are strain-improved, either by natural selection or by deliberate genetic manipulation. EPA has determined that it is appropriate to utilize these testing guidelines in the context of PIPs.

The Tier I screening maximum hazard dose (MHD) approach to environmental hazard assessment is based on some factor (whenever possible >10) times the maximum amount of active ingredient expected to be available to terrestrial and aquatic non-target organisms in the environment (EEC)[5]. Tier I tests serve to identify potential hazards and are conducted in the laboratory at high dose levels that increase the statistical power to test the hypotheses. Elevated test doses, therefore, add certainty to the assessment, and such tests can be well standardized. The Guidelines call for initial screening testing of a single group or several groups of test animals at the maximum hazard dose level. The Guidelines call for testing of one treatment group of at least 30 animals or three groups of 10 test animals at the screening test concentration. The Guidelines further state that the duration of all Tier I tests should be approximately 30 days.

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maximum concentrations expected to occur under realistic exposure scenarios. This has customarily allowed an endpoint of 50% mortality to be used as a trigger for additional higher-tier testing. Lower levels of mortality under these conditions of extreme exposure suggest that population effects are likely to be negligible given realistic exposure scenarios. Thus, it follows that the observed proportion of responding individuals can be compared to a 50% effect to determine if the observed proportion is significantly lower than 50%. For example, using a binomial approach, a sample size of 30 individuals is sufficient to allow a treatment effect of 30% to be differentiated from a 50% effect with 95% confidence using a one-sided Z test. A one-sided test is appropriate because only effects of less than 50% indicate that further experiments are not needed to evaluate risk.

[4] http://www.epa.gov/opptsfrs/publications/OPPTS_Harmonized/885_Microbial_Pesticide_Test_Guidelines/Series/

[5] The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits or other factors. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the test concentration used must exceed 1X EEC.

Some test species, notably non-target insects, may be difficult to culture and the suggested test duration has been adjusted accordingly. Control and treated insects should be observed for at least 30 days, or in cases where an insect species cannot be cultured for 30 days, until negative control mortality rises above 20 percent.

Failing the Tier I (10 X EEC) screening does not necessarily indicate the presence of an unacceptable risk in the field but it triggers the need for additional testing.[6] A less than 50% mortality effect at the MHD is taken to indicate minimal risk. Greater than 50% mortality does not necessarily indicate the existence of unacceptable risk in the field, but it does trigger the need to collect additional dose-response information and a refinement of the exposure estimation before deciding if the risk is acceptable or unacceptable. Where potential hazards are detected in Tier I testing (i.e. mortality is greater than 50%), additional information at lower test doses is required which can serve to confirm whether any effect might still be detected at more realistic field [1X EEC] concentrations and routes of exposure[7].

When screening tests indicate a need for additional data, the OPPTS Harmonized Guidelines call for testing at incrementally lower doses in order to establish a definitive LD_{50} and to quantify the hazard. In the definitive testing, the number of doses and test organisms evaluated must be sufficient to determine an LD_{50} value and, when necessary, the Lowest Observed Effect Concentration (LOEC), No Observed Adverse Effect Level (NOAEL) , or reproductive and behavioral effects such as feeding inhibition, weight loss, etc. In the final analysis, a risk assessment is made by comparing the NOAEL to the EEC; when the EEC is lower than the NOAEL, a no risk conclusion is made. These tests offer greater environmental realism, but they may have lower statistical power. Appropriate statistical methods, and appropriate statistical power, must be employed to evaluate the data from the definitive tests. Higher levels of replication, test species numbers or repetition are needed to enhance statistical power in these circumstances.

Data that show less than 50 % mortality at the maximum hazard dosage level – (i.e., LC_{50}, ED_{50}, or LD_{50} >10 X EEC) is sufficient to evaluate adverse effects, making lower field exposure dose definitive testing unnecessary. It is also notable that the recommended >10X EEC maximum hazard dose level is a highly conservative factor. The published EPA Level of Concern [LOC] is 50% mortality at 5X EEC [8].

[6] It is notable that that the 10 X EEC MHD testing approach is not equivalent to what is commonly known as "testing at a 10X SAFETY FACTOR" where any adverse effect is considered significant. Tier I screen testing is not 'safety factor testing'. In a "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

[7] The 1X EEC test dose is based on plant tissue content and is considered a high worst case dose (sometimes referred to as HEEC). This 1X EEC is still much greater than any amount which any given non-target organism may be ingesting in the field because most non-target organisms do not ingest plant tissue.

[8] Environmental Protection Agency (USEPA) (1998). "Guidelines for Ecological Risk Assessment." EPA 630/R-95-002F. Washington, DC, USA. [Federal Register, May 14, 1998. 63(93): 26846-26924.] The established peer and EPA Science Board reviewed guidance on screening test levels of concern is 50% mortality at 5X environmental concentration. The appropriate endpoints in high dose limit/screening testing are based on mortality of the treated, as compared to the untreated (control) non-target organisms. A single group of 30 test animals may be tested at the maximum hazard dose.

Validation: The tiered hazard assessment approach was developed for EPA by the American Institute of Biological Sciences (AIBS) and confirmed, in 1996, as an acceptable method of environmental hazard assessment by a FIFRA Scientific Advisory Panel (SAP) on microbial pesticides and microbial toxins. The December 9, 1999, SAP agreed that the Tiered approach was suitable for use with plant-incorporated protectants; however, this panel recommended that, for PIPs with insecticidal properties, additional testing of beneficial invertebrates closely related to target species and/or likely to be present in GM crop fields should be conducted. Testing of *Bt* Cry proteins on species not closely related to the target insect pest was not recommended, although it is still performed to fulfill the published EPA non-target species data requirements. In October 2000, another SAP also recommended that field testing should be used to evaluate population-level effects on non-target organisms. The August 2002 SAP, and some public comments, generally agreed with this approach, with the additional recommendation that indicator organisms should be selected on the basis of potential for field exposure to the subject protein.[9]

Chronic studies: Since delayed adverse effects and/or accumulation of toxins through the food chain are not expected to result from exposure to proteins, protein toxins are not routinely tested for chronic effects on non-target organisms. The 30 day test duration requirement does, however, amount to subchronic testing when performed at field exposure test doses. Proteins do not bioaccumulate. The biological nature of proteins makes them readily susceptible to metabolic, microbial, and abiotic degradation once they are ingested or excreted into the environment. Although there are reports that some proteins (Cry proteins) bind to soil particles, it has also been shown that these proteins are degraded rapidly by soil microbial flora upon elution from soil particles.

Conclusion: The tiered approach to test guidelines ensures, to the greatest extent possible, that the Agency requires the minimum amount of data needed to make scientifically sound regulatory decisions. EPA believes that maximum hazard dose Tier I screening testing presents a reasonable approach for evaluating hazards related to the use of biological pesticides and for identifying negative results with a high degree of confidence. The Agency expects that Tier 1 testing for short-term hazard assessment will be sufficient for most studies submitted in support of PIP registrations. If long range adverse effects must be ascertained, then higher-tier longer-term field testing will be required. The Agency has been frequently asking the registrants to

[9] EPA-SAP. February 4, 2000. Characterization and non-target organism data requirements for protein plant-pesticides. SAP report No. 99-06A for FIFRA Scientific Advisory Panel Meeting held December 8, 1999, held at the Sheraton Crystal City Hotel, Arlington, VA.

EPA-SAP. November 6, 2002. Corn rootworm plant-incorporated protectant insect resistance management and non-target insect issues. Transmittal of meeting minutes of the FIFRA Scientific Advisory Panel Meeting held August 27-29 at the Marriott Crystal City Hotel, Arlington, VA.

EPA-SAP. March 12, 2001. *Bt* plant-pesticides risk benefit assessments. SAP report No. 2000-07 for FIFRA Scientific Advisory Panel Meeting held October 18-20, 2000 at the Marriott Crystal City Hotel, Arlington, VA.

EPA-SAP. August 19, 2004. Product characterization, human health risk, ecological risk, and insect resistance management for *Bacillus thuringiensis* (*Bt*) cotton products. Transmittal of meeting minutes of the FIFRA Scientific Advisory Panel Meeting held June 8-10 at the Holiday Inn Ballston, Arlington, VA.

conduct post-registration long term invertebrate population/community and Cry protein accumulation in soils studies as a condition of registration. As noted above, the October 2000 SAP and the National Academy of Sciences[10] (NAS 2000) recommended testing non-target organisms directly in the field. This approach, with an emphasis on testing invertebrates found in crop fields, was also recommended by the August 2002 SAP and was supported by several public comments. The issue of long range effects of cultivation of currently registered Cry proteins on the invertebrate community structure in *Bt* crop fields has since been adequately addressed by a meta analysis of field studies performed during the last 10 years. No unexpected adverse effects on invertebrate community structure were reported.[11] The meta analysis of short term and long term field study effects on invertebrate populations in *Bt* corn and cotton fields indicate that no unreasonable adverse effects are taking place as a result of wide scale *Bt* crop cultivation. The Agency is in agreement with these conclusions. Slight reductions in some invertebrate predator populations are an inevitable result of all pest management practices which result in reductions in the abundance of the pests as prey. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

2. Environmental Exposure Assessment

The EPA risk assessment is centered only on adverse effects at the field exposure rates (1X EEC), and not on adverse effects at greater concentrations. The dose margin can be less than 10x where uncertainty in the system is low or where high concentrations of test material are not possible to achieve due to test organism feeding habits. High dose testing also may not be necessary where many species are tested or tests are very sensitive, although the concentration used must exceed 1X EEC. It is important to note that Tier I screen testing is not "safety factor testing." In a traditional "10X safety factor" test any adverse effect noted is a "level of concern", whereas in the EPA environmental risk assessment scenario any adverse effect is viewed as a concern only at 1X the field exposure.

For the purposes of the nontarget organism (NTO) studies submitted in support of the MON 89034 registration, test material dose levels were based on the estimated concentration of Cry1A.105 and/or Cry2Ab2 protein expressed in the tissue(s) that NTO would most likely be exposed to in the environment (see Edelstein, 2007 for protein expression levels). Whenever possible, a targeted margin of exposure (MOE) of greater than 10X the maximum environmental exposure was used in the tests. The primary route of Cry1A.105 and Cry2Ab2 protein exposure for honeybee, ladybird beetle, parasitic wasp, and minute pirate bug is corn pollen. Consequently, test material dose levels were based on the maximum level of measured protein

[10] *Environmental Effects of Transgenic Plants: The Scope and Adequacy of Regulation* is available from the National Academy Press, 2101 Constitution Avenue, N.W., Lockbox 285, Washington, DC 20055; (800) 624-6242 or (202) 334-3313 (in the Washington metropolitan area); http://www.nap.edu.

[11] Marvier, M., McCreedy, C., Regetz, J. & Kareiva, P. A meta-analysis of effects of *Bt* cotton and maize on nontarget invertebrates. Science 316, 1475–1477 (2007).

Sanvido,O., Romeis, J., Bigler, F. (2007). Ecological Impacts of Genetically Modified Crops: Ten Years of Field Research and Commercial Cultivation. Adv Biochem Engin/Biotechnol 107: 235–278

expression in pollen (8.8 ug/g fwt for Cry1A.105 and 0.47 ug/g fwt for Cry2Ab2). The principal route of Cry1A.105 and Cry2Ab2 protein exposure for soil-dwelling organisms, such as Collembola and earthworms, is assumed to be from decomposing plant tissue and plant exudates in soil. Consequently, the test material dose levels were based on the maximum level of estimated protein concentration in the soil environment.

3. Non-Target Wildlife Hazard Assessment for MON 89034 corn

Two separate SAP reports (October 2000 and August 2002) recommended that non-target testing of *Bt* Cry proteins should focus on invertebrate species exposed to the crop being registered. Following the SAP recommendations, EPA determined that non-target organisms with the greatest exposure potential to Cry protein in transgenic corn fields are beneficial insects, which feed on corn pollen and nectar, and soil invertebrates, particularly Coleopteran species. Therefore, maximum hazard dose toxicity testing on representative beneficial organisms from several taxa was performed in support of this Section 3 FIFRA registration. The toxicity of the Cry1A.105 and Cry2Ab2 proteins has been evaluated on several species of invertebrates including the lady beetle, minute pirate bug, parasitic hymenoptera, collembola, daphnia, honey bee, and earthworm. Developmental observations were also made in the lady beetle, minute pirate bug and honeybee studies. Observations of possible reproductive effects were made in the collembola studies.

Although the Cry1A.105 and Cry2Ab2 proteins in MON 89034 are known to be very host specific, conferring toxic effects on ECB, CEW, FAW, BCW and closely related species, and despite the October 2000 and August 2002 SAP's recommendations against testing of non-target species not related to susceptible target pests, EPA has done a risk assessment on a range of non-target wildlife to comply with the Agency's published non-target data requirements (in the absence of PIP-specific risk assessment guidance, EPA requires applicants for PIP registrations to meet the 40 CFR Part 158 data requirements for microbial toxins). These requirements include birds, mammals, plants and aquatic species. In addition, an earthworm study was voluntarily submitted to the Agency to ascertain the potential effects of the Cry1A.105 and Cry2Ab2 proteins on beneficial decomposer species.

Test substances used for studies submitted in support of the event MON 89034 registration included bacterially produced purified Cry1A.105 and Cry2Ab2 proteins and MON 89034 corn grain. The October 2000 SAP recommended that while actual plant material is the preferred test material, bacterially-derived protein is also a valid test substance, particularly in scenarios where test animals do not normally consume corn plant tissue and where large amounts of Cry protein (Cry protein concentrations that exceed levels present in plant tissue) are needed for maximum hazard dose testing. An insect feeding study, which compared the relative potency of plant produced Cry1A.105 and Cry2Ab2 proteins to the microbe produced proteins, indicated that plant produced protein was similar in toxicity to the microbe produced protein (Edelstein Memo, November 7, 2007).

Potential Interaction between the Cry1A.105 and Cry2Ab2 proteins was addressed in a memorandum for the MON 89034 Experimental Use Permit accompanying the Agency's review of "Evaluation of the Potential for Interactions Between the *Bacillus thuringiensis* Proteins Cry1A.105 and Cry2Ab2," (Hunter, M., July 6, 2006). The purpose of this study was to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2. The study provides evidence that the proteins do not interact in an antagonistic, or synergistic manner and that there will not be any unexpected interaction with regard to target and non-target insects. New data on the potential interaction between combined Cry1A.105, Cry2Ab2 with the Cry3Bb1 protein was submitted. The results from the study demonstrated that combined Cry1A.105 and Cry2Ab2 activity was not affected by the Cry3Bb1 protein and that Cry3Bb1 activity was not affected by combined Cry1A.105 and Cry2Ab2 activity (MRID 469513-05 & 469513-06).

The results of ecological effects studies submitted in support of the MON 89034 Section 3 FIFRA registration are summarized in Table 5 and presented in a more descriptive format in subsequent sections of this risk assessment document. Full reviews of each study can be found in the individual Data Evaluation Reports (DERs/MRID#s) and accompanying memos.

Table 5. Summary of environmental effects studies and waiver justifications submitted to comply with data requirements published in 40 CFR § 158.2150(d).

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
Avian oral	885.4050* 154-16**	Acceptable	MON 89034 corn grain†	A 42-day dietary study showed that Event MON 89034 did not adversely affect broiler chickens.	469514-12
Avian injection	885.4100 154-17	Acceptable waiver rationale	N/A	N/A	N/A
Avian acute oral	850.2100	Acceptable	MON 89034 corn grain	An eight-day dietary study showed that the LC_{50} for MON 89034 is >500,000 ppm in the diet northern bobwhite quail.	469514-27
Wild mammal	885.4150 154-18	Acceptable waiver rationale	N/A	N/A	N/A
Freshwater fish	885.4200 154-19	Acceptable waiver rationale	N/A	Freshwater fish studies were not required because of the low potential that fish will be exposed to high levels of the Cry1A.105 and Cry2Ab2 proteins	N/A
Freshwater aquatic invertebrate *Daphnia magna*	885.4240 154-20	Unacceptable [The 885 Series Guidelines call for a 7-14 day study. The submitted 48 hour acute study is inadequate.]	MON 89034 corn Pollen	A 48-hour static renewal limit bioassay resulted in 17% mortality compared with 0% mortality in the control groups (120 mg/L). A 48-hour static renewal dose-response bioassay was conducted and no mortality or adverse effects were observed at any concentration (6.3-120 mg/L). The acute EC50 was estimated to be >120 mg/L and the NOEC was 100 mg/L.	469514-17

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
Freshwater aquatic invertebrate *Daphnia magna*	885.4240 154-20	Acceptable	MON 89034 corn Pollen	It was determined that the study is acceptable and satisfies the condition of registration for additional aquatic invertebrate acute toxicity testing. No unreasonable adverse effects to aquatic invertebrates are expected from exposure to MON 89034 corn.	478388-01
Estuarine and marine animal	885.4280 154-21	Acceptable waiver rationale	N/A	N/A	N/A
Non-target plant	885.4300 154-22	Acceptable waiver rationale	N/A	N/A	N/A
Non-target insect testing, minute pirate/insidious flower bug *Orius insidiosus*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	*Orius* nymphs were fed a pollen diet containing 100 µg Cry2Ab2 protein/diet for 14 days. No adverse effects were observed.	469514-24
Non-target insect testing, minute pirate/insidious flower bug *Orius insidiosus*	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	*Orius* nymphs were fed a diet containing 30 to 240 µg Cry1A.105/g diet for 14 days. In an initial maximum dose test (240 µg) the survival rate was 47% compared to 88% in the control groups. In the three subsequent dose-response tests, the mean survival rate of the 240 µg group was 55% compared to 91% and 89% in the control groups. No statistically significant effects on survival or development were seen at concentrations less than or equal to 120 µg Cry1A.105/g diet.	469514-23
Non-target insect testing, parasitic wasp, *Ichneumon promissorius*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	Adult female wasps were fed a sucrose solution containing 100 µg Cry2Ab2 protein/mL for 21 days. Mortality in the Cry2Ab2 group was 3% and the LC50 was determined to be >100 µg/L.	469514-26
Non-target insect testing, parasitic wasp *Ichneumon promissorius*	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Adult female wasps were fed a sucrose solution containing 240 µg Cry1A.105 protein/mL for 21 days. Mortality in the Cry1A.105 group was 7% and the LC50 was determined to be >240 µg/L.	469514-25
Non-target insect testing, ladybird beetle *Coleomegilla maculata*	885.4340 154-23	Acceptable	Cry2Ab2 protein (Lot No. 20-100071)	*C. maculata* larvae were fed a diet containing 120 µg Cry2Ab2 protein/g diet for 17-20 days. No statistically significant difference in survival or development to adult was found between the test and control groups. A slight (~5%) statistical decrease in mean adult	469514-22

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
				body weight was found between the test and buffer control groups; however, this difference was not observed between the test and assay control group.	
Non-target insect testing, ladybird beetle *Coleomegilla maculata*	885.4340 154-23	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Ladybird beetle larvae were fed a diet containing 240 µg Cry1A.105 protein/g diet for 14 days. No statistically significant differences in survival, development, or adult beetle weight were found between the test and control groups.	469514-21
Non-target insect testing, collembolan *Folsomia candida*	885.4340 154-23	Acceptable	MON 89034 Leaf Tissue (80 µg Cry1A.105 and 70 µg Cry2Ab2/g diet)	Collembola were fed a diet containing 50% Brewer's yeast and 50% lyophilized leaf tissue for 28 days. No statistically significant effects on survival or reproduction were found among the test and negative control groups.	469514-16
Honeybee testing, Adult Honeybee, *Apis mellifera*	885.4380 154-24	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Adult honeybees were fed a 30% sucrose solution containing 550 µg Cry1A.105 protein/mL for 19 days. No statistically significant differences in mortality were observed between the test group and negative controls. The NOEC was determined to be at least 550 µg Cry1A.105 protein/mL.	469514-20
Honeybee testing, Honeybee larvae, *Apis mellifera*	885.4380 154-24	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Two-to-three day old honeybee larvae in brood frames were administered a single 10 µL dose of Cry1A.105 protein per brood cell (equivalent to 12 µg total protein/cell). On day 18 after dosing mean survival of the test group was 95%. The NOEC was determined to be at least 12 µg Cry1A.105 protein per brood cell	469514-20
Earthworm subchronic toxicity, *Eisenia fetida*	850.620	Acceptable	Cry1A.105 protein (Lot No. 20-100073)	Adult earthworms were exposed to artificial soil containing 178 mg Cry1A.105 protein/kg dry soil for 14 days. No mortality was observed in the test group. The LC50 was determined to be >178 mg Cry1A.105/kg dry soil and the NOEC was 178 mg Cry1A.105 mg/kg dry soil.	469514-18

Data Requirement	Guideline	Classification	Test Substance	Results Summary	MRID #
Soil fate	885.5200	Acceptable	Purified Cry1A.105 (Lot No. 20-100073) and Cry2Ab2 protein (Lot No. 20-100071)	Results of this degradation study indicate that Cry1A.105 and Cry2Ab2 proteins do not persist in soil beyond approximately three weeks.	469514-28

Note: Earthworm and honey bee studies for Cry2Ab2 protein were submitted and reviewed with previously registered products. The interaction study between Cry1A.105 and Cry2Ab2 was reviewed for the MON 89034 Experimental Use Permit

*OPPTS Microbial pesticide test guidelines

**Microbial pesticide test guidelines identified in the 40 CFR data tables.

† Cry1A.105 and Cry2Ab2 are the active ingredients (a.i.) in MON 89034 corn.

a) Non-target Wildlife Study Summaries

i. Avian species

Published data and studies on file at EPA show that consumption of *Bt* corn has no measurable deleterious effects on avian species. To comply with published data requirements, the following studies were submitted to EPA in support of the MON 89034 product registration. These studies were GLP compliant and, when considered together, meet EPA data requirements for avian species.

(a) Broiler (MRID 469514-12)

For the first 42 days of life, commercial broiler chickens (*Gallus domesticus*) were fed a corn and soybean diet that contained up to 59% ground corn grain. Treatments consisted of soybean meal with MON 89034, a similar isoline (negative control), or one of four different commercial hybrid corn varieties. At test end, chickens were processed in order to obtain performance and carcass yield data. Breast and thigh meat were also analyzed for moisture, protein, and fat content. Among treatments, there were no biologically significant differences in broiler performance, carcass, or meat quality.

(b) Northern Bobwhite Quail (MRID 469514-27)

In this eight-day dietary study, 10-day-old northern bobwhites (*Colinus virginianus)* were fed a corn and game bird ration containing 50% ground corn grain. Treatments consisted of game bird ration with MON 89034, a similar isoline (negative control), or one of three different commercial hybrid corn varieties. At test end, no mortality was seen in the MON 89034 treatment group, all birds appeared normal for test duration, and feed consumption was comparable to that of the control group. The dietary LC_{50} of MON 89034 corn grain was determined to be >500,000 ppm in the diet.

ii. Wild mammalian species

Mammalian wildlife exposure to Cry1A.105 and Cry2Ab2 proteins is considered likely; however, mammalian toxicology information gathered to date on *Bt* Cry proteins does not show a hazard to wild mammals. In addition, acute oral toxicity studies submitted to EPA in support of the MON 89034 registration indicated that no significant toxicity was seen when rodents were exposed to Cry1A.105 or Cry2Ab2 at the maximum hazard dose level. Therefore, no hazard to mammalian wildlife is anticipated and data on wild mammal testing is not required for this registration.

iii. Aquatic species

There is no evidence for sensitivity of aquatic species to anti-coleopteran Cry proteins. A published laboratory study with lepidopteran-active Cry proteins has revealed that the leaf shredding (caddis fly) trichopteran, *Lepidostoma liba*, had 50% lower growth rate when fed *Bt* corn litter (Rosi-Marshall, et al. 2007). Two previous field study reports by the same authors did not find adverse effects on head stream invertebrates. The Agency's position on this matter is that until Tier III and Tier IV field studies are performed, there is not enough information to assert that sufficient corn plant litter enters streams to cause unreasonable adverse effects on stream invertebrate populations or communities (See Section D. 1. above - Tiered Hazard and Risk Assessment Process). Two years ago the Iowa State University and the University of Maryland received Research grants to study the effects of *Bt* corn cultivation on streams and to develop methods for aquatic hazard assessment. The results of these studies are pending. When the study reports are reviewed the Agency will respond with action commensurate with the outcome of the studies.

The Agency's current position is that there is no evidence to conclude that there is sufficient aquatic exposure to Cry proteins in corn plant litter to result in adverse effects on stream invertebrate populations or communities. Aquatic animal exposure to *Bt* crops is extremely small.

(a) Freshwater fish-*Waiver granted*

Freshwater fish studies were not required for this product, because of the low potential that aquatic systems will be exposed to the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 corn plant tissues.

(b) Freshwater aquatic invertebrates (MRID 469514-17)

The objective of this study was to determine the potential for acute effects to the aquatic organism, *Daphnia magna*, during a static renewal exposure to MON 89034 corn pollen. The test was initially conducted as a limit test using one test concentration. Slight effects were noted at the limit concentration. In response, a dose-response test was conducted. The test substance, MON 89034 pollen expressing Cry1A.105 and Cry2Ab2 proteins, was evaluated for potential

adverse effects to neonate *Daphnia.* Test organisms were < 24 hours old at test initiation and came from in-house cultures at the test facility.

Limit Test

In the initial test, daphnids were exposed to a single nominal test concentration of 120 mg pollen/L for 48 hours with renewal of the test solution at approximately 24 hours. Two control groups were included: a group in well water exposed to pollen (120 mg/L) from conventional corn with a genetic background similar to MON 89034, and an assay control group exposed to well water only. Each treatment was replicated three times and each replicate contained 10 neonate daphnids. Test chambers consisted of 600-mL glass beakers containing 300 mL of the appropriate treatment solution. Observations of mortality, immobility and other clinical signs were made at approximately 3.5, 24 and 48 hours after test initiation. At test termination there was 17% immobility in the 120 mg/L treatment group, with two daphnids exhibiting lethargy. All daphnids in the assay control group and 120 mg pollen/L control group appeared normal throughout the testing period.

Dose-Response Test

In the dose-response test, daphnids were exposed to six concentrations of MON 89034 pollen for 48 hours. The concentrations tested were 6.3, 13, 25, 50, 100, and 120 mg pollen/L. Two control groups were included: a group in well water exposed to pollen (120 mg pollen/L) from conventional corn with a genetic background similar to MON 89034, and an assay control group exposed to well water only. The test and control solutions were renewed at approximately 24 hours. Each treatment was replicated two times and each replicate contained 10 neonate daphnids. Test chambers consisted of 600-mL glass beakers containing 300 mL of the appropriate treatment solution. Observations of mortality, immobility and other clinical signs were made at approximately 5, 24, and 48 hours after test initiation. The NOEC was estimated by visual interpretation of the mortality, immobility and observation data. At test termination there were no mortalities, immobile daphnids or signs of toxicity noted in any control or test substance group during the 48 hour exposure period.

Conclusions: Based on the results of the dose-response test, the 48-hour EC_{50} was estimated to be greater than 120 mg MON 89034 pollen/L. Based on the results of both studies, the 48-hour NOEC was 100 mg MON 89034 pollen/L. This study is unacceptable because it is an 850 Series OPPTS Guideline study. The 48 hour test duration is not sufficient to show mortality for *Bt* toxins. The 48 hours test duration is not considered to be sufficient duration to assess the potential for adverse effects to non-target organisms. Consistent with the 885 Series OPPTS Guidelines, a 7 to 14 day *Daphnia* study is necessary. The study may be submitted as a condition of registration. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, may be performed and submitted in lieu of the *Daphnia* study.

(c) Freshwater Aquatic Invertebrates (MRID 478388-01)

When *Bt* Cry1A.105, and Cry2Ab2 Protein in corn (EPA Reg. No. 524-575, and 524-576) were initially registered, the Agency issued registration notices to Monsanto Company that contained the following requirement for further Environmental Assessment information:

> "A 7-14 day *Daphnia* study as per the 885 Series OPPTS Guidelines needs to be performed. Alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams, can be performed and submitted in lieu of the *Daphnia* study."

Due to the fact that there was a statistically significant reduction in survival rate in the pollen control group when compared to the assay control group, the study author concluded the exposure to high concentrations of conventional corn pollen resulted in impacts on the overall health of daphnids. It was therefore considered most appropriate to compare performance by the MON 89034 treatment groups to the pollen control group. Based on the immobility and sublethal signs of toxicity noted in the pollen control group, effects on survival noted in the 8.3 and 42 mg/L pollen from MON 89034 treatment groups were not considered to be related to toxicity of MON 89034, but were considered to be due to physical toxicity caused by high concentrations of pollen in the solutions. Reductions in survival in the 8.3 and 42 mg/L pollen from MON 89034 treatment groups at the Day 14 interval when compared to the pollen control group were not statistically significant ($p > 0.05$). The 14-day median effect concentration (EC_{50}) and no observed effect concentration (NOEC) values for adult immobility when compared to the pollen control were determined to be >42 mg/L and 42 mg/L pollen from MON 89034, respectively.

From the results, there was no indication of a delay in the onset of reproduction in any of the treatment groups. Observations of immobile neonates in the 42 mg/L pollen control and 42 mg/L treatment group may have been related to high pollen concentrations in the test solutions, but were not considered by the study author to be related to toxicity of MON 89034. The NOEC for reproduction was considered to be 42 mg/L pollen from MON 89034 when compared to the pollen control group.

Conclusions: It was determined that the study is acceptable and satisfies the condition of registration for additional aquatic invertebrate acute toxicity testing. No unreasonable adverse effects to aquatic invertebrates are expected from exposure to MON 89034 corn.

(d) Estuarine and marine animals-*Waiver granted*

Estuarine and marine animal studies were not required for this product, because of the low probability that estuarine or marine systems will be exposed to the Cry1A.105 and Cry2Ab2 proteins produced in MON 89034 corn plant tissues.

iv. Terrestrial and aquatic plant species-*Waiver granted*

Plant toxicity studies were not required for this product because the active ingredient is an insect toxin (*Bt* endotoxin) that has never shown any toxicity to plants.

v. Terrestrial Invertebrate species

The Cry1A.105 and Cry2Ab2 proteins are meant to target species within the order Lepidoptera (moths and butterflies). *Bt* toxins are known to have a limited host range, however, to address any unforeseen change in activity spectrum and to fulfill the published registration data requirements EPA requires that test species used for non-target insect evaluations should include several species that are not related to the target pests. Earthworm studies are also recommended.

(a) Ladybird beetle

MRID 469514-21
The purpose of this study was to determine the potential dietary effects of the Cry1A.105 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata*. The test substance, Cry1A.105 protein, was produced by a recombinant *E. coli* fermentation system. The test substance was incorporated at 240 µg Cry1A.105 protein/g of diet. The diet consisted of an artificial agar-based diet. Three control treatments were included in the experiment. The buffer control contained 25 mM CAPS buffer, which was the buffer used for storage of the test material. The assay control (purified water) was used to generate a diet-only treatment and a positive control treatment was also tested, containing potassium arsenate. Ladybird beetle larvae were less than 48 hours old at test initiation. The larvae were contained in individual test arenas (inverted 60 x 15 mm Petri dishes) and were allowed to feed *ad libitum* on the appropriate test diet. Each treatment was replicated six times and each replicate contained 15 or 16 larvae. All six replicates met the acceptance criteria of less than or equal to 20% mortality; the mortality of larvae ranged from 0 to 18.8% in the assay control treatment groups. The diet treatments were replaced with fresh diet approximately every 48 to 72 hours. The larvae were monitored every 24 to 72 hours for survival and development to the adult stage. Adults were weighed within 30 hours of eclosion and each adult was dissected and sexed. Any abnormal behavior or development was noted during feedings and observational evaluations. The study duration ranged from 17 to 20 days depending on adult emergence. Samples were taken to test the biological activity, homogeneity, and stability of the Cry1A.105 protein. Results showed that there were no differences in the mean survival percentage of *C. maculata* between the Cry1A.105 protein, buffer control, and assay control treatments (88.5%, 87.5%, 91.6%). The survival rate was 2.08% for the positive control treatment. There were no significant differences in the mean percent of *C. maculata* that developed to adults when comparing the Cry1A.105, buffer control, and assay control treatments (88.5%, 85.4%, 90.6%). None of the larvae developed to adults in the positive control treatment. Further, there were no significant differences in the mean weight of *C. maculata* adults between the Cry1A.105, buffer control, and

assay control. No significant interaction was found between insect sex and treatment. In the bioactivity confirmation assay, results indicated equivalent biological activity among the Cry1A.105 used in the test diets and the reference standard. In addition, the homogeneity and stability study confirmed that Cry1A.105 was homogeneous in the test substance diet and was stable under the storage conditions employed in the study.

Conclusions: This study is acceptable. The results indicate that Cry1A.105 protein had no adverse effect on the survival, development, and growth of the ladybird beetles at a dietary concentration of 240 µg/g of diet.

MRID 469514-22

The objective of this study was to determine the potential dietary effects of Cry2Ab2 protein on the mortality and development of the ladybird beetle, *Coleomegilla maculata*. The test substance, Cry2Ab2, was produced by recombinant *E. coli* fermentation system. The endpoints evaluated were survival and development through 20 days (some replicates were completed before 20 days if all insects had developed to adults). The Cry2Ab2 protein was incorporated in to an agar-based artificial diet at a concentration of 120 µg Cry2Ab2/g diet. Three control treatments were included in the study: 1) buffer control, 2) assay control (purified water), and 3) positive control (potassium arsenate). The ladybird beetle larvae were less than 48 hours old when testing began and the larvae were allowed to feed *ad libitum*. The diet treatments were replaced approximately every 48 to 72 hours. Each treatment was replicated six times and each replicate included 14 to 16 ladybird beetle larvae. Each larva was contained in its own test arena which consisted of an inverted 60 x 15 mm Petri dish. The larvae were monitored every 24 to 72 hours for survival and development to the adult stage. Adults were weighed within 30 hours of eclosion and adults were sexed. The biological activity, homogeneity, and stability of the Cry2Ab2 protein in the diet were confirmed in a separate bioassay using *Helicoverpa zea*. The mean survival for *C. maculata* was 94.7% for the Cry2Ab2 treatment, 88.8% for the buffer control treatment, 91.6% for the assay control, and 2.08% for the positive control. The mean percent of larvae that developed to adults was 92.6% in the Cry2Ab2 treatment, 85.3% for the buffer control treatment, and 90.6% for the assay control. None of the larvae developed to adults in the positive control treatment. The mean adult weights for the test material and groups were about 5% lower buffer control than those of the assay control group, which was a statistically significant difference. However, there was no significant difference in adult weight of the test material and buffer control groups.

Conclusions: This study is acceptable. No adverse effects were seen in *C. maculata* fed 120 µg Cry2Ab2 protein/g diet. Although the mean adult weight of the Cry2Ab2 protein treatment group was statistically significantly lower than that of the assay control group, the difference was slight (~5%) and there was no significant difference between the weight of the Cry2Ab2 protein group and buffer control groups.

(b) Minute pirate bug

MRID 469514-23

The purpose of this study was to determine the potential dietary effects of Cry1A.105 protein on mortality and development of *Orius insidiosus*, the minute pirate bug or insidious flower bug. The Cry1A.105 protein (*E. coli*-produced) was incorporated into a pollen-based diet for treatment of the test group. Both a buffer control diet and an assay control diet (pollen diet only) were included in the study. A positive control group was fed a diet treated with potassium arsenate. The initial test involved dosing the insects with a single maximum dose level (240 µg Cry1A.105/g diet) for 14 days, resulting in 47% survival. The assay and buffer control resulted in 88% survival. A total of 75 *Orius* were tested in each treatment. Based on the results of the maximum hazard dose assay, three 14-day dose-response tests were conducted with test substance exposure levels of 30, 60, 120 and 240 µg Cry1A.105/g diet. Again, a buffer control, assay control, and positive control were included in each of the three tests. Each exposure test was conducted independently at a different time using separate groups of *Orius*. During the test, *Orius* were supplied with a capsule (50 µL) of the appropriate diet and the capsules were replaced every other day. The test arenas consisted of 1-ounce plastic cups with plastic covers and each cup contained one *Orius*. For each dose-response exposure, 25 test arenas were included for each diet treatment. Observations and feeding behavior were recorded each feeding day for each test arena. Results of the first replicate resulted in percent survival in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet was 88, 84, 88 and 56%, respectively. Percent survival in the assay, buffer, and positive control was 92, 88, and 36%, respectively. The percent of nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and potassium arsenate control organisms was 92, 100, 96, 92, 96, 96 and 40%, respectively. No statistically significant differences were detected between the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet. Percent survival in the second replicate in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet was 88, 92, 92, and 52%, respectively. Percent survival in the assay and buffer controls was 88% and percent survival in the positive control was 32%. The percent of nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and positive control organisms was 100, 92, 96, 92, 96, 92 and 96%, respectively. The mean number of days to development for all treatments was 6.0 days. Percent survival in the third replicate in the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet treatments was 92, 80, 80, and 56%, respectively. Percent survival in the assay, buffer and positive controls was 92 and 28%, respectively. The percent of nymphs developing to adults for the 30, 60, 120 and 240 µg Cry1A.105 protein/g diet, assay control, buffer control and positive control organisms was 100, 96, 100, 92, 100, 100 and 80% respectively. The mean number of days to development for all treatments was 6.0 days. Throughout the study samples of the test and control substances were taken to be used in a bioassay with *Helicoverpa zea* to confirm the presence of the test substance, homogeneity of the test substance in the diet, diet stability and bioactivity of the test material. The bioassay confirmed that the test substance was stable throughout the study, was biologically active at anticipated levels and was appropriately mixed in the test diet.

Conclusions: This study is acceptable. *Orius insidiosus* were exposed for 14 days to a range of dietary concentrations of Cry1A.105. For the three dose-response replicates the mean survival for the 240 µg Cry1A.105/g diet treatments was 55%. Therefore, the LC_{50} value was empirically determined to be greater than 240 µg Cry1A.105/g diet. No adverse effects were observed at concentrations less than or equal to 120 µg Cry1A.105/g diet.

MRID 469514-24
The purpose of this study was to determine the potential dietary effects of Cry2Ab2 protein on mortality and development of *Orius insidiosus*. The Cry2Ab2 protein was incorporated into a pollen-based diet at a concentration of 100 µg Cry2Ab2 protein/g diet. The protein was produced by a recombinant *E. coli* fermentation system. Both a buffer control treatment and an assay control (pollen-based diet only) were included in the study. In addition, a positive control was included which consisted of the pollen-diet treated with potassium arsenate. The duration of the experiment was 14 days which was long enough to observe the *Orius* develop from nymph to adult. The test insects were approximately 3 days old at test initiation. Each treatment contained seventy-five insects and each insect was contained in its own test arena. During the exposure period, one capsule of approximately 50 µL of the appropriate test diet was provided in each test arena on Day 0 and every other day thereafter for the duration of the test. Observations and feeding behavior were recorded each day for each test arena. The biological activity, homogeneity, and stability of Cry2Ab2 protein in the test diet were tested and confirmed in a separate bioassay using *Helicoverpa zea*. The percent survival of insects exposed to the 100 µg Cry2Ab2 protein/g diet treatment was 91%, which was similar to the percent survival of the insects in the buffer and assay control groups. The percent of nymphs developing to adults in the 100 µg Cry2Ab2 protein/g diet treatment, assay control, buffer control and positive control was 93, 95, 91, and 73%, respectively. The mean number of days to develop to adult for insects exposed to the 100 µg Cry2Ab2 protein/g diet, assay control, buffer control and positive control treatments was 6.1, 7.1, 8.0 and 6.0 days, respectively.

Conclusions: This study is acceptable. No adverse effects were observed for *Orius insidiosus* at the concentration level of 100 µg Cry2Ab2 protein/g diet. Therefore, the LC_{50} is greater than 100 µg Cry2Ab2 protein/g diet.

(c) Parasitic hymenoptera

MRID 469514-25
This study was conducted to evaluate the potential effects of acute exposure of Cry1A.105 protein to the parasitic wasp, *Ichneumon promissorius*. The Cry1A.105 protein was administered to the wasps at a concentration of 240 µg/mL in a 30% sucrose solution. The protein was produced by a recombinant *E. coli* fermentation system. Three control treatments were included in the experiment: 1) buffer control, 2) assay control (sucrose solution only), and 3) positive control (potassium arsenate). The positive control substance was tested at two concentrations (100 and 400 ppm). There were three replications per treatment and each replication contained 10 female wasps. The wasps were 3 to 6 days old at the time of test

initiation. The test chambers were disposable 64 ounce containers. Observations of mortality and clinical signs were conducted once within the hour of test initiation and then continued daily until Day 21 of the test. Samples of the assay control, control substance and protein group diets were collected for analysis by bioassay to test for bioactivity and stability of the Cry1A.105 protein. Mortality in the assay control, buffer control, and test material treatments was 10%, 8%, and 7% respectively. All surviving wasps in those groups appeared normal in appearance and behavior. There was no statistically significant difference in mean mortality in the Cry1A.105 treatment and buffer control treatments. The biological activity and stability of the Cry1A.105 protein was confirmed in a seven-day bioassay using the corn earworm (*Helicoverpa zea*).

Conclusions: This study is acceptable. The LC_{50} for *Ichneumon promissorius* was greater than 240 µg Cry1A.105 protein/mL and the NOEC was at least 240 µg Cry1A.105 protein/mL.

MRID 469514-26

A laboratory bioassay was conducted to evaluate the potential effects of acute exposure to Cry2Ab2 protein to the parasitic wasp *Ichneumon promissorius*. The Cry2Ab2 protein used was produced by an *E. coli* fermentation system. Wasps were exposed to the Cry2Ab2 at a concentration of 100 µg/L in a 30% sucrose solution. Three control groups were utilized, including: 1) buffer control, 2) negative assay control group (sucrose solution only) and 3) two positive controls using two concentrations of potassium arsenate. The test diets were prepared by diluting 60% (w:v) sucrose solution with equal amounts of solutions containing test and control substances in deionized water to o*Bt*ain diets with approximately 30% sucrose. Test diet containing Cry2Ab2 protein was prepared weekly. The wasps were given fresh diet daily. Three replicate test chambers were used for each treatment and control group and 10 female wasps were contained in each test chamber. The test chambers consisted of disposable 64 oz. polypropylene containers. The wasps were approximately 3 to 6 days old at test initiation. Observations were made once during the hour of test initiation and once daily until Day 21 of the test. Samples of the assay control, control substance and protein group diets were collected for analysis by bioassay. The biological activity relative to a reference standard and stability of the Cry2Ab2 protein in the test diet was confirmed in a seven-day corn earworm (*Helicoverpa zea*) bioassay. At test termination (Day 21), mortality in the assay control, buffer substance, and test substance groups was 10%, 3%, and 3%, respectively. All surviving wasps were normal in appearance and behavior. No statistically significant differences in mean mortality were found between the Cry2Ab2 treatment group and the negative control group.

Conclusions: This study is acceptable. The LC_{50} for *Ichneumon promissorius* was determined to be >100 µg Cry2Ab2 protein/mL and the NOEC was at least 100 µg Cry2Ab2 protein/mL.

(d) Collembola (MRID 469514-16)

The objective of this study was to determine the potential effect of chronic exposure of lyophilized corn leaf tissue from MON 89034 maize on the survival and reproduction of *Folsomia candida*. The study sponsor verified the identity and the concentrations of Cry1A.105

and Cry2Ab2 in the lyophilized leaf material. The concentration of Cry1A.105 was 160 µg/g lyophilized leaf dry weight and the concentration of Cry2Ab2 was 140 µg/g lyophilized leaf dry weight. The lyophilized test material was incorporated in to a diet containing 50% Brewer's yeast and 50% test material (0.500g leaf tissue with 0.500 g yeast). Therefore, the test diet contained Cry1A.105 at a nominal concentration of 80 µg/g lyophilized leaf dry weight and Cry2Ab2 at a nominal concentration of 70 µg/g lyophilized leaf dry weight. A control diet was prepared by mixing 50% control leaf tissue, by weight, with 50% Brewer's yeast. An additional control treatment consisted of a test diet containing only Brewer's yeast. Three positive control treatments were included. The three treatments included three treatments of thiodicarb, representing nominal concentrations of 1.0, 10 and 100 mg a.i./kg. Collembola were provided enough food such that an excess was always available. Each treatment contained four replicates and each replicate initially contained 10 juvenile Collembola (12 days old). Each replicate was contained in a glass jars containing a water saturated substrate consisting of plaster of Paris and charcoal at a ration of 8:1 by weight. The biological activity and concentration of Cry1A.105 protein were confirmed in samples collected at the end of dosing. The bioassay confirmed that the test material was biologically active against CEW and the level of activity was not significantly different from that of the reference standard. Mortality and observations of sublethal effects of the surviving Collembola were recorded on day 28 (test termination). Collembola were removed from the test arenas and the number of adult and young Collembola were counted. Among the yeast-only diet control organisms, mean survival was 98% and mean reproduction was 170 offspring per arena. Mean survival of Collembola exposed to the control diet (control leaf tissue) and MON 89034 diet was 100 and 98%, respectively. The mean number of offspring produced in the control substance diet (control leaf tissue) and the MON 89034 diet was 260 and 257 offspring per arena, respectively. Mean survival in the positive control substance treatments 1.0, 10 and 100 mg thiodicarb/kg diet was 95, 63 and 35% respectively. The mean number of offspring produced in the 1.0, 10 and 100 mg thiodicarb/kg diet treatments was 136, 93, and 16 offspring per arena, respectively. Statistical analysis demonstrated no significant reduction in survival or reproduction among Collembola exposed to the MON 89034 diet when compared to either negative control.

Conclusions: The study is acceptable. The NOEC for *Folsomia candida* is at least 80 µg Cry1A.105 and 70 µg Cry2Ab2 per gram of diet.

(e) Honeybee

MRID 469514-19

The objective of this study was to evaluate potential dietary effects of Cry1A.105 protein when administered to honeybee larvae (*Apis mellifera*). Honeybees were approximately 2 to 3 days old during the experiment. The test substance was Cry1A.105 protein produced by an *E. coli* fermentation system. The protein was used to prepare a test solution at a concentration of 1200 µg/mL. This concentration is equivalent to 12 µg total protein per cell. Additional treatment groups included an assay control, buffer control, and two reference substance concentrations (potassium arsenate at low and high doses). Each treatment included four replications of 20 bees

for a total of 80 bees per treatment. The larvae were exposed to a single dose (10 mL) of the appropriate dosing solution at initiation and observed during larval and pupal development. Survival of larvae was assessed at study completion (Day 18) by observing adult emergence. The Cry1A.105 treatment group resulted in 95% survival. Survival in the assay control and buffer control was 92.5%. Adult emergence in the low and high dose potassium arsenate treatments was 26.5% and 5.0%, respectively. To verify test concentration and bioactivity of the Cry1A.105 protein, samples of the test material were taken at test initiation. A bioassay using *Helicoverpa zea* was conducted to test for bioactivity and no significant difference was observed between the test substance and the Cry1A.105 reference standard.

Conclusions: This study is acceptable. The NOEC for Cry1A.105 protein to honey bee larvae was determined to be at least 12 µg Cry1A.105 protein/cell.

MRID 469514-20

An acute bioassay was conducted to determine the effects of Cry1A.105 protein on adult honeybees (*Apis mellifera*). The study was initiated a total of five times, with the first four attempts resulting in early termination (high control mortality). Adult bees were approximately five days old at the start of the bioassay. The test material was *E. coli*-produced Cry1A.105 protein supplied by the study sponsor. The protein was used to prepare a 30% sucrose solution containing 550 µg Cry1A.105 protein/mL. A buffer control diet was prepared by combining the buffer solution with stock sucrose solution producing a 12.5 mM buffer in a 30% sucrose solution. An assay control diet was also prepared and consisted of only the 30% sucrose solution. A positive control diet was prepared and contained 100 µg/mL potassium arsenate in a solution of 30% sucrose. Honeybees were maintained in cages that were approximately 12.7 cm on each side. To induce clustering, a small cone of beeswax was attached to the cage cover and extended down into the cage. The diet was provided via an inverted 12 mL glass vial fitted with a plastic screw cap containing two ~1.0 mm holes. Each treatment group included 270 adult honeybees in six replicates of 45 adult honeybees per replicate. The number of dead bees in each cage was assessed on a daily basis. The study acceptance criteria stipulated that the assay be terminated at 30 days or when the adult control mortality reached 30%. The 30% criterion was met between Day 18 and 19 and the study was terminated on Day 20. On Day 18, the buffer control treatment produced significantly higher mortality (37.41%) than either the sucrose (20.00%) or the Cry1A.105 treatments (20.37%). Mortality in the Cry1A.105 treatment was not statistically different than the sucrose treatment on Day 18. On Day 19, no significant differences were detected among the three treatment groups (buffer, sucrose, and Cry1A.105 protein) with mortalities of 52.22%, 51.48%, and 47.04%, respectively. The potassium arsenate treatment resulted in 100% mortality by Day 2 of the study. A diet incorporation assay using the Cry1A.105 test diet was conducted to confirm the bioactivity of the protein. The biological activity of the test substance was evaluated using *Helicoverpa zea* and compared with the biological activity of a Cry1A.105 reference standard. In addition, the control substance and the assay control substance were evaluated in the diet incorporation assay. The bioassay confirmed the test diet contained the expected level of Cry1A.105 activity.

Conclusions: This study is acceptable. The NOEC for Cry1A.105 protein fed to adult honey bees is at least 550 µg/mL.

(f) Earthworm (MRID 46954-18)

The objective of this study was to evaluate the potential effects of acute exposure of Cry1A.105 protein administered to earthworms (*Eisenia fetida*) during a 14-day exposure period. In the test, earthworms were exposed to a single concentration of Cry1A.105 protein that was incorporated into an artificial soil substrate. The Cry1A.105 protein used in this study was *E. coli* produced. The concentration of the test substance was 178 mg Cry1A.105 protein/kg soil dry weight. A total of four control treatments were included in the study, including: 1) buffer solution control, 2) assay control group containing neither test substance or buffer solution, 3) positive control group exposed to 15 mg chloroacetamide/kg dry soil and 4) additional positive control group exposed to 30 mg chloroacetamide/kg dry soil. Each treatment was replicated four times and each replicate contained 10 earthworms. Test chambers consisted of one-liter glass beakers covered with plastic wrap what was perforated for air exchange. The artificial soil was prepared in bulk by blending 70% sand, 20% kaolin clay and 10% sphagnum peat. Each test container contained 750 grams of prepared soil. The worms were not provided food during the test period. At test initiation (Day 0), the worms were placed on the surface of the soil and observed for 30 minutes to assess burrowing behavior. On Days 7 and 14, the contents of each test chamber were removed to determine the number of surviving worms. On Day 7, following observations, the test soil was returned to the test chambers and the worms were placed on the soil surface in order to observe burrowing behavior. On Day 14, following observations and body weight determinations, surviving earthworms were euthanized. Samples of soil were collected from each treatment (except positive controls) and saved to verify the presence or absence of biological activity. This was done by conducting a bioassay with *Helicoverpa zea*. The bioactivity of the Cry1A.105 treated soil was also compared against a reference standard of Cry1A.105 provided by the study sponsor. There were no mortalities in the assay control group, buffer control group, or Cry1A.105 protein group. In the 15 mg chloroacetamide/kg reference group there was 48% mortality and in the 30 mg chloroacetamide/kg reference group there was 100% mortality at test termination. A slight loss in average individual body weight from test initiation to test termination was noted in all test groups and was expected since the worms were not fed during the 14-day test. Losses in body weight in the Cry1A.105 protein test substance group were not statistically significant when compared to the control substance group. Analysis of the test soil showed that Cry1A.105 was present in the soil and was biologically active against *Helicoverpa zea*.

Conclusions: This study is acceptable. The 14-day LC_{50} for earthworms was determined to be greater than 178 mg Cry1A.105 protein/kg dry soil. The NOEC was determined to be greater than 178 mg Cry1A.105 protein/kg dry soil.

vi. Soil Fate (MRID 469514-28)

Soil organisms may be exposed to Cry1A.105 and Cry2Ab2 protein through contact with corn plant roots (by direct feeding), corn plant root exudates, incorporation of above-ground plant tissues into soil following harvest, or by soil-deposited pollen. Some evidence suggests that soils which are high in clays and humic acids are more likely to bind Cry protein. However, neutral pH soils (above pH 5.6), that are typical of corn production sites, tend to have high microbial activity and microbes contribute to Cry protein degradation. Despite evidence that soils high in clay and humic acids may bind Cry proteins, and thus interfere with the microbial degradation processes, the weight of evidence indicates that Cry proteins do not accumulate in soil to arthropod-toxic levels. Nonetheless, the Agency required the following soil fate evaluations to support the MON 89034 *Bt* corn registration.

A study of Cry protein degradation in soil evaluated clay, silt loam, and loamy sand soils that were spiked with Cry2Ab2 (0.60 µg/g) or Cry1A.105 (0.062 µg/g) protein and incubated under controlled conditions for four months. The soils were dosed with an approximately 500-fold excess of the maximum calculated protein concentrations in the field. Samples of the treated soils were collected eleven times during the incubation period and analyzed for protein content using western blot analysis (Cry2Ab2 only) and a corn earworm bioassay. Results indicated that Cry2Ab2 protein concentration decreased by 50% in about 1 to 6 days, and by 90% in about 3 to 14 days in the three soils. The amount of Cry1A.105 protein decreased by 50% in about 2 to 7 days and by 90% in about 7 to 19 days in the same soils.

This study utilized field soil spiked with purified insecticidal protein. This approach is useful because dose responses can be easily quantified. But, the degradation and accumulation of Cry proteins found within decaying plant tissue may behave differently than proteins in artificially spiked soil. Thus, the presence of low levels of Cry protein in the soil (at or below the level of detection) is anticipated until all plant tissue is 'mineralized'. The data reviewed here do, however, show that Cry proteins will be quickly degraded upon release from decaying plant tissue. More specifically, a study that evaluated Cry1Ab protein accumulation in a field with three years of continuous Cry1Ab field corn production showed that the protein had not accumulated in soil to a level that would elicit a toxic response from ECB larvae, a species that is highly susceptible to Cry1Ab protein (Milofsky, 2006).

As a result of FIFRA Scientific Advisory Panel recommendations and public comments, the Agency has been receiving three year soil fate studies for the currently registered Cry protein producing crops grown in a variety of soils and environmental conditions. The results of these studies show that there is no detectable Cry protein accumulation in agricultural soils during commercial planting of currently registered Cry protein producing crops. Therefore no additional long term soil degradation studies are required for Cry2Ab2 or Cry1A.105 proteins.

vii. Effects on Soil Microorganisms

Numerous published studies indicate that exposure to Cry protein produced in *Bt* PIP crop plants does not adversely affect soil microorganisms (Sanvido *et al*. 2007; Oliveira *et al*. 2008). In addition, *Bt* toxin released from root exudates and biomass of *Bt* corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil (Saxena and Stotzky 2001). Other research findings conclude no *Bt*-related risks have evolved from the decomposition of *Bt*-corn leaves for the meso- and macrofauna soil community (Hönemann *et al*. 2008). Although a minimal transient increase and shift in microbial populations may result from the presence of transgenic plant tissue in soil, no adverse effects have been attributed to the Cry protein.

In addition, there are several ongoing U.S. Department of Agriculture and EPA Office of Research and Development funded research projects evaluating the effects of *Bt* crops on soil microbial flora. If adverse effects are seen from this or any other research, the Agency will take appropriate action to mitigate potential risks.

With regard to the impact of genetically engineered crops on soil, it is important to note that agricultural practices themselves cause large changes in soil and soil microbial composition. Furthermore, factors such as variations in seasons and weather, plant growth stage, and plant varieties, independent of being genetically engineered, are also responsible for significant shifts in soil microbial communities. To date, most studies with genetically engineered crops have shown minor or no effects on soil microbes beyond the variation caused by the factors listed above.

4. Horizontal Transfer of Transgenes from *Bt* Crops

EPA has evaluated the potential for horizontal gene transfer (HGT) from *Bt* crops to soil organisms and has considered possible risk implications if such a transfer were to occur. Genes that have been engineered into *Bt* crops are mostly found in, or have their origin in, soil-inhabiting bacteria. Soil is also the habitat of anthrax, tetanus, and botulinum toxin-producing bacteria. Transfer of these genes and/or toxins to other microorganisms or plants has not been detected. Furthermore, several experiments (published in scientific journals), that were conducted to assess the likelihood of HGT, have been unable to detect gene transfer under typical environmental conditions. Horizontal gene transfer to soil organisms has only been detected with very promiscuous microbes under laboratory conditions designed to favor transfer.

As a result of these findings, which suggest that HGT is at most an artificial event, and the fact that the *Bt* toxins engineered into *Bt* Cry1A.105, and Cry2Ab2 Protein in corn are derived from soil-inhabiting bacteria, EPA has concluded that there is a low probability of risk from HGT of transgenes found in *Bt* Cry1A.105, and Cry2Ab2 Protein in corn.

5. Gene Flow and Weediness Potential

The movement of transgenes from the host plant into weeds has been a significant concern for the Agency due to the possibility of novel exposures to the pesticidal substance. The Agency has determined that there is no significant risk of gene capture and expression of Cry34/35Ab1 protein by wild or weedy relatives of corn in the U.S., its possessions, and/or its territories. In addition, the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) has made this same determination under its statutory authority under the Plant Pest Act.

Under FIFRA, the Agency has reviewed the potential for gene capture and expression of *Bt* endotoxins by wild or weedy relatives of corn, cotton, and potatoes in the U.S., its possessions, and/or its territories. *Bt* plant-incorporated protectants that have been registered to date have been expressed in agronomic plant species that, for the most part, do not have a reasonable possibility of passing their traits to wild native plants. Feral species related to these crops, as found within the United States, cannot be pollinated by these crops (corn, potato, and cotton) due to differences in chromosome number, phenology (i.e., periodicity or timing of events within an organism's life cycle as related to climate, e.g., flowering time) and habitat. The only exception, however, is the possibility of gene transfer from *Bt* cotton to wild or feral cotton relatives in Hawaii, Florida, and the Caribbean.

The Scientific Advisory Panel meeting held on October 18–20, 2000 further discussed the matter of gene flow and offered some issues for consideration in this matter. The panel agreed that the potential for gene transfer between corn (maize) and any receptive plants within the U.S., its possessions, and/or its territories was of limited probability and nearly risk free.

Concern over the potential for species related to maize (*Zea mays* ssp. *mays*), such as *Tripsacum* species and the teosintes, as potential recipients of gene flow from genetically modified *Zea mays* indicated a need for review of what is known related to gene flow potential of *Z. mays*. Some *Zea* species, such as the teosintes, are known to be interfertile with maize and are discussed as potential recipients of pollen-directed gene flow from maize. This issue is of particular concern based upon the increased planting of genetically modified maize. Therefore, the Agency conducted a reevaluation in early 2000, the results of which are reported here.

a. *Zea mays* ssp. *mays* - Maize - General Biology

Zea mays is a wind-pollinated, monoecious, annual species with imperfect flowers. This means that spatially separate tassels (male flowers) and silks (female flowers) are found on the same plant, a feature that limits inbreeding. A large variety of types are known to exist (e.g., dent, flint, flour, pop, sweet) and have been selected for specific seed characteristics through standard breeding techniques. Maize cultivars and landraces are known to be diploid (2n = 20) and interfertile to a large degree. However, some evidence for genetic incompatibility exists within the species (e.g., popcorn x dent crosses; Mexican maize landraces x Chalco teosinte). *Zea mays*

has been domesticated for its current use by selection of key agronomic characters, such as a non-shattering rachis, grain yield, and resistance to pests. The origin of corn is thought to be in Mexico or Central America, based largely on archaeological evidence of early cob-like maize in indigenous cultures approximately 7,200 years ago.

A recent study has indicated that cross-pollination of commercial maize cultivars at 100 feet downwind from the source of genetically modified maize was 1%, and this proportion declined exponentially to 0.1% at 130 feet and further declined to 0.03% at 160 feet. At 1,000 feet, the farthest distance measured, no cross-pollination was detected (Jemison and Vayda 2000). For production of Foundation Seed, a distance of 660 feet has been generally required to mitigate outcrossing between different genotypes. The relatively large size of corn pollen and its short viability period under most conditions reduce long distance transfer for purposes of outcrossing (Schoper, personal communication, 1999). Under conditions of high temperature or low humidity, corn pollen may only survive for a matter of minutes. Under more favorable conditions in the field or with controlled handling in the laboratory, pollen life may be extended to several hours.

b. *Tripsacum* species - Gama Grass - General Biology

Close relatives of corn or maize are found in the genus *Tripsacum*. Sixteen species of *Tripsacum* are known worldwide and generally recognized by taxonomists and agrostologists; most of the 16 different *Tripsacum* species recognized are native to Mexico, Central America, and South America, but three occur within the U.S. Hitchcock (1971) reports the presence of three species of *Tripsacum* in the continental United States: *Tripsacum dactyloides*, *Tripsacum floridanum*, and *Tripsacum lanceolatum*. Of these, *T. dactyloides*, Eastern Gama Grass, is the only species of widespread occurrence and of any agricultural importance. It is commonly grown as a forage grass and has been the subject of some agronomic improvement (i.e., selection and classical breeding). *T. floridanum* is known from southern Florida, and *T. lanceolatum* is present in the Mule Mountains of Arizona and possibly southern New Mexico.

For the species occurring in the United States, *T. floridanum* has a diploid chromosome number of 2n = 36 and is native to Southern Florida; *T. dactyloides* includes 2n = 36 forms, which are native to the central and western U.S., and 2n = 72 forms, which extend along the Eastern seaboard and along the Gulf Coast from Florida to Texas but which have also been found in Illinois and Kansas; these latter forms may represent tetraploids (x = 9 or 18)(Lambert, personal communication, 1999); and *T. lanceolatum* (2n = 72), which occurs in the southwestern U.S. *Tripsacum* differs from corn in many respects, including chromosome number (*T. dactyloides* n = 18; *Z. mays* n = 10). Many species of *Tripsacum* can cross with *Zea*, or at least some accessions of each species can cross, but only with difficulty and the resulting hybrids are primarily male and female sterile (Duvick, personal communication, 1999; Galinat 1988; Wilkes 1967). *Tripsacum*/maize hybrids have not been observed in the field but have been accomplished in the laboratory using special techniques under highly controlled conditions.

Eastern Gama Grass is considered by some to be an ancestor of *Z. mays* or cultivated maize (Mangelsdorf 1947), while others dispute this (Galinat 1983; Iltis 1983; Beadle 1980), based largely on the disparity in chromosome number between the two species (maize n = 10; Gama Grass x = 9 or 18, with diploid, triploid, and tetraploid races existing; 2n = 36 or 72), as well as radically different phenotypic appearance. Albeit with some difficulty, hybrids between the two species have been made (Mangelsdorf and Reeves 1939; DeWald, personal communication, 1999). In most cases, these progeny have been sterile or viable only by culturing with *in vitro* "embryo rescue" techniques.

Even though some *Tripsacum* species occur in areas where maize is cultivated, gene introgression from maize under natural conditions is highly unlikely, if not impossible (Beadle 1980). Hybrids of *Tripsacum* species with *Z. mays* are difficult to o*Bt*ain outside of the controlled conditions of laboratory and greenhouse. Seed obtained from such crosses are often sterile or progeny have greatly reduced fertility. Approximately 10–20% of maize-*Tripsacum* hybrids will set seed when backcrossed to maize, and none are able to withstand even the mildest winters. The only known case of a naturally occurring *Zea - Tripsacum* hybrid is a species native to Guatemala known as *Tripsacum andersonii*. It is 100% male and nearly 99% female sterile and is thought to have arisen from gene flow to teosinte, but the lineage is uncertain (Doebley, personal communication, 2000). *Z. mays* is not known to harbor properties that indicate it has weedy potential and, other than occasional volunteer plants in the previous season's corn field, maize is not considered as a weed in the U.S.

In a telephone conversation with Dr. Chester "Chet" DeWald (Agricultural Research Service of the USDA; Woodward, Oklahoma), a geneticist working on improvement of grasses, he stated that relatively few accessions of *T. dactyloides* will cross with maize, and the majority of progeny are not fertile or viable even in those that do. In controlled crosses, if the female parent is maize, there is a greater likelihood of obtaining viable seed. When these hybrids have been backcrossed to maize in attempts to introgress *Tripsacum* genes for quality enhancement or disease resistance, the *Tripsacum* chromosomes are typically lost in successive generations. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the chromosomal complements of one of the parent species in subsequent generations.

Only recently has Dr. DeWald (or anyone else) succeeded in obtaining a true *Tripsacum* cytoplasm with a maize nuclear background. This was done by using gama grass as the female parent and maize as the male or pollen donor. Numerous accessions were tested and crosses made before this came to fruition. The *Tripsacum*-derived mitochondrial chondrome and chloroplast plastome in these hybrids contribute to the seed qualities of the plants, but the nuclear genome appears to be totally maize in origin (DeWald *et al*. 1999).

Dr. DeWald concluded that the possibility of maize contributing genetic material to Eastern Gama Grass through random pollen flow in agricultural or natural situations is extremely remote based upon his experience trying to create hybrids under the best of conditions. He also felt that

no other known grass species present in the continental U.S. would interbreed with commercial maize populations (i.e., be recipients of pollen-directed gene flow). This is in agreement with Holm *et al*. (1979), who determined that none of the sexually compatible relatives of corn in the U.S. are considered to be serious, principal, or common weeds in the U.S.

c. *Zea* species - Teosintes - General Biology

Teosintes—specifically *Zea mays* ssp. *mexicana* (Schrader) Iltis, *Zea mays* ssp. *parviglumis* Iltis and Doebley, *Zea mays* ssp. *huehuetenangensis* (Iltis and Doebley) Doebley, *Zea luxurians* (Durieu and Ascherson) Bird, *Zea perennis* (Hitchc.) Reeves and Mangelsdorf, and *Zea diploperennis* Iltis, Doebley and Guzman—have co-existed and co-evolved in close proximity to maize in the Americas over thousands of years; however, maize and teosinte maintain distinct genetic constitutions despite sporadic introgression (Doebley 1990).

The teosintes retain a reduced cob-like fruit/inflorescence that shatters more than cultivated maize but still restricts the movement of seeds as compared to more widely dispersed weedy species. Hence, the dispersal of large numbers of seeds, as is typical of weeds, is not characteristic of teosintes or maize. In their native habitat, some teosintes have been observed to be spread by animals feeding on the plants. Teosintes and teosinte-maize hybrids do not survive even mild winters and could not propagate in the U.S. Corn Belt. Additionally, some types have strict day length requirements that preclude flowering within a normal season (i.e., they would be induced to flower in November or December) and, hence, seed production under our temperate climate (Beadle 1980; Iltis, personal communication, 2000; Wilkes, personal communication, 2000; Wilkes 1967).

Since both teosinte and *Tripsacum* are included in botanical gardens in the U.S., the possibility exists (although unlikely) that exchange of genes could occur between corn and its wild relatives. The Agency is not aware, however, of any such case being reported in the United States. Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time within cultivated corn hybrids and landraces. Plant architecture and reproductive capacity of the intercrossed plants will be similar to normal corn, and the chance that a weedy type of corn will result from gene flow with cultivated corn is extremely remote.

Like corn, *Z. mays* ssp. *mexicana* (annual teosinte) and *Z. diploperennis* (diploid perennial teosinte) have 10 pairs of chromosomes, are wind pollinated, and tend to outcross but are highly variable species that are often genetically compatible and interfertile with corn, especially when maize acts as the female parent. *Z. perennis* (perennial teosinte) has 20 pairs of chromosomes and forms less stable hybrids with maize (Edwards *et al*. 1996; Magoja and Pischedda 1994). Corn and compatible species of teosinte are capable of hybridization when in proximity to each other. In Mexico and Guatemala, teosintes exist as weeds around the margins of corn fields. The F_1 hybrids have been found to vary in their fertility and vigor. Those that are fertile are capable of backcrossing to corn. A few isolated populations of annual and perennial teosinte were said to exist in Florida and Texas, respectively (USDA APHIS 1997). The Florida populations were

presumably an escape from previous use of *Z. mays* ssp. *mexicana* as a forage grass, but local botanists have not documented any natural populations of this species for approximately twenty-five years (Bradley, personal communication, 2000; Hall, personal communication, 2000; Wunderlin, personal communication, 2000).

Consultation with botanists and agronomists familiar with Texas flora suggested that no teosinte populations exist in the state (Benz, personal communication, 2000; Read, personal communication, 2000; Orzell, personal communication, 2000; Wilson, personal communication, 2000). Further, given the day length characteristics of *Z. diploperennis*, it is highly unlikely a sustaining population would result from introduction of this species. *Z. mays* ssp. *mexicana*, *Z. mays* ssp. *parviglumis*, *Z. luxurians*, and *Z. diploperennis* may cross with maize to produce fertile hybrids in many instances (Wilkes 1967). None of these teosinte species have, however, been shown to be aggressive weeds in their native or introduced habitats (Schoper, personal communication, 1999). Except for special plantings as noted above, teosinte is not present in the U.S. or its territories. Its natural distribution is limited to Mexico, Honduras, Nicaragua, El Salvador, and Guatemala.

Given the cultural and biological relationships of various teosinte species and cultivated maize over the previous two millennia, it would appear that significant gene exchange has occurred (based upon morphological characters) between these two groups of plants, and that no weedy types have successfully evolved as a result. More recent cytogenetic, biochemical, and molecular analyses have indicated that the degree of gene exchange is far less than previously thought (Doebley 1984; Doebley *et al*. 1987; Kato 1997a; Kato 1997b; Smith *et al*. 1985). Partial and complete gametophytic incompatibility has been documented among cultivated maize, landraces, and teosinte (Kermicle 1997). The former is demonstrated by differential pollen growth and a skewed recovery of alleles linked to incompatibility genes. Complete incompatibility mechanisms serve to isolate a species or subspecies and are evidenced as pollen exclusion or non-functioning of pollen types on certain genotypes. Attempts to cross six collections of Z. mays ssp. *mexicana* with U.S. maize cultivars (W22, W23) yielded no or few seeds in five of the six groups (Kermicle and Allen 1990).

Based on the ability of maize to hybridize with some teosintes, the suggestion of previous genetic exchange amongst these species over centuries, and their general growth habits, any introgression of genes into wild teosinte from *Z. mays* is not considered to be a significant agricultural or environmental risk. The growth habits of teosintes are such that the potential for serious weedy propagation and development is not biologically plausible in the United States.

d. Conclusion

The potential for pollen-directed gene flow from maize to Eastern Gama Grass is extremely remote. This is evidenced by the difficulty with which *T. dactyloides* x *Z. mays* hybrids are produced in structured breeding programs. Additionally, the genus does not represent any species considered as serious or pernicious weeds in the United States or its territories. Any introgression

of genes into this species as a result of cross fertilization with genetically modified maize is not expected to result in a species that is weedy or difficult to control. In many instances where hybridization has been directed between these two species, the resultant genome is lacking in most or all of the maize chromosomal complement in subsequent generations.

Many of the *Zea* species loosely referred to as "teosintes" will produce viable offspring when crossed with *Z. mays* ssp. *mays*. None of these plants are known to harbor weedy characteristics and none of the native teosinte species, subspecies, or races are considered to be aggressive weeds in their native or introduced habitats. In fact, many are on the brink of extinction where they are indigenous and will be lost without human intervention (i.e., conservation measures). Further, none of the landraces or cultivated lines of *Z. mays* are considered to have weedy potential and are generally considered to be incapable of survival in the wild as a result of breeding practices (i.e., selection) during domestication of the crop.

6. Impacts on Endangered Species

The primary route of exposure to Cry1A.105 and Cry2Ab2 proteins in corn is through ingestion of corn tissue. There are no reports of threatened or endangered species feeding on corn plants, therefore such species would not be exposed to corn tissue containing Cry protein. Since Cry1A.105 and Cry2Ab2 proteins have not been shown to have toxic effects on mammals, birds, plants, aquatic species, insects outside the order Lepidoptera and other invertebrate species at the Estimated Environmental Concentration (EEC), a "may affect" situation for endangered land and aquatic species is not anticipated. In addition, EPA does not expect that any threatened or endangered plant species will be affected by outcrossing to wild relatives or by competition with such entities. Hybrid corn does not exist in the wild, nor are there wild plants that can interbreed with corn in the United States.

Because of the selectivity of Cry1A.105 and Cry2Ab2 proteins for lepidopteran species, endangered species concerns are mainly restricted to the order Lepidoptera. Examination of an overlay map showing the county level distribution of endangered/threatened lepidopteran species (currently listed by the U.S. Fish and Wildlife Service) relative to corn production counties in the United States clearly indicated that any potential concern regarding range overlap with corn production was mainly restricted to the Karner blue butterfly (*Lycaeides melissa samuelis*). Research demonstrates that the Cry1A.105 and Cry2Ab2 proteins are selectively toxic to lepidopteran larvae at field concentrations and that the Karner Blue butterfly is the only endangered lepidopteran species that may be exposed to MON 89034 (via pollen). A model developed to assess the risk of *Bt* corn to Monarch butterfly larvae was used to assess the risk of MON 89034 to Karner blue larvae. Based on the LC_{50} value for larvae of the most sensitive known lepidopteran species (ECB) and the maximum estimated level of Cry protein in pollen-contaminated food (9.27 µg/g fresh weight), the margin of safety was calculated to be >10X maximum estimated exposure of Karner blue larvae to corn pollen. These results indicate that cultivation of MON 89034 is not likely to pose a risk to endangered species.

After careful review of available data, EPA determined that exposure of the Karner blue butterfly to harmful levels of MON 89034 corn plant tissues is not expected. Likewise, a review of the preferred habitats of other lepidopteran species listed as endangered by the U.S. Fish and Wildlife Service indicated that exposure to harmful levels of Cry1A.105 or Cry2Ab2 protein would not take place. The main reasons for the lack of exposure are geographical and habitat limitations. These species are located in non-corn production areas and/or their habitat does not encompass agricultural areas.

Likewise, other insect species in the orders Diptera, Hemiptera, Lepidoptera, Odonata and Orthoptera that are listed as endangered/threatened species are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production, often times due to the specificity of the habitat of their host plants. The reviewed toxicological data shows the relative insensitivity of a range of insects in non-lepidopteran orders to the Cry1A.105 and Cry2Ab2 proteins, indicating that MON 89034 maize hybrids are not likely to have detrimental effects on non-lepidopteran insects included on the endangered/threatened species list.

In light of the above considerations (based on no spatial and temporal overlap), the Agency has determined that registered uses of MON 89034 corn will have No Effects (NE), direct or indirect, on endangered and threatened species or their habitat as listed by the United States Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS), including mammals, birds or terrestrial and aquatic plants and invertebrate species. Therefore, no consultation with the USFWS is required under the Endangered Species Act.

Current ecological effects data and EPA reviews of *Bt* Cry1A.105, and Cry2Ab2 Protein in corn support the Agency's determination that adverse effects will not occur to nontarget organisms. Due to a demonstrated lack of toxicity and/or exposure, no effects from *Bt* Cry1A.105, and Cry2Ab2 Protein in corn are anticipated for any nontarget species, including federally-listed threatened and endangered ("listed") lepidopteran and coleopteran species and their designated critical habitats. EPA has also determined that there are no indirect effects on endangered and threatened plant species, such as impacts on lepidopteran pollinators that are important and/or essential to an endangered or threatened plant. The Agency is therefore upholding its determination that the registered uses of *Bt* Cry1A.105, and Cry2Ab2 Protein in corn will have "No Effect," direct or indirect, on endangered or threatened terrestrial or aquatic species as listed by the U.S. Fish and Wildlife Service (USFWS) and the National Marine Fisheries Services (NMFS).

7. MON 89034 Corn Environmental Risk Assessment Conclusions

The EPA uses a Maximum Hazard Dose Tiered system for biopesticide non-target wildlife hazard assessment. When no adverse effects at the maximum hazard screening dose are observed on representative non-target species, the Agency concludes that there are no unreasonable adverse effects on non-target populations from the use of the pesticide.

a) Direct Effects

At present, the Agency is aware of no identified significant adverse effects of Cry protein on the abundance of non-target organisms in any population in the aquatic or terrestrial field environment, whether they are animals, plants, pest parasites, pest predators, or pollinators. Further, EPA believes that cultivation of MON 89034 corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, MON 89034 corn requires substantially fewer applications of chemical pesticides, compared to production of non-*Bt* corn. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers. In addition, no adverse effect on Federally listed endangered and threatened species is expected from the proposed lepidopteran-resistant corn registration. Further, EPA has determined that there is no significant risk of gene capture and expression of Cry1A.105 or Cry2Ab2 proteins by wild or weedy relatives of corn in the U.S., its possessions, or territories (see Section 5. "Gene Flow and Weediness Potential" above), available data do not indicate that Cry proteins have any measurable adverse effect on microbial populations in the soil (see Section vii. "Effects on Soil Microorganisms" above), nor has horizontal transfer of genes from transgenic plants to soil bacteria been demonstrated (see Section 4. **"**Horizontal Transfer of Transgenes from *Bt* Crops**"** above). In conclusion, this risk assessment finds no hazard to the environment at the present time from cultivation of MON 89034 corn for a time-limited registration.

b) Indirect Effects:

The purpose of using PIP plants is the same as for any other pest management tactic, i.e., to reduce pest populations below economic injury levels. As a result the abundance of pest insects should be significantly reduced and this will have corresponding implications for those organisms that exploit these pests as prey and hosts. Thus, the potential for these indirect ecological effects on biological control organisms should not be regarded as a unique ecological risk associated with the PIP crop. Some reductions, however, should be expected if the pest management strategy is effective. Since PIP crops are often grown in vicinity with conventional crops to prevent resistance build-up by the target pest(s), specialist antagonists can persist in these 'refuges', in other crops and in non-crop habitats and retain the potential for recolonization of the PIP crop area. Based on these considerations, regulatory testing of the specialist predators and parasitoids of target pests may eventually be considered unnecessary.

c) Supplemental Data Needed to Confirm MON 89034 Non-Target Hazard Assessment

The Agency has sufficient information to believe that there is no risk from the proposed uses of MON 89034 corn to non-target terrestrial wildlife, aquatic, and soil organisms. The Agency has been frequently asking the registrants to conduct post-registration long term invertebrate population/community and Cry protein accumulation in soils studies as a condition of

registration. The issue of long range effects of cultivation of these Cry proteins on the invertebrate community structure in corn fields has since been adequately addressed by the analysis of field studies performed during the last 10 years (Marvier, et al. 2007; Sanvido, et al. 2007). No unexpected adverse effects on invertebrate community structure were reported. The Agency is in agreement with these conclusions. Similarly, no unexpected accumulation of Cry proteins in agricultural soils was seen in published studies (Icoz and Stotzky 2007; Sanvido, et al. 2007) and in numerous studies submitted directly to the EPA for the currently registered Cry proteins. (Milofsky, 2006; See Section vi. "Soil Fate" above).

In light of published laboratory studies showing reduced growth in shredding caddis flies exposed to anti-lepidopteran Cry1A protein corn litter (Rosi-Marshall, et al. 2007), additional aquatic invertebrate data were required when these products were initially registered The submitted study (MRID 478388-01) satisfies this requirement. As discussed earlier, no unreasonable adverse effects to aquatic invertebrates are expected from exposure to MON 89034 corn.

8. Potential Interaction Between Cry1A.105, Cry2Ab2 and Cry3Bb1 Proteins (MRID 469513-05 & 469513-06)

The purpose of these studies was to characterize the potential for interaction between the lepidopteran-active proteins Cry1A.105 and Cry2Ab2 and the coleopteran-active protein Cry3Bb1. The Cry1A.105 and Cry2Ab2 proteins were tested alone and in combination with either the Cry3Bb1 protein against European corn borer (ECB, *Ostrinia nubilalis*) and corn ear worm (CEW, *Helicoverpa zea*) in diet incorporation studies. Also, the Cry3Bb1 protein was tested alone and with the Cry1A.105 and/or the Cry2Ab2 proteins, against the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*). The activity of Cry1A.105 and Cry2Ab2 proteins was not significantly altered by the presence of Cry3Bb1, and the activity of Cry3Bb1 was not significantly altered by the presence of Cry1A.105 and/or Cry2Ab2. Collectively these data provide evidence that the proteins do not interact in an antagonistic or synergistic manner. This study, along with the interaction study between Cry1A.105 and Cry2Ab2 reviewed for the MON 89034 Experimental Use Permit indicate that MON 89034 x MON 88017 maize will not result in any unexpected interaction in an antagonistic, or synergistic manner with regards to target insects. It is therefore extremely unlikely that the Cry1A.105, Cry2Ab2 and Cry3Bb1 proteins contained in a single plant will impart any hazard to non-target organisms exposed to these hybrids in the environment.

E. INSECT RESISTANCE MANAGEMENT (IRM)

1. IRM Assessment for the Initial Registration of MON 89034 and MON 89034 x MON 88017

This section presents the original assessment for MON 89034 (based on BPPD's review -- see BPPD, 2007). Subsequent to registration, Monsanto submitted an amendment request with additional IRM data to support a 5% lepidopteran refuge. This amendment and BPPD's assessment are detailed in section 2 of the Insect Resistance Management Assessment. Please note that the assessment in section 1 contains some IRM program elements that have been superseded by the amendment discussed in section 2.

Monsanto developed through the use of genetic engineering, MON 89034, a corn product that produces the *Bacillus thuringiensis* (*Bt*)-derived insecticidal proteins Cry1A.105 and Cry2Ab2. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton. MON 89034 is protected from damage caused by larval feeding of *Ostrinia nubilalis* (European corn borer; ECB), *Diatraea grandiosella* (southwestern corn borer; SWCB) and *Diatraea saccharalis* (sugarcane borer; SCB), *Spodoptera frugiperda* (fall armyworm; FAW), and *Helicoverpa zea* (corn earworm; CEW). Monsanto presented data to support its proposed IRM plan for MON 89034. Monsanto wished to demonstrate that: (1) resistance to Cry1A.105 and Cry2Ab2 proteins in MON 89034 is expected to be at least partially recessive; (2) the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low; and (3) the level of both Cry1A.105 and Cry2Ab2 produced in MON 89034 confer high level of control of susceptible target pests (*in vitro* and *in planta).* Monsanto originally proposed a 5% structured refuge in the U.S. Corn Belt (currently, a 20% structured refuge) and a 20% structured refuge in cotton growing regions (currently, a 50% structured refuge) to mitigate insect resistance to the Cry1A.105 and Cry2Ab2 proteins. Simulation modeling was provided to support this plan. BPPD's technical analysis of Monsanto's proposed IRM plan for MON 89034 is described below.

a) Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins

The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton. There are a number of *Bt* corn products on the market that produce the insecticidal proteins, Cry1Ab and Cry1Fa (potential cross resistance with Cry1A.105/Cry2Ab2 and Cry1Ab is discussed in section c, "Impact of Prior Use of Cry1Ab-Expressing *Bt* Corn Products on MON 89034"). There are also *Bt* cotton products that produce the Cry1Ac, Cry1F, and Cry2Ab2 insecticidal proteins. Mathematical models indicate that the IRM values of a *Bt* corn product with two insecticidal proteins, like MON 89034, would be the greatest if there is a low probability of cross-resistance (See Roush 1998). Cross-resistance is most likely when proteins share key

structural features, which allows one resistance mechanism to confer resistance to more than one protein (Tabashnik, 1994; Gould et al., 1995).

There are three models that have been proposed to explain the mode of action of Cry1A toxin mode of action (see discussion in Piggott and Ellar, 2007). The most accepted Bravo model proposes that both the cadherin and aminopeptidase (APN) receptors are required for full Cry1A toxicity. This model suggests that receptor binding is sequential: 1) ingestion of the protein inclusions by a susceptible insect larva, 2) solubilization of the protein in the insect midgut, 3) cleavage of the protoxin by host proteases and release of the active toxin, 4) binding of the active toxin to specific receptors on the midgut epithelieum, 5) oligomerization of toxin subunits to form pore structures that inject into the membrane, 6) passage of ions and water through the pores, resulting in swelling, lysis, and the eventual death of the host. Differences in any of these steps will reduce the probability of cross-resistance between any two Cry proteins. The more controversial Zhang model suggests that receptor binding activates an Mg^{+}-dependent signaling cascade that promotes cell death. The Jurat-Fuentes model suggests that cytotoxicity is due to the combined effects of osmotic lysis and cell signaling. The latter two models are, at present, more speculative.

Resistance associated with modification of the binding site receptor has been the primary *Bt* resistance mechanism reported to date (reviewed in Ferré & Van Rie 2002). Other *Bt* resistance mechanisms have been reported that are based on alterations in the proteases that cleave the protoxin, processing it into a smaller active toxin (Candas et al. 2003) and most recently, the discovery that esterases can bind and detoxify *Bt* toxins (Gunning et al. 2005). Only the binding reduction mechanism has a demonstrated causal link between the biochemical modification and resistance (Ferré and Van Rie 2002). Ferré and Van Rie (2002) indicate that in all cases of binding site modification, resistance is due to a recessive or partially recessive mutation in a major autosomal gene, and cross-resistance extends only to Cry proteins sharing binding sites. Cry proteins that do not share high levels of sequence similarity tend to have different binding sites and different modes of action. Analyses of resistance to *Bt* Cry proteins indicate that cross-resistance occurs most often with proteins that are similar in structure (Tabashnik, 1994; Gould et al., 1995).

With this information in mind, Monsanto has assessed the probability of cross-resistance between Cry1A.105 and Cry2Ab2 on three levels: 1) structural similarity between the proteins, which is indicative of mode of action; 2) characterization of elements of the mode of action, such as the biophysical nature of binding of the *Bt* proteins to the target insect midgut; and 3) demonstration that the individual proteins are effective in controlling resistance to the other protein. Results of these efforts are discussed below.

The first piece of the analysis relates to whether the Cry1A.105 protein has high sequence similarity with the Cry2Ab2 protein. Monsanto provided BPPD with a summary of current information about the structural and functional similarities of the Cry1A.105 protein to other *Bt* Cry1 proteins. The Cry1A.105 protein is a chimeric protein with overall amino acid

sequence identity to the Cry1Ac, Cry1Ab and Cry1Fa proteins of 93.6, 90.0 and 76.7%, respectively. The Cry1A.105 protein expressed in MON 89034 corn plants results in increased activity against FAW, SCB, and CEW compared to Cry1Ab expressed in MON 810 corn plants (see BPPD review of efficacy data, Matten, 2007; Monsanto study MRID# 46951415). A structural model of the Cry1A.105 protein was developed using the X-ray crystal structure of the Cry1Aa protein. This model demonstrated high overall main chain structural similarity with Cry1Aa. Models of Cry1Ab and Cry1Ac were also prepared using the Cry1A.105 model. Comparison of the aligned folds of all three proteins showed that Cry1Ab and Cry1A.105 have essentially the same main chain structure (i.e., similar three domain structures) and that Cry1Ac differs slightly in its main chain structure from the other two in domain III. Thus, comparison of the modeled crystal structures of the Cry1A.105, Cry1Ab, and Cry1Ac with the experimental Cry1Aa X-ray crystal structure demonstrated high three-dimensional structural similarity between the four proteins (i.e., Cry1A.105, Cry1Ab, Cry1Ac, and Cry1Fa).

In the case of Cry1A.105 and Cry2Ab2 proteins, however, there is only a 14% amino acid sequence similarity. Based on the available data, Monsanto has sufficiently demonstrated that there is low sequence similarity between the Cry1A.105 protein and the Cry2Ab2 protein. Lack of sequence similarity would suggest that cross-resistance between the Cry1A.105 and Cry2Ab2 proteins would be unlikely. On the other hand, high sequence similarity between the Cry1A.105 protein and Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa proteins is one indicator that cross-resistance may be a concern for these proteins. This is important because Cry1A.105 is composed of domains I and II and the C-terminus of Cry1Ac and domain III of Cry1Fa. This subject will be discussed further in the review.

Previous studies have shown that Cry1A proteins are activated by proteolytic cleavage of the C-terminal domain and the N-terminus of domain I in the insect gut. In contrast, Cry 2A proteins are activated by cleavage of the N-terminus of domain I and the C-terminal part of domain III. Different activation mechanisms would tend to decrease the likelihood of cross-resistance between the Cry1A and Cry2A proteins.

Assessment of binding characteristics is one way of determining the potential for cross-resistance between the two proteins. As noted above, changes in the nature of protein binding to the insect midgut is the mode of action step that has most often been associated with insect resistance to *Bt* Cry proteins (for reviews, see Tabashnik, 1994; Baxter et al., 2005). Biacore is used to quantify the interaction kinetics of *Bt* proteins with the insect brush border membranes (BBM). Competitive and non-competitive binding may not always be distinguished by Biacore and other analyses, such as ligand blotting, may be used. Ligand blotting is a qualitative tool used to identify protein bands that have the specific secondary modification to bind *Bt* proteins. Monsanto used both Biacore and ligand blotting to characterize Cry1A.105 and Cry2Ab2 binding to ECB brush border membranes (studies by

Li and Guzov, 2006 were provided in Appendix 1 of Monsanto submission, MRID# 469514-30, Head, 2006 and are discussed below).

Binding constants for the interaction of Cry1A.105 and Cry2Ab2 with immobilized BBMV (ECB) differed by more than an order of magnitude with essentially no BBMV-specific binding being observable for Cry2Ab2. The Biacore system could not distinguish unique aspects of non-competitive binding for Cry1A.105 and Cry2Ab2 on BBMV. This result suggests that there are very different binding sites for Cry1A.105 and Cry2Ab2 in the ECB midgut. Additional Biacore analyses indicated that Cry1A.105 and Cry2Ab2 bound to different glycosyl moieties linked to bovine serum albumin (BSA). Cry1A.105 preferentially bound to galactosamine (K_D=1.5x10^{-8}M and R_{max}=2419 RU). Cry2Ab2 preferentially bound to N-acetyl glucosamine (K_D=7.0x10^{-11}M and R_{max}=32 RU), but also bound galactosamine with a K_D =2.0 x10^{-8}M and R_{max}=625 RU. Furthermore, Cry1A.105 binding to galactosamine filled a two-binding-site model as evidenced by the reduction in the Chi^2 value from 2583 to 53, but the fit of Cry2Ab2 binding was similar for both models suggesting that the Cry2Ab2 and Cry1A.105 proteins not only bind to different sugars but also differ in their binding kinetics.

The ligand blotting analysis demonstrated that the Cry1A.105 and Cry2Ab2 proteins bound to different components on ECB brush border membrane filaments (BBMF) separated by SDS-Page and immobilized on a nitrocellulose membrane. Trypsin-treated Cry1A.105 protein was shown to bind to a ~150 kDa band while the trypsin-treated Cry2Ab2 protein was shown to a ~130 kDa band, but weakly to a ~150 kDa band. The trypsin-treated Cry2Ab2 protein had a greater rate of binding than the Cry1A.105 protein. Overall these results support the conclusion, as Monsanto has described, that the Cry2Ab2 and Cry1A.105 proteins displayed different binding components and different kinetics in binding to ECB BBMF. These results are consistent with the differences in binding affinity for Cry1A.105 and Cry2Ab2 proteins observed with Biacore. In addition, Monsanto noted that Cry2Aa did not bind to a specific, high affinity Cry1Ac receptor in work performed by English et al. (1994).

In conclusion, Biacore and ligand blotting analyses demonstrate that Cry1A.105 and Cry2Ab2 proteins bind to some unique components on ECB brush border membranes. They also share many common binding sites. Screening a limited number of glycosylated BSAs, indicated that galactosamine is recognized by Cry1A.105 only, while Cry2Ab2 demonstrated a high affinity for both N-acetylglucosamine and galactosamine. These data support the conclusion that *Bt* protein binding to carbohydrate moieties is the principal basis of the specific interactions between the Cry1A.105 and Cry2Ab2 proteins and the ECB brush border membrane. Specific binding of *Bt* proteins to the target insect gut membrane is a key step in their mode of action. Differences in the Cry1A.105 and Cry2Ab2 protein interactions with the BBM suggest that these two proteins have differences in mode of action. BPPD

agrees with Monsanto that these differences should minimize the development of cross-resistance by the target insect pests to these two proteins.

Monsanto also provided evidence to show that there is a lack of cross-reactivity between Cry1A.105 and Cry2Ab2 antibodies. The homologous primary-secondary antibody pairs recognized only their corresponding antigens (i.e., trypsin-treated Cry1A.105 or Cry2Ab2) with no cross-reactivity. Similarly, Monsanto previously demonstrated that anti-Cry2Ab antibodies do not cross-react with the Cry1Ac proteins, nor do the anti-Cry1Ac antibodies cross-react with the Cry2Ab2 protein (Head and Reding 2001, MRID# 455457-01). The lack of cross-reactivity shows that the epitope binding sites for antibody recognition are different and therefore the tertiary structure is different. Lack of similar tertiary structure supports the conclusion that there will be a very low likelihood of high levels of cross-resistance in the target insect pests for the Cry1A.105 (and all Cry1A proteins) and Cry2Ab proteins. Monsanto provided indirect information (i.e., there are no colonies of lepidopteran corn pests resistant to either Cry1A.105 or Cry2Ab2 proteins) to indicate that insects resistant to one of the two insecticidal proteins, Cry1A.105 or Cry2Ab2, will be controlled by the other insecticidal protein. First, Monsanto cited to studies provided in support of the Bollgard II cotton registration (i.e, Cry2Ab2 and Cry1Ac *Bt* plant-incorporated protectants as expressed in cotton) that indicated that Cry1Ac-resistance did not confer Cry2Ab2 resistance to tobacco budworm, cotton bollworm, and pink bollworm (Head and Reding 2001; EPA 2007). In addition, Monsanto shared information that a Cry2Ab2-resistant colony (called SP15) of *Helicoverpa armigera* (Dr. Rod Mahon, CSIRO, Australia) showed little or no cross-resistance to Cry1Ac and the microbial insecticide, DiPel®, that contains the Cry1Ab, Cry1Ac, and Cry2Aa proteins. Monsanto tested this Cry2Ab2-(SP15) resistant colony against purified Cry1A.105, Cry1Ac, and Cry2Ab2 protein relative to a susceptible laboratory colony of *H. armigera*. The SP15 colony was found to be highly resistant to the Cry2Ab2 protein, but showed little or no cross-resistance to the Cry1Ac and Cry1A.105 proteins. Other published research indicates that there is evidence for broad cross-resistance (low levels of resistance) to Cry1A and Cry2A proteins in laboratory-selected strains of beet armyworm (Moar et al. 1995) and tobacco budworm (Gould, et al., 1992). Collectively, results of resistant colony studies indicate that there is some low potential for cross-resistance, but that high levels of cross-resistance to Cry1A.105 and Cry2Ab2 is unlikely. In the field, this would translate to the efficacy of MON 89034 being maintained even though resistance might occur to one of the proteins.

b) Dose

The determination of dose, or the amount of toxin expressed by the transgenic crop relative to the susceptibility of the target pests, is a critical component of IRM. Models have shown that a high dose of toxin, coupled with a non-transgenic refuge to provide a supply of susceptible insects, is the most effective strategy for delaying resistance in *Bt* crops. The high dose/refuge strategy assumes that resistance to *Bt* is recessive and is conferred by a

single locus with two alleles resulting in three genotypes: susceptible homozygotes (SS), heterozygotes (RS), and resistant homozygotes (RR). It also assumes that there will be a low initial resistance allele frequency and that there will be extensive random mating between resistant and susceptible adults. In practice, a high dose PIP should express sufficient quantities of toxin to kill all susceptible insects (SS) as well as heterozygous insects with one resistance allele (RS). Lower dose PIPs might allow for survival of insects with at least one susceptibility allele (SS or RS), although effective IRM may still be possible with a suitable refuge strategy.

The 1998 Science Advisory Panel (SAP) defined high dose as a level of toxin 25 times greater than is needed to kill all susceptible insects. The SAP also outlined five techniques to determine high dose: 1) Serial dilution bioassay with artificial diet containing lyophilized tissues of *Bt* plants using tissues from non-*Bt* plants as controls; 2) Bioassays using plant lines with expression levels approximately 25-fold lower than the commercial cultivar determined by quantitative ELISA or some more reliable technique; 3) Survey large numbers of commercial plants in the field to make sure that the cultivar is at the $LD_{99.9}$ or higher to assure that 95% of heterozygotes would be killed (see Andow & Hutchison 1998); 4) Similar to #3 above, but would use controlled infestation with a laboratory strain of the pest that had an LD_{50} value similar to field strains; and 5) Determine if a later larval instar of the targeted pest could be found with an LD_{50} that was about 25-fold higher than that of the neonate larvae. If so, the later stage could be tested on the *Bt* crop plants to determine if 95% or more of the later stage larvae were killed.

It must be noted that both the high dose definition and verification techniques were developed in 1998 when all of the registered *Bt* crops were single toxin products targeted against lepidopteran pests. In recent years, PIPs (in *Bt* cotton) have been approved that contain two genes targeted at the same insect pest. These "pyramided" products can be beneficial for IRM, since target pests must overcome two toxins to develop field resistance to the PIP. The benefits are greatest for two toxins with unrelated modes of action (i.e. binding to different *Bt* receptor sites in the midgut) that are expressed at high doses in the plant (Roush 1994).

For pyramided products, the dose of each toxin should be evaluated separately. This can be easily accomplished if the pyramided product is created through conventional breeding -- in this case, the dose of the single toxin products has already been established and the combined dose in the pyramided PIP can be determined with comparative efficacy studies. But, for pyramids created by non-conventional breeding (e.g. recombinant DNA techniques), defining the dose can be more complicated since single toxin lines may not be available (or commercialized) for comparisons. The dual toxins can also be evaluated collectively to determine an "effective" high dose. In some examples, each toxin by itself may not supply a high dose, but in combination a sufficient control (>95% of heterozygotes) is provided to be considered high dose.

MON 89034 was created with recombinant DNA technology (and not conventional breeding) to express the Cry1A.105 and Cry2Ab2 toxins. Both of the toxins are located on the same plasmid in the MON 89034 plant genome. Because of this, there are no originating single gene lines (i.e., expressing Cry1A.105 or Cry2Ab2 only) for dose comparisons, although single gene events were separately engineered. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa (domain III). By creating this chimera, Monsanto hoped to improve efficacy against several target pests including fall armyworm and corn earworm. The Cry2Ab2 protein is exactly the same as that currently expressed in Monsanto's Bollgard II cotton.

To evaluate dose, Monsanto conducted a number of laboratory and field studies with diet bioassays and MON 89034 plant material. Three sets of experiments were conducted: 1) bioassays with purified toxin incorporated into artificial diet to determine pest susceptibility, 2) leaf disk or kernel testing conducted in the laboratory, and 3) field tests with whole plants (artificial infestation of small corn plots) compiled over a several year period. Four target pests were evaluated including European corn borer (ECB), southwestern corn borer (SWCB), fall armyworm (FAW), and corn earworm (CEW). A description of the test procedures is included in Monsanto's submission (Head 2006; MRID# 469514-30). Toxin expression data was also obtained from MON 89034 leaf tissue and other tested lines.

Laboratory bioassays (Head 2006; MRID# 469514-30, section 2.2.1) were conducted using purified protein in diet to determine susceptibility (molting inhibitory concentration, MIC_{90}) to the MON 89034 toxins. Molting inhibition is often used instead of straight mortality (i.e. an LC_{50} or LC_{90}) because it can be assumed that insects that fail to develop as larvae will be functionally dead in the field. A MIC_{90} bioassay can also reduce the amount of purified toxin needed for the testing relative to an LC_{90} determination, though it is unclear whether Monsanto had insufficient purified protein to determine LC_{90} values. The MIC_{90} tests showed that all four target species were more susceptible to Cry1A.105 than Cry2Ab2 (as measured in ppm). ECB was more sensitive to both Cry1A.105 and Cry2Ab2 than the other tested lepidoptera by at least an order of magnitude. For Cry1A.105, BPPD agrees with Monsanto that the amount expressed in plant leaf tissue is high relative to the susceptibility of the target insects. Toxin levels in leaf tissue measured throughout the growing season (V2 - Pre-VT) exceeded the MIC_{90} for all four species (both measured in ppm). On the other hand, the amount of Cry2A2b expressed in MON 89034 exceeded the MIC_{90} value only for ECB. For the other three pests, the level of Cry2Ab2 was at (for SWCB) or below (CEW and FAW) the MIC_{90} level. These data suggest that the Cry1A.105 component of MON 89034 may be expressed at a sufficient level for all four pests to be considered "high dose" while the Cry2Ab2 expression is less certain. However, BPPD concurs with Monsanto's contention that the results of laboratory bioassays are difficult to correlate with natural field systems and larval survival on plant tissue is more challenging than on artificial diet.

Unlike the artificial diet bioassays, the tests with plant material (leaf disks and whole plant) directly assessed the performance of MON 89034 against the target pests. Since MON

89034 expresses both Cry1A.105 and Cry2Ab2 simultaneously, the tests with MON 89034 plant material evaluate the "effective dose" of both toxins together. However, Monsanto was also able to include single gene lines producing either Cry1A.105 or Cry2Ab2, though none of these were ultimately commercialized or used to create MON 89034. To relate the single gene isolines to MON 89034, Monsanto supplied some plant expression data for the isolines which could be compared to the known toxin expression of the stacked product. For the leaf disk/kernel tests, two Cry1A.105 isolines were used; one (LAJ138) with toxin expression equivalent to that of MON 89034 and another (LAJ129) with less than half the expression. Both of the Cry2Ab2 lines that were used (70774 and 67620) had less toxin expression than in MON 89034. Other single gene lines were used for the field tests, although no expression data were reported for those hybrids.

The results of the leaf disk tests generally supported the conclusions derived from the susceptibility diet bioassays (i.e., high efficacy against the target pests). Two-toxin MON 89034 was highly effective against all four target pests with at least 90% mortality among exposed larvae and significant growth inhibition in the survivors. On the other hand, mortality was more variable for the single gene isolines that were also tested. For ECB, both MON 89034 and the single gene (Cry1A.105 and Cry2Ab2) isolines killed nearly all exposed larvae. Low survival (4%) was noted only on a Cry2Ab2 isoline (67620) and on MON 89034, though the surviving larvae were stunted (< 41% the mass) relative to larvae on control leaf disks. The highest level of survival was noted for SWCB with some survival (up to 41% of the control group) observed on both the isolines and MON 89034, although the surviving larvae showed growth inhibition in all cases. For FAW and CEW, no survival was noted on MON 89034 or the isolines (though CEW survival on the control was only 26%, presumably due to CEW preference for feeding on corn ears instead of leaf tissue).

A second trial using kernels instead of leaf disks was performed for CEW. This test revealed relatively high survival (up to 35%) on the lower expressing isolines (LAJ129 and 67620) and 9% survival on MON 89034 (growth inhibition was not recorded).

Several sets of field tests (conducted in 2000 and 2002) showed high efficacy, though they provided less information on dose. The field tests were targeted primarily at ECB (one study was designed for SWCB) and assessed plant damage (as opposed to directly evaluating mortality). Single gene isolines were used, but no expression data were given (they were claimed to be lower than MON 89034) and MON 89034 was not included in the trials. The trials showed that Cry1A.105 and Cry2Ab2 isolines significantly reduced ECB and SWCB leaf and tunneling damage relative to the non-*Bt* control groups. Feeding damage was comparable to the commercial product MON 810, which is known to express a high dose for ECB and SWCB. While these studies demonstrated field efficacy of the Cry1A.105 and Cry2Ab2 isolines, they provide limited information for the assessment of MON 89034 dose. This is because 1) MON 89034 was not evaluated in any of the trials, 2) mortality was not assessed, and 3) CEW and FAW were not included in the trials. Monsanto recognized the

limitations of the field work, but indicated that they should be considered *in toto* with the laboratory bioassays and leaf disk tests.

Overall, the dose studies presented a mixed picture of the dose profile for MON 89034. Dose and efficacy data indicated that: (1) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide essentially 100% control of ECB; (2) the CrylA.105 protein in MON 89034 provides approximately 95% control of SWCB, while the Cry2Ab2 protein provides 80-90% control; (3) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide >95% control of FAW; and (4) the CrylA.105 and Cry2Ab2 proteins in MON 89034 each provide 90-95% control of CEW. Clearly, the hybrid offers a high level of control against the four major target pests including greater than 95% control of ECB and FAW and greater than 90% control of CEW and SWCB. The actual level of control may be even higher due to growth inhibition among survivors that would likely preclude developmental completion. As demonstrated in the diet bioassays, the target pests appear to be somewhat more sensitive to Cry1A.105 than to Cry2Ab2. However, much of the dose information is circumstantial; the leaf disk assays were the only trial phase that directly evaluated MON 89034. The other data were obtained from susceptibility assays with purified protein (that were compared to MON 89034 expression data) and tests with (non-commercialized) single gene isolines.

BPPD agrees with Monsanto that MON 89034 provides strong control; these tests demonstrate that MON 89034 will likely kill >90% of susceptible insects. On the other hand, the data did not support a high dose under the definition put forth by the 1998 SAP (a level of toxin 25 times greater than needed to kill susceptible larvae; i.e. a dose greater than the LC_{99} of the pest). Some survival of MON 89034 plant tissue was noted for ECB, SWCB, and CEW. Monsanto assumed that the survivors would not reach adulthood due to growth inhibition (and therefore are functionally dead), but that assumption was not tested due to the short time frame of the experiment.

Monsanto's dose studies did not directly evaluate the effect of MON 89034 on potentially heterozygous larvae (i.e. with one copy of a resistance allele). Since heterozygotes may be more tolerant of *Bt* toxins than susceptible homozygous larvae, the 1998 SAP indicated that a high dose product should kill at least 95% of homozygous susceptibles. Roush's modeling (1998) specifies that 70% of heterozygotes should be killed by the toxins expressed in the dual gene PIP. Monsanto assumes that MON 89034 meets the criteria for the Roush model (i.e. 95% susceptible and 70% heterozygote mortality), but no empirical evidence was presented regarding potential heterozygote mortality. Given that the major support for Monsanto's proposal to reduce corn refuge from 20% to 5% is the Roush model, BPPD recommended that Monsanto further investigate whether MON 89034 consistently has high mortality of susceptible homozygotes (>95%) and further investigate heterozygote mortality for MON 89034. BPPD recognizes that direct evaluations of heterozygote effects can be difficult, particularly if resistant colonies for the target pests are unavailable and given that there is no field resistance to either protein. Monsanto has not provided enough information to determine the "killing power" of each individual protein -- it would be useful to assess

whether Cry1A.105 and Cry2Ab2, individually, will kill greater than 95% of the susceptible homozygotes. The 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e. heterozygotes) (EPA 1998). These techniques included testing larger, later instar larvae that may be less susceptible to the toxins or with PIPs expressing lower levels of toxin than the commercial event (see the discussion of the SAP recommendations at the beginning of this section).

c) Impact of Prior Use of Cry1Ab-Expressing *Bt* Corn Products on MON 89034

Monsanto examined the impact of prior use of Cry1Ab-expressing *Bt* corn products on MON 89034 *Bt* corn. Cry1Ab-expressing *Bt* corn products have been on the U.S. market since 1997 and planted on millions of acres. This selection pressure could result in increased Cry1Ab-resistant allele frequencies in lepidopteran corn pests, particularly those that are more dependent on corn as a primary host such as ECB and SWCB. Should there be Cry1Ab-resistant insects that are cross-resistant to either the Cry1A.105 protein and/or Cry2Ab2 protein then IRM value of MON 89034 would be significantly reduced. Given that there is very high amino acid similarity between the Cry1Ab and Cry1A.105 proteins, the potential for cross-resistance between Cry1Ab and Cry1A.105 is an important consideration. In an earlier section of this assessment, BPPD concluded that there is a low likelihood of cross-resistance between Cry1A.105 (Cry1A proteins) and Cry2Ab2 proteins.

European corn borer (ECB) populations have been monitored for susceptibility to Cry1Ab since the 1995 growing season (diagnostic concentration information has been collected since 1999). Since 1998, monitoring has also been required for corn earworm (CEW), southwestern corn borer (SWCB), and fall armyworm (FAW, sweet corn only) susceptibility to Cry1Ab. All of the Cry1Ab monitoring data through the 2000 growing season were reviewed by the Agency during the 2001 *Bt* crops reassessment (EPA 2001). Data for the 2001 through 2005 growing seasons were independently reviewed by BPPD (see Reynolds 2004a, 2004b, 2006; Milofsky 2007). Estimates of the frequency of Cry1Ab resistance in ECB indicate that Cry1Ab-resistant alleles capable of conferring ECB survival on a *Bt* corn plant are very rare (Andow et al. 2000; Bourguet et al. 2003, Stodola et al. 2006). The highest estimate of Cry1Ab resistance allele frequency in U.S. ECB populations was $<4 \times 10^{-4}$ at the 95% confidence level. The Cry1Ab resistance allele frequency has not increased significantly in frequency even with the ten years of widespread use of Cry1Ab-expressing corn products. There have been no instances of Cry1Ab resistance capable of conferring survival on Cry1Ab-expressing *Bt* corn plants in annual monitoring of ECB, SWCB, and CEW (see ABSTC 2006 and BPPD's technical review in Milofsky 2007). BPPD agrees with Monsanto that the frequency of Cry1Ab-resistance is very low and that it has not increased significantly in over ten years of widespread use of Cry1Ab-expressing corn products.

Current knowledge about *Bt* toxin receptors is summarized in a recent review by Piggott & Ellar (2007). By far the most studied receptors have been lepidopteran receptors associated

with Cry1A toxins. These authors summarized Cry toxins for which a putative receptor has been identified (see Table 2 in Pillar & Ellar 2007). The Cry1A proteins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Fa) all have aminopeptidase N receptors (APNs) that can serve as Cry-binding proteins that mediate pore formation, but their relevance to toxin susceptibility has not been demonstrated. Cry1Aa, Cry1Ab, and Cry1Ac also have cadherin-like receptors that have been shown to mediate Cry1A toxicity. Other putative receptors, i.e., alkaline phosphatases, glycolipids, *BT*R-270, P252, may also play a role in Cry1A toxicity, but further study is needed. While there has been progress in what is known about Cry1A toxicity, little is known about other Cry families, such as the Cry2A family. How pore formation confers toxicity requires further study.

Monsanto characterized binding of Cry1A.105 and Cry1Ab proteins to ECB brush border membrane vesicles using Biacore. These studies (Li & English 2006) indicated that Cry1A.105 and Cry1Ab occupy different binding sites on the ECB midgut epithelial membrane and therefore have distinct membrane binding mechanisms. Cry1Ab binding data suggest that the binding patterns are much more complex for Cry1Ab than for Cry1A.105 despite these two proteins having 90% amino acid sequence homology. BPPD agrees with Monsanto that differences in binding mechanisms lessen the likelihood of Cry1A.105 and Cry1Ab cross-resistance.

Monsanto also summarized the laboratory studies examining ECB colonies selected for resistance to the Cry1Ab protein. While these colonies are imperfect tools for predicting what will happen in the field, they are the best tools available for looking at potential resistance mechanisms. In particular, Monsanto discussed a series of studies conducted on three ECB colonies selected for resistance to Cry1Ab by Blair Siegfried at the University of Nebraska (Siegfried & Spencer 2001). Two of the colonies were created by laboratory selection, the Europe colony was established from larvae collected in Lombardia region of northern Italy and a second colony was created from larval collected in Nebraska. A third colony was created from survivors of diagnostic bioassays from both the Europe and Nebraska populations. All three colonies, along with two susceptible colonies, were assayed for their response to purified Cry1Ab, Cry1Ac, Cry2Ab2, and a version of the Cry1A.105 protein. Results of the bioassays indicated that all three Cry1Ab-resistant colonies were resistant to Cry1Ab and Cry1Ac, but remained susceptible to Cry1A.105 and Cry2Ab2 with no evidence of cross-resistance.

As noted earlier, Cry1A.105 is a chimeric protein consisting of domains I and II and the C-terminus of Cry1Ac and domain III of Cry1Fa. Several pieces of evidence suggest that there is at least some likelihood of cross-resistance of Cry1Fa and Cry1Ac/Cry1Ab. Denolf et al. (1993) conducted Cry1Ab, Cry1Ac, and Cry1B proteins binding experiments with isolated brush border membrane vesicles (BBMV) and gut tissue sections from ECB. These studies indicated that Cry1Ab and Cry1Ac proteins recognized the same membrane receptor with different binding affinities while the Cry1B protein recognized a separate receptor. More recent binding studies with BBMV from ECB conducted by Hua et al. (2001) indicated that

there was limited shared binding between Cry1Fa and Cry1Ab/Cry1Ac proteins. Pereira et al. (2008) showed that there was little cross-resistance to Cry1Ac (6.9-fold) in a Cry1Fa-resistant line of ECB (>3,000-fold). Jurat-Fuentes & Adang (2001) demonstrated that Cry1Fa (and Cry1Ja) share the Receptor A binding site with the Cry1A toxins in *Heliothis virescens* (tobacco budworm), but they also have unique binding sites. These researchers proposed a model that suggests that Cry1Fa, Cry1Ab, and Cry1Ac all bind to the Cry1Aa binding site (called Receptor A, although with different binding affinities) as well as to unique binding sites. An altered Cry1Aa binding site may cause resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1Fa proteins, but the unique binding sites also play a role in toxicity. Competition binding experiments performed by Hernández & Ferré (2005) showed the occurrence of a common receptor for Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera, H. virescens*, and *Spodoptera exigua*. So far, all available information on binding site competition suggests that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja share a common binding site in most, if not all, Lepidoptera. These authors suggest that Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja protein binding to a common site explains, perhaps, the biochemical basis of multiple resistance and cross-resistances among these five proteins in some insect species. Jurat-Fuentes & Adang (2006) recently demonstrated that a cadherin-like protein, HevCaLP, is the functional receptor for Cry1Ac binding in a highly-resistant (>300,000-fold) tobacco budworm colony (YHD2) although it is not a receptor for Cry1Fa (130-fold resistant). These results suggest that the Cry1Fa and Cry1Ac shared binding site is not a cadherin-like protein and that cross-resistance would be due to modification of some other receptor. Collectively, the availability information indicates that there is some likelihood of cross-resistance to both the Cry1Fa and Cry1Ab/Cry1Ac proteins through modification of a single shared receptor site. Hernández & Ferré (2005) suggest that neither transgenic plants expressing stacked combinations of Cry1Ac (and by extension Cry1Ab), Cry1Fa, and Cry1Ja nor rotations of *Bt* crops containing single genes of these three (four) proteins would be a good resistance management strategy. In the case of corn, primary pests susceptible to Cry1Ab and Cry1Fa, such as ECB (and SWCB and CEW), would necessitate the importance of establishing the binding site model for this species in order to develop an appropriate resistance management strategy.

Monsanto has shown, using the weight-of-evidence approach, that there is a low likelihood of cross-resistance between Cry1A.105 and Cry2Ab2 (see section **a.,** "**Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins"** of this IRM assessment). It is assumed that the primary mechanism of resistance will be that of binding site modification, which is a reasonable assumption based on studies with other *Bt*-resistant insect populations (laboratory and field) (see Ferré & Van Rie 2002). Similarly, Monsanto has adequately demonstrated that there is a low likelihood of cross-resistance of Cry1A.105 and Cry1Ab. On the other hand, Monsanto has not addressed the likelihood of cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac. The Cry1A.105 protein is a chimeric protein consisting of Domains I and II and the C-terminus of Cry1Ac and Domain III of Cry1Fa.

It was recommended that Monsanto provide BPPD with additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034.

d) Proposed IRM Plan for MON 89034

Monsanto stated that the introduction of MON 89034 would significantly decrease the risk of lepidopteran pests evolving resistance to *Bt* corn. Monsanto's Insect Resistance Management (IRM) plan for MON 89034 focused on three key assumptions: (1) resistance to Cry1A.105 and Cry2Ab2 is expected to be at least partially recessive; (2) the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low; and (3) the level of both Cry1A.105 and Cry2Ab2 produced in MON 89034 confer high level of control of susceptible target pests (high dose defined as at least 90% and preferably >95% control). Should these assumptions be met, MON 89034 would have significantly more durability than all existing single-gene products for lepidopteran control in the U.S., including MON 810 (Cry1Ab), *BT*11 (Cry1Ab), Herculex I (Cry1Fa), and their respective stacked products. The primary focus of the MON 89034 IRM plan was on management of ECB resistance and to a lesser extent, SWCB and CEW in regions where these pests are economically important. FAW and sugarcane (SCB) were not a focus of Monsanto's IRM for MON 89034.

Monsanto's proposed IRM plan for MON 89034 originally consisted of the following elements.

1. A 5% structured non-lepidopteran *Bt* corn refuge for the Corn Belt based on two independent (minimal cross-resistance), highly effective modes of action of Cry1A.105 and Cry2Ab2;
2. A 20% structured non-lepidopteran *Bt* corn refuge for cotton-growing areas;
3. Annual resistance monitoring, grower education, and compliance monitoring programs; and
4. A remedial action plan that describes a series of action to investigate suspected resistance, confirms actual resistance, and mitigates the resistant population(s).

Each of these elements will be discussed below.

i. 5% Structured Refuge for Field Corn Uses of MON 89034 in the Corn Belt

The critical question is whether Monsanto has provided sufficient data/information to indicate that the durability of a 5% structured refuge (as Monsanto has proposed) is equal to or greater than durability of a 20% structured refuge (the current structured requirement for lepidopteran-protected *Bt* corn products) for management of resistance to MON 89034. Monsanto's MON 89034 IRM plan for field corn uses focused on ECB, though the issue of

whether MON 89034 has consistently high mortality of susceptible homozygotes for all of the primary target species also has to be considered.

In the case of MON 89034, two *Bt* genes, *cry1A.105* and *cry2Ab2*, were engineered into *Bt* corn plants to provide even better control (than first-generation, single *Bt* protein products) of ECB, CEW, SWCB, and FAW. Two proteins are expressed in MON 89034 corn plants: Cry1A.105, a chimeric protein consisting of domains of Cry1Ac and Cry1Fa; and Cry2Ab2, the same protein that is expressed in Bollgard II cotton (a Monsanto product). Monsanto has provided sufficient efficacy data to demonstrate that MON 89034 provides good control of ECB, CEW, SWCB, FAW, and SCB (see Matten 2007 for BPPD's review of Monsanto's submission, Headrick et al. 2006, MRID# 469514-15). The level of control of MON 89034 for these pests was equal to or greater than YieldGard (MON 810, Monsanto's single *Bt* (Cry1Ab) trait corn product).

Monsanto's first assumption was that Cry2Ab2 and Cry1A.105 have different modes of action and therefore the potential for cross-resistance is low. As discussed earlier in this review, Cry2Ab2 and Cry1A.105 have low sequence homology (14%), different activation mechanisms and binding characteristics, unique antibody binding sites, and resistant insects to one protein will be controlled by the other protein. Therefore, it can be concluded that Cry2Ab2 and Cry1A.105 have different modes of action and therefore it is expected that there will be a low likelihood of cross-resistance (see earlier discussion in Section a. **"Assessment of the Probability of Cross-Resistance to the Cry1A.105 and Cry2Ab2 Proteins"**). Lack of cross-resistance would increase the durability of MON 89034. These two proteins, Cry2Ab2 and Cry1A.105, therefore, seem to be good candidate proteins for pyramiding. BPPD agrees with Monsanto that the probability of cross-resistance between Cry1A.105 and Cry2Ab2 is low and these two proteins have different modes of action.

On the other hand, Roush (1998) cautions that proteins that have already shown significant levels of cross-resistance in resistant insect strains (e.g., *H. virescens*, *H. armigera*, *S. exigua*, *O. nubilalis*, *Plutella xylostella*), such as between Cry1A, Cry1Fa and Cry1J proteins, should not be used in pyramiding. This same warning was also given by Hernández & Ferré (2005)**.** Cry1A.105 is a chimera that consists of binding domains of Cry1Ac and Cry1Fa**.** There are commercial *Bt* crops that express Cry1Ac, Cry1Ab, Cry1Fa proteins and these products have been in the marketplace for nearly a decade. Should there be insect populations resistant to Cry1Ac, Cry1Ab, and/or Cry1Fa that are cross-resistant to Cry1A.105 then the durability of MON 89034 would be significantly reduced and a 5% structured refuge would be insufficient to maintain high levels of durability.

Given that there is very high amino acid similarity between Cry1Ab, Cry1Ac, and Cry1A.105 proteins then the potential for cross-resistance between Cry1Ab, Cry1Ac and Cry1A.105 is an important consideration. Cry1A.105 and Cry1Fa have about 76% amino acid similarity. However, what is really important is the similarity of the binding domain III of Cry1Fa and Cry1A.105 which is presumed to be very high. Cross-resistance is a real

possibility for these two proteins. There are several lines of evidence that indicate that Cry1Fa and Cry1Ab/Cry1Ac share a common binding receptor although each of these proteins has unique binding receptors as well (Denolf et al. 1993, Hua et al. 2001, Jurat-Fuentes & Adang 2001; Hernández & Ferré, 2005). Evidence for a shared binding receptor would increase the likelihood of cross resistance should resistance evolve through modification of the shared binding receptor.

Evidence provided by Monsanto indicates that there is little cross-resistance of Cry1A.105 and Cry1Ab. One cannot, however, infer much about the likelihood of cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac based on the binding patterns of Cry1A.105 and Cry1Ab because binding patterns are unique to each species (e.g., ECB, SWCB, and CEW) and each protein. Monsanto did not address the likelihood of cross-resistance of Cry1A.105 and Cry1Fa, a protein already in existing *Bt* corn and *Bt* cotton products, and what impact cross-resistance would have on the durability of MON 89034. BPPD recommends that Monsanto provide additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034.

Monsanto's second assumption was that resistance will be recessive. Ten years of resistance monitoring data indicate that the frequency of Cry1Ab alleles in ECB is very low ($<4 \times 10^{-4}$) and that this frequency has not changed significantly during this time. The 20% structured refuge requirement for single-gene *Bt* corn products has been in place for over a decade and, as noted earlier in this review, there is no evidence of field resistance to Cry1Ab and Cry1Fa in ECB, SWCB, and CEW during that period in the continental U.S. There is also no evidence of Cry2Ab2 resistance (CEW) after five years of widespread use of Bollgard II cotton. The absence of any cases of field resistance to *Bt* crops after a decade of use indicates that any relatively common *Bt*-resistant alleles must be recessive (Tabashnik et al., 2003). This evidence provides a strong indicator that resistance to the *Bt* proteins expressed in MON 89034 would also be recessive. BPPD agrees with this line of reasoning. Pyramids are considerably more effective when resistance frequencies are low provided that the susceptible homozygotes are all killed by each of the toxins used separately (Roush 1998; Figure 4.).

Monsanto's third assumption for its proposed 5% structured refuge depended on whether the amount of Cry1A.105 and Cry2Ab2 produced in MON 89034 confers a high level of control of susceptible target pests (defined as at least 90% and preferably >95% mortality). It is this third assumption that is the most difficult to prove.

Resistance simulation models predict that the greatest benefits of combining toxins in single plants by "pyramiding" or "stacking" are achieved when no cross-resistance occurs, when there are no fitness costs, when resistance to each toxin is rare and recessive, and when a refuge of plants without toxins are present. Modeling simulations of two-gene products

predict that the resistance risk associated with a two-gene product will be significantly less than for a single-gene product (for example, Caprio 1998; Roush 1998). Pyramiding two or more proteins increases the chance that at least one of the proteins will be especially favorable to resistance management. Modeling simulations predict that pyramids (without cross-resistance) can reduce the need for larger refuges (Roush 1998).

Pyramiding relies on the idea that each protein is used individually in a way that would kill all insects susceptible to that protein, and in so doing, kills insects that are resistant to the companion protein (Roush 1998). This has been described as "redundant killing" in the sense that most of the population is susceptible to both proteins and thus is killed twice. The extent to which the individuals that are resistant to one protein are killed by the other is central to the effectiveness of the pyramiding strategy.

Monsanto relied on the Roush (1998) model to support the need for a 5% structured refuge rather than a 20% structured refuge in the Corn Belt. Roush's model (figure 2 in the publication) indicated that a 5% structured refuge is equal to or greater than a 20% structured refuge for a highly effective, high dose single-gene product when a two-gene product (MON 89034 in this case) achieves at least 95% control of susceptible homozygotes **and** 70% control of heterozygotes assuming there is no cross-resistance. Monsanto's dose studies, as discussed earlier, presented a mixed picture for MON 89034 (see Section b., **"Dose"**). Dose and efficacy data indicated that MON 89034 has a high level of control against the four major target pests (as described in Head 2006)**: "**(1) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide essentially 100% control of ECB; (2) the CrylA.105 protein in MON 89034 provides approximately 95% control of SWCB, while the Cry2Ab2 protein provides 80-90% control; (3) the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide >95% control of FAW; and (4) the CrylA.105 and Cry2Ab2 proteins in MON 89034 each provide 90-95% control of CEW." The actual level of control may be even higher due to growth inhibition among survivors that would likely preclude developmental completion. The target pests appear to be somewhat more sensitive to Cry1A.105 than to Cry2Ab2.

Monsanto's dose testing indicated that MON 89034 has a high level of control (greater than 90%) of susceptible homozygotes (ECB, SWCB, CEW, FAW), one of two thresholds needed to support the durability of a 5% structured refuge for a two-gene pyramided *Bt* corn product (as equal to or better than that of a single-gene *Bt* corn product expressing a high dose of control against the target pests). However, it was not easily discernable as to whether each individual toxin kills greater than 95% of susceptible individuals. This is important for prediction of the durability of MON 89034: Roush's simulations (1998; Figure 3) showed that the greatest gains of pyramiding two proteins are when the mortality of susceptible insects is considerably greater than 95%, especially if resistance allele frequencies are quite low.

On the other hand, Monsanto's dose studies did not directly evaluate whether MON 89034 kills at least 70% of the heterozygotes, the other threshold needed to support a 5% structured refuge for a two-gene pyramided *Bt* corn product. Monsanto did not provide enough

information to determine the mortality of susceptible (SS) homozygotes and heterozygotes (RS) on MON 89034 plants. It is important to know whether Cry1A.105 and Cry2Ab2 are: 1) both high dose proteins; 2) one high dose and one moderate dose protein (and which one is high and which one is moderate); or 3) two moderate dose proteins to control ECB (and SWCB) in the Corn Belt. In other words, one has to establish whether each protein can kill potentially resistant individuals to the other protein. To evaluate MON 89034 in the context of Roush's model, it must be determined whether Cry1A.105 and Cry2Ab2 are produced at high levels to kill at least 95% of susceptible homozygotes and 70% of the heterozygotes. Because of this, BPPD recommended that Monsanto further investigate heterozygote mortality for MON 89034. BPPD recognizes that direct evaluations of heterozygote effects can be difficult, particularly if resistant colonies for the target pests are unavailable. However, the 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e., heterozygotes). These techniques included testing larger, later instar larvae that may be less susceptible to the either the Cry1A.105 or Cry2Ab2 proteins or with PIPs expressing less protein (less Cry1A.105 or Cry2Ab2) than MON 89034. As Roush (1998) cautioned, "…small refuges remain risky…" when mortalities of heterozygotes are lower than expected. For MON 89034, it has only been assumed (but not verified) that the heterozygote mortality will be at least 70% for each protein.

Cross-resistance between Cry1A.105 and Cry1Ac and Cry1Fa is not known, but published studies indicate that there is at least some potential for cross-resistance between Cry1A and Cry1Fa proteins in a number of insect species (see earlier discussion). The impact of this potential cross-resistance on the durability of MON 89034 is not known.

Monsanto's use of the Roush (1998) model as a guide to predict the durability of MON 89034 was very useful, but it is only a first step. Roush encouraged researchers to further investigate the points raised in his 1998 paper with additional modeling and experiments (see Roush 1998). However, this was not done by Monsanto. Additional modeling using a species-specific (e.g., ECB and SWCB for the Corn Belt), spatially-explicit, preferably stochastic, landscape model of available *Bt* crops expressing many different Cry proteins (needs to be a multiple gene model, a more complex model) needs to be performed to more precisely predict the evolution of ECB resistance (or SWCB) to MON 89034. This new model would need to consider the impact of other *Bt* proteins in which there may be some cross-resistance. This is analogous to the species-specific simulation modeling that EPA required Monsanto do to support the use of natural refuge (instead of a structured refuge) for management of *H. virescens* and *H. zea* to the Cry1Ac and Cry2Ab2 proteins expressed in Bollgard II cotton. In conclusion, Monsanto's initial data and modeling do not support a 5% structured refuge for MON 89034 for field corn uses in the Corn Belt.

Given the uncertainties in the dose determination for ECB and SWCB (SS and RS mortality) (note: CEW and FAW are lesser pests in the Corn Belt), cross-resistance likelihood of Cry1A.105, Cry1Ac, and Cry1Fa, and limitations of the simulation modeling, BPPD recommended that the current 20% structured refuge requirement for field corn uses of MON 89034 in the Corn Belt be maintained until such time as Monsanto could address these

uncertainties associated with the durability of a 5% structured refuge. There are many *Bt* corn and *Bt* cotton products in the landscape. Cross-resistance conferred by any of these proteins may negatively affect the durability of MON 89034. Studies indicate that there is at least some potential for cross-resistance between Cry1A.105, Cry1Fa, and Cry1Ac proteins in a number of insect species (see earlier discussion). Monsanto needs to examine the potential of Cry1A.105, Cry1Ac, and Cry1Fa cross-resistance and what impact it has on the durability of MON 89034.

ii. 20% Structured Refuge for Field Corn Uses of MON 89034 in Cotton-Growing Areas

Monsanto has proposed that a 20% structured refuge rather than the current 50% structure refuge requirement for single-gene lepidopteran-control products be used to manage insect resistance to MON 89034 in cotton-growing areas. The major pest of concern for *Bt* corn in cotton-growing areas is CEW (also known as cotton bollworm when it feeds on cotton), although ECB, FAW, SCB (sugar cane borer) are also sporadic corn pests in cotton-growing areas. As described earlier in this assessment (Section b **"Dose"**), Cry1A.105 and CryAb2 proteins have at least 90% control of CEW. Previous studies submitted by Monsanto (Head & Reding 2001; reviewed in BPPD 2007) demonstrated the low likelihood of cross-resistance between the Cry2Ab2 and Cry1Ac proteins. Both Cry1A.105 and Cry2Ab2 have a low likelihood of cross-resistance with Cry1Ab (see earlier discussion in Section c, "**Impact of Prior Use of Cry1Ab-Expressing *Bt* Corn Products on MON 89034**").

Monsanto used its deterministic, non-spatial model (Gustafson & Head 2005) to examine whether planting a 20% structured non-*Bt* corn refuge with MON 89034 was sufficient to manage the risk of resistance evolution to *Bt* corn and *Bt* cotton products. In this model, it was assumed that all cotton planted consisted of Bollgard II cotton, with no non-*Bt* cotton in the system, and that 80% of the corn planted in the region consisted of MON 89034 and 20% non-*Bt* corn. The modeling was focused on estimation of the likelihood of CEW resistance in the Mississippi region because of the relatively higher risk of CEW resistance evolution in this review. Monsanto estimated the effective (all non-*Bt* hosts of CEW, including current levels of non-*Bt* cotton and 20% non-*Bt* corn refuge associated with MON 89034) and natural refuge (only non-cotton hosts of CEW, including 20% structured non-*Bt* corn refuge associated with MON 89034 and other unmanaged hosts) available for CEW in this region as described in Gustafson & Head (2005). These estimates were used as parameter values in the model. One model scenario assumed that MON 89034 is fully cross-resistant with Bollgard II cotton (i.e., Cry1A.105 and Cry1Ac are fully cross-resistant). Resistance was assumed to be complete with no associated fitness costs. Using these assumptions, the simulation modeling predicted that a 20% non-*Bt* corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to *Bt* corn and *Bt* cotton products. Resistance to Cry2Ab2 protein evolved first and took >24 modeling years to evolve (modeling time was 25 years). It is not clear from Monsanto's discussion whether Cry1A.105 and Cry1Fa cross-resistance was included in the modeling. The current landscape has both Cry1Fa- and Cry1Ab-corn and Cry1Fa- and Cry1Ac- and Cry2Ab2 +

Cry1Ac-cotton products. Should there be substantial cross-resistance, then the value of MON 89034 would be dramatically reduced.

A 20% non-*Bt* corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to *Bt* corn and *Bt* cotton products assuming there is no cross-resistance. Monsanto did not, however, sufficiently address the cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac in the cotton-growing landscape and how such cross-resistance may impact the durability of MON 89034. Should cross-resistance be of concern then the durability of MON 89034 in the southern cotton-growing areas might be compromised. Monsanto needs to address this potential in subsequent simulation modeling.

e) Sweet Corn Uses

As stated in Monsanto's submission (Head 2006): "In the U.S., sweet corn is grown on approximately 500,000 acres, with California, Florida, Georgia, New York, Ohio, and Pennsylvania accounting for 62% of the acres. The insecticide use per acre on sweet corn is approximately 35-fold that of field corn (2.7 lb/A versus 0.76 lb/A) (USDA, 2006) and typically, 12 - 40 applications of insecticides may be applied to a single crop of sweet corn in the southern U.S (Adams 1996). Therefore, planting of MON 89034 has the ability to drastically reduce the amount of synthetic insecticides used for sweet corn production."

Monsanto has proposed the use of MON 89034 as a sweet corn product to control certain lepidopteran insect pests in conjunction with no structured refuge. While sweet corn has a similar pest spectrum to field corn, agronomic practices differ between sweet corn and field corn. This makes pest management different between the two crops. As described in Monsanto's submission (Head 2006): "Sweet corn is harvested approximately 18 to 23 days after silk emergence, compared to field corn in which the grain is allowed to mature and dry in the field. For sweet corn, the ears are harvested while still wet and placed in cold storage for fresh market corn or processed immediately. Shortly after harvest, corn stalks are typically destroyed in the field by disking, chopping or plowing. Previous work by Lynch et al. (1999), show that these harvest and post-harvest practices make it unlikely that any surviving/resistant larvae could survive, complete its development, and contribute any resistant allele to the next generation in sweet corn. Even if a larva was to survive, sweet corn farmers, including home gardeners, typically grow sweet corn in small plots along with many other vegetables that serve as alternative hosts for these polyphagous lepidopteran pests. Therefore, sufficient non-corn refuge should be present due to the typical practices of planting multiple host crops."

BPPD requested in a January 17, 2007 letter to Monsanto that the company provide additional (dose) data to support the sweet corn use. Monsanto responded to BPPD's request for supplemental data on March 9, 2007. Monsanto provided data that compared the estimated Cry1A.105 and Cry2Ab2 protein levels in leaf tissues collected from MON 89034

sweet corn varieties with field corn varieties (see Table 4 in Bogdanova 2007; MRID# 470794-02). Sweet corn data came from one site and field corn data came from five sites. The mean levels of the Cry1A.105 and Cry2Ab2 proteins were comparable between field and sweet corn MON 89034 hybrids.

BPPD agrees with Monsanto that no structured refuge is needed in conjunction with the sweet corn use based on the destruction of potential resistant larvae through cultivation practices.

f) Popcorn Use of MON 89034

Monsanto proposed to use the same IRM plan described for field corn with popcorn uses for MON 89034. Monsanto stated that there are approximately 291,000 acres of popcorn grown annually in the U.S., with Illinois, Indiana, Iowa, Nebraska, and Ohio accounting for 86% of the planted acres (Pike 2003). Popcorn, like field corn, is allowed to mature and dry in the field, and the pest spectra are essentially identical in popcorn and field corn.

Monsanto provided no additional dose and/or efficacy data to what was provided for field corn to support the use of MON 89034 on popcorn. Without these data, the popcorn use cannot be supported.

g) Other Elements of IRM for MON 89034

Monsanto proposed to have resistance monitoring, grower education and compliance monitoring as necessary parts of the IRM program. They proposed to implement a program similar to what is currently carried out for MON 810 and other single-gene *Bt* corn products. In particular, the educational and compliance assurance programs for MON 89034 would follow the structure established through consultations between EPA and the industry, and will involve working closely with NCGA and other interested stakeholders.

Similarly, post-commercial resistance monitoring programs would be established as an extension of existing programs to track the susceptibility of the key lepidopteran corn pests to the Cry1A.105 and Cry2Ab2 proteins. In the monitoring program, insect populations would be collected and each protein will be tested separately, rather than a mixture of the two proteins, because resistance to one protein could be masked by the activity of the other. As part of this program, baseline susceptibility studies are planned for the Cry1A.105 protein against ECB (through Dr. Blair Siegfried at the University of Nebraska), and for the Cry1A.105 and Cry2Ab2 proteins against SWCB (through Dr. Qisheng Song at the University of Missouri), and CEW (through Bruce Lang of Custom Bio-Products). The baseline susceptibility of ECB to Cry2Ab2 has already been assessed over a two year period (see Appendix 4 - Siegfried & Spencer 2001 in Monsanto's submission, MRID# 469514-30). In the case of CEW, baseline studies and annual monitoring have been conducted for Cry2Ab2 protein as part of the Bollgard II cotton IRM program, and the resulting data will

be useful for MON 89034. In addition to the formal monitoring program, any unusual damage from lepidopteran pests will be monitored by the routine scouting of corn fields and be reported to Monsanto or local extension agents.

A remedial action plan has been developed and approved by EPA for MON 810 and other single-gene *Bt* corn products (EPA 2001). This plan describes a series of actions to investigate suspected resistance, confirm actual resistance, and mitigate the resistant population. The basis of this plan also is appropriate for MON 89034. However, because MON 89034 contains both the Cry1A.105 and Cry2Ab2 proteins, this product has the advantage of having a "built-in" mitigation program if resistance evolves to one of the Cry proteins but not the other. Therefore, Monsanto indicated that the remedial action plan should only be implemented for MON 89034 if a field population evolves resistance to both the CrylA.105 and Cry2Ab2 proteins.

Monsanto's proposed program for resistance monitoring, grower education and compliance monitoring as part of the MON 89034 IRM program was determined to be "acceptable." No Cry1A.105 baseline susceptibility studies have been conducted at the time of the registration application, but are planned by Monsanto. Monsanto has indicated that baseline susceptibility information for ECB to Cry2Ab2 has been collected over a two-year period (summarized in Monsanto's submission). For each protein, a discriminatory concentration (diagnostic dose) will have to be determined for use in the annual resistance monitoring program. Annual reporting to the Agency of the results of the resistance monitoring, grower education, and compliance monitoring is needed (as is required for all other *Bt* PIPs). If there is confirmed resistance to either protein then it must be reported to the Agency (see FIFRA 6(a) incident reporting requirements and the requirements as part of the Remedial Action plan).

h) Conclusions for Initial Registration[12]

MON 89034 field corn uses in the Corn Belt

1) Pyramids can reduce the need for large refuges. Monsanto had originally proposed that a 5% structured refuge, rather than the current 20% structured refuge, be used with the field corn uses of MON 89034. However, Monsanto's initial data and modeling do not support a 5% structured refuge for MON 89034 for field corn uses in the Corn Belt. There are uncertainties in the dose determination for ECB, SWCB, CEW, FAW (SS and RS mortality), cross-resistance likelihood of Cry1A.105, Cry1Ac, and Cry1Fa and its impact on the durability of MON 89034, and limitations of the simulation modeling. The current 20% structured refuge requirement for field corn uses of MON 89034 in the Corn Belt will be maintained until such time as Monsanto can address these uncertainties.

[12] The assessment for the amendment to reduce lepidopteran refuge requirements modified these conclusions. (See section II. E. 2.)

2) Monsanto relied on the Roush (1998) model to support the need for a 5% structured refuge rather than a 20% structured refuge in the Corn Belt. Roush's model (1998; Figure 2) indicated that a 5% structured refuge is equal to or greater than a 20% structured refuge for a highly effective, high dose single-gene product when a two-gene product (MON 89034 in this case) achieves at least 95% control of susceptible homozygotes **and** 70% control of heterozygotes assuming there is no cross-resistance. The dose information provided by Monsanto is not sufficient to demonstrate that each protein will kill 95% of the homozygous susceptible insects and 70% of the heterozygotes. To support a 5 % refuge Monsanto will have to further investigate whether MON 89034 consistently has high mortality of susceptible homozygotes (>95%) and whether the heterozygote mortality is at least 70% for MON 89034 against the target pests (for the Corn Belt – ECB and SWCB). The 1998 SAP suggested several ways to estimate mortality for less susceptible larvae (i.e., heterozygotes) (EPA 1998). These techniques included testing larger, later instar larvae that may be less susceptible

3) Monsanto has demonstrated that Cry1A.105 and Cry2Ab2 have different modes of action and, therefore, a low likelihood of cross-resistance. Cry1A.105 and Cry2Ab2 would be suitable partners in a pyramided product. Monsanto has also shown that there is a low likelihood of cross-resistance between Cry1A.105 and Cry1Ab. Monsanto has previously demonstrated that there is a low likelihood of cross-resistance between Cry2Ab2 and Cry1Ac.

4) However, Monsanto did not address the likelihood of cross-resistance of Cry1A.105, Cry1Ac, Cry1Fa, proteins already in existing *Bt* corn and *Bt* cotton products, and what impact such cross-resistance would have on the durability of MON 89034. Monsanto must provide additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034. The Cry1A.105 protein is a chimeric protein consisting of Domains I and II and the C-terminus of Cry1Ac and Domain III of Cry1Fa. It is important to address not only the likelihood of cross-resistance potential of Cry1A.105 and Cry1Ab and, similarly, Cry1A.105 and Cry2Ab2 (which was done by Monsanto), but also that of Cry1A.105 and Cry1Ac and Cry1Fa.

5) Additional species-specific (e.g., ECB and SWCB for the Corn Belt), spatially-explicit, landscape modeling is recommended to explore the durability of MON 89034 versus single-protein *Bt* corn products. Modeling would need to consider the impact of other *Bt* proteins in the landscape that may confer some cross-resistance (to Cry1A.105, in particular) and how such cross-resistance would impact the durability of MON 89034 in the Corn Belt (use of simulation modeling). This is analogous to the species-specific simulation modeling that EPA required Monsanto do to support the use of natural refuge (instead of a structured refuge) for management of *H. virescens* and *H. zea* to the Cry1Ac and Cry2Ab2 proteins expressed in Bollgard II cotton.

6) *MON 89034 field corn use in cotton-growing areas.* A 20% non-*Bt* corn refuge for MON 89034 in the southern cotton-growing areas would be sufficient to manage the risk of resistance evolution to *Bt* corn and *Bt* cotton products assuming there is no cross-resistance. However, Monsanto did not sufficiently address the cross-resistance of Cry1A.105, Cry1Fa, and Cry1Ac in the cotton-growing landscape and how cross-resistance may impact the durability of MON 89034. Should cross-resistance be of concern then the durability of MON 89034 in the southern cotton-growing areas might be compromised. Monsanto must address this potential in subsequent simulation modeling. (See item 4 above.)

7) *Sweet corn.* No structured refuge is needed in conjunction with the MON 89034 sweet corn use based on the destruction of potential resistant larvae through cultivation practices. Grower agreements (also known as stewardship agreements) will specify that growers must adhere to the following refuge requirements or, in the case of sweet corn, harvest practices, as described in the grower guide/product use guide and/or in supplements to the grower guide/product use guide:

   For MON 89034 sweet corn, growers are required to destroy any MON 89034 sweet corn stalks that remain in the field following harvest via rotary mowing, discing, or plow-down within one (1) month of harvest.

8) *Popcorn.* Monsanto provided no additional dose and/or efficacy data to what was provided for field corn to support the use of MON 89034 on popcorn. Without these data, the popcorn use cannot be supported.

9) *Other Important Elements of the IRM Plan.* Monsanto's proposed program for resistance monitoring, grower education and compliance monitoring as part of the MON 89034 IRM program is "acceptable." No Cry1A.105 baseline susceptibility studies have been conducted, but are planned by Monsanto. Monsanto has indicated that baseline susceptibility information for ECB to Cry2Ab2 has been collected over a two-year period (summarized in Monsanto's submission). For each protein, a discriminatory concentration (diagnostic dose) will have to be determined for use in the annual resistance monitoring program. Annual reporting to the Agency of the results of the resistance monitoring, grower education, and compliance monitoring is needed (as is required for all other *Bt* PIPs). If there is confirmed resistance to either protein then it must be reported to the Agency (see FIFRA 6(a) incident reporting requirements and the requirements as part of the Remedial Action plan).

i) Insect Resistance Management Plan for MON 89034 X MON 88017 *Bt* Corn

MON 89034 x MON 88017 expresses the Cry1A.105, Cry2Ab2, and Cry3Bb1 *Bt* toxins and is targeted against lepidopteran corn pests including European corn borer (ECB), southwestern corn borer (SWCB), corn earworm (CEW), and fall armyworm (FAW) as well

as the coleopteran corn rootworm sp. pest complex (CRW). MON 89034 (Cry1A.105 and Cry2Ab2) provides activity against the lepidopteran corn stalk and ear insects while MON 88017 (Cry3Bb1) is active against root-feeding CRW. The product was created by conventional breeding in which the previously-registered MON 88017 (EPA Reg. No. 524-551) was crossed with MON 89034 (EPA Reg. No. 524-LTL). The Cry3Bb1 toxin in MON 88017 is the same as expressed by MON 863 corn (Yieldgard Rootworm, EPA Reg. No. 525-528), which was registered by Monsanto for the 2003 growing season.

1) Monsanto has provided information to demonstrate that the dose of MON 89034 x MON 88017 against the major target pests should be comparable to the dose of the MON 89034 and MON 88017 isolines. Therefore, the IRM considerations (dose, refuge, cross resistance) for MON 89034 and MON 88017 are applicable to the stacked MON 89034 x MON 88017 product.

2) Monsanto has proposed a 5% lepidopteran refuge as part of the "Separate Refuge" option for MON 89034 x MON 88017 corn. Due to uncertainties in the review of the MON 89034 IRM plan (see BPPD 2007a), a 5% refuge cannot be supported at the present time. Instead, BPPD recommends that the separate refuge option include a 20% lepidopteran refuge (as has been required for other *Bt* corn products). However, BPPD notes that a 20% refuge can be supported for MON 89034 x MON 88017 in cotton-growing regions in southeastern U.S. where a 50% refuge has been previously required.

3) Monsanto's proposal for a combined refuge (covering both coleopteran and lepidopteran pests) is acceptable. This option calls for a 20% refuge throughout the U.S. (as described in #2 above, a 20% refuge can be supported in southern cotton-growing regions).

4) The other aspects of Monsanto's IRM plan for MON 89034 x MON 88017 including resistance monitoring, remedial action plans, grower education, compliance, and annual reporting are acceptable. Resistance monitoring (sampling, bioassays, and data reporting) and remedial action should be conducted under the terms and conditions of registration for MON 89034 and MON 88017.

2. Amendment to Reduce Lepidopteran Refuge to 5% (2008).

After the registration of MON 89034 was granted (with a 20% lepidopteran refuge in the Corn Belt, as described in the preceding section), Monsanto submitted an amendment with supporting data to request a reduction in refuge to 5% in the Corn Belt. This section contains BPPD's assessment of this proposal (based on the review contained in BPPD 2008a).

As part of the IRM proposal for MON 89034 corn, Monsanto proposed a 5% lepidopteran structured refuge for non-cotton growing regions instead of the 20% refuge that has been required for all other *Bt* corn registrations. Monsanto reasoned that the combination of two

toxins targeting lepidopteran corn pests with no cross resistance allowed for a reduced refuge with little risk of resistance. As described in section 1 above, BPPD's review of the IRM proposal (BPPD 2007) agreed with much of Monsanto's justification but determined that there were a number of uncertainties in the request for lower refuge. Specifically, there were three areas of concern: (1) Cry1A.105 and Cry2Ab2 dose determination for the major target pests (ECB, CEW, SWCB, and FAW); (2) cross resistance potential between Cry1A.105 and Cry1F and Cry1Ac (toxins expressed in previously-registered PIPs); and (3) species-specific (e.g., ECB and SWCB for the Corn Belt), spatially-explicit, landscape modeling to explore the durability of MON 89034 versus single-protein *Bt* corn products. Given the uncertainty of the reduced refuge request, EPA registered MON 89034 with a 20% structured refuge requirement, similar to other *Bt* corn products. Separately, EPA did agree with Monsanto's request to reduce refuge in cotton-growing areas from 50% to 20% (see discussion in section 1 above and in BPPD 2007). As a condition of registration, Monsanto was required to address cross resistance in existing *Bt* corn and *Bt* cotton products for Cry1A.105, Cry1Fa and Cry1Ac.

Monsanto subsequently provided materials to address these three areas of uncertainty as part of a new amendment request for a reduced 5% refuge for non-cotton regions. The response, including a discussion of cross resistance and a new model, is included in a study titled "Assessment of the Impact of MON 89034 Introduction on *Bt* Resistance Development in European and Southwestern Corn Borer" (MRID# 474748-01).

a) Monsanto's Proposed Amendment to Support a 5% Refuge for MON 89034

Monsanto's proposal for a 5% refuge with MON 89034 included two major components: (1) a discussion of the cross resistance potential between the toxins in MON 89034 and (2) a deterministic model to simulate a 5% refuge and the risk of resistance for ECB and SWCB. Each of these sections is described and reviewed individually below.

In lieu of submitting new dose determination data for Cry2Ab2 and Cry1A.105 for the major target pests, Monsanto has used the existing dose information (submitted for the original registration) in the new simulation model. Therefore, Monsanto's response to the dose determination uncertainties (detailed in BPPD 2007 and section 1 above) will be discussed and reviewed in the modeling portion (section ii.) below.

i) Cross Resistance Potential

MON 89034 contains both Cry1A.105 and Cry2Ab2, which target the same lepidopteran corn pest complex. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1Fa while the Cry2Ab2 protein is the same as that currently expressed in Monsanto's Bollgard II cotton. Monsanto has sufficiently demonstrated that the cross resistance potential between these two proteins should be low, primarily due to differing modes of action (see discussion in BPPD 2007). In evaluating new PIP traits, the landscape of previously registered toxins in the same crop must be taken into

account. In addition, for corn PIPs, cotton must also be considered because one of the key target pests, corn earworm (also referred to as cotton bollworm, CBW, when a pest on cotton), is a pest of both crops. As a condition of registration, Monsanto was required to address cross resistance in existing *Bt* corn and *Bt* cotton products for Cry1A.105, Cry1Fa and Cry1Ac.

Monsanto's amendment submission for MON 89034 contained a discussion of cross resistance including an analysis of previous studies as well as a summary of recently developed data. Analysis of existing data was conducted for four toxin combinations: 1) Cry1Ab vs. Cry1Ac; 2) Cry1F vs. Cry1Ab and Cry1Ac; 3) Cry2Ab2 vs. Cry1 proteins; and 4) Cry1A.105 vs. Cry1Ab and Cry1Ac. New data were presented for comparisons between Cry1A.105 and Cry2Ab2 vs. Cry1F.

Cry1Ab vs. Cry1Ac: Based on a literature review of binding studies with numerous lepidopteran species, Cry1Ac is known to have strong cross resistance with Cry1Ab. Both toxins share a high affinity binding site in ECB, CEW/CBW, SWCB, FAW, and others (references cited in MRID# 474748-01).

Cry1F vs. Cry1Ab and Cry1Ac: Cry1F also shares a binding site with Cry1Ab/Cry1Ac, though the level of cross resistance between Cry1F and Cry1A is not as strong as Cry1Ab vs. Cry1Ac. ECB resistant to Cry1Ab have been shown to be partially resistant to Cry1F although Cry1F resistant ECB were not cross resistant to Cry1Ab and only slightly resistant to Cry1Ac. Similar trends have also been shown with tobacco budworm (*Heliothis virescens*, TBW) (references cited in MRID# 474748-01). Overall, Cry1F can be considered partially cross resistant to Cry1Ab and Cry1Ac. The availability of binding sites may explain the partial cross resistance: Cry1Ab and Cry1Ac could have more different sites to bind with than Cry1F so that resistance to Cry1F still allows for some binding of Cry1Ab or Cry1Ac.

Cry2Ab vs. Cry1 proteins: A literature review suggests that Cry2Ab has no cross resistance potential with any of the currently registered Cry1 proteins including Cry1Ab and Cry1Ac. Studies have been conducted with numerous cotton pests including CEW, TBW, pink bollworm (*Pectinophora gossypiella*, PBW), and *Helicoverpa armigera* that revealed no shared binding sites between Cry2A and Cry1Ab or Cry1Ac proteins. Additional studies with Cry1Ac-resistant TBW, CEW/CBW, and PBW found no cross resistance with Cry2Ab (references cited in MRID# 474748-01). Previously submitted data by Monsanto for MON 89034 (Head 2006; reviewed in BPPD 2007) demonstrated that Cry1Ab-resistant ECB were not found to be cross resistant with Cry2Ab while Cry2Ab2-resistant *H. armigera* were not cross resistant with Cry1A.105 or Cry1Ac.

Cry1A.105 vs. Cry1Ab and Cry1Ac: For Cry1Ab, a previously submitted binding study with ECB (Head 2006; reviewed in BPPD 2007) showed that the protein has a distinct binding site from Cry1A.105. This was confirmed by studies with Cry1Ab-resistant ECB and sugarcane borer (*Diatraea saccharalis*, SCB) that showed no cross resistance with Cry1A.105. Monsanto argues that due to similar characteristics between Cry1Ab and Cry1Ac (i.e., mode of action), it is

reasonable to assume that Cry1Ac should not be cross resistant with Cry1A.105. However, no binding studies or experiments with resistant colonies were described to verify that assumption.

Cry1A.105 and Cry2Ab2 vs. Cry1F: New data were cited by Monsanto (Schlenz et al. 2008) to assess the cross resistance potential between Cry1A.105/Cry2Ab2 and Cry1F using Cry1F-resistant ECB and FAW colonies. Artificial diet bioassays were used to test Cry1A.105, Cry2Ab2, and control groups against ECB and FAW colonies previously selected for high-level Cry1F resistance as well as unselected control colonies. A range of five concentrations was used and the test was conducted over a seven day period to determine growth inhibition (GI_{50}) for each colony. The results showed that, as expected, Cry1F-resistant ECB and FAW were not cross resistant with Cry2Ab2 -- the GI_{50} resistance ratios (Cry1F-resistant : Cry1F-susceptible) were 1.4 for ECB and 0.11 for FAW. With Cry1A.105, the GI_{50} resistance ratios were > 3.9 for ECB and 7.0 for FAW, indicating low level cross resistance.

Table 7: Cross resistance potential of MON 89034 (Cry1A.105 and Cry2Ab2) with previously registered *Bt* corn toxins.

	Bt toxins in MON 89034	
Existing *Bt* toxins	**Cry1A.105**	**Cry2Ab2**
Cry1Ab	No cross resistance (ECB, SCB)	No cross resistance (ECB)
Cry1Ac	Unlikely cross resistance, but unverified experimentally	No cross resistance (TBW, PBW, CEW/CBW)
Cry1F	Low level cross resistance (ECB, FAW)	No cross resistance (ECB, FAW)

BPPD Review - Cross Resistance

BPPD agrees with Monsanto's characterization of the cross resistance potential for the Cry1A.105 and Cry2Ab2 toxins with (1) each other (previously demonstrated in Head 2006), (2) Cry1F, and (3) Cry1Ab. Binding and resistant colony work conducted by Monsanto and other researchers clearly show that no cross resistance can be expected between Cry1A.105, Cry2Ab2 and Cry1Ab (see Table 7 above). New data referenced in Monsanto's amendment request also experimentally demonstrate the cross resistance potential between Cry1F and Cry2Ab2 (no cross resistance) and Cry1A.105 (low cross resistance).

Nonetheless, BPPD still has reservations about Cry1Ac. While Monsanto has made the case that Cry1Ac should be expected to behave like Cry1Ab due to a similar mode of action, no experimental data (i.e., binding studies or bioassays with resistant insect colonies) were provided either in the original MON 89034 IRM submission (Head 2006) or the follow-up amendment request (MRID# 474748-01). BPPD notes that Cry1A.105 (a chimeric protein) contains domains I and II and the C-terminal from Cry1Ac. Cross-resistance could result when proteins share key

structural features, which allows one resistance mechanism to confer resistance to more than one protein (Tabashnik 1994; Gould et al. 1995).

BPPD recognizes that at the present time there are no registered *Bt* corn products containing Cry1Ac. Therefore, exposure to ECB and SWCB to Cry1Ac is unlikely, as neither is known as a cotton pest. FAW may occasionally feed on cotton, but favors corn and is also unlikely to have much exposure to Cry1Ac. On the other hand, successive generations of CEW may feed on both corn and cotton during the same growing season. This could result in a potential "double" exposure to *Bt* cotton (including Cry1Ab) and *Bt* corn (including Cry1A.105) and increased selection pressure for resistance, particularly if there is a risk of cross resistance.

Given that Monsanto has proposed to substantially reduce refuge for MON 89034 from 20% to 5%, cross resistance is an important consideration even for Cry1Ac. Although improbable, BPPD cannot rule out that a CEW/CBW population could develop Cry1Ac resistance in cotton and then encounter MON 89034 corn. [Tabashnik et al. (2008) have argued that Cry1Ac resistance has already evolved in CBW in the south, although this conclusion has been disputed (Moar et al. 2008).] Should there be a degree of cross resistance between Cry1Ac and Cry1A.105, MON 89034 might functionally have only Cry2Ab2 remaining as an effective toxin against CEW. With a reduced refuge (5%), selection pressure could be increased for resistance to MON 89034 and Cry2Ab2 (which also is expressed in Bollgard II cotton). So that BPPD can fully assess the cross resistance potential of Cry1A.105 with Cry1Ac in CEW/CBW, it is recommended that Monsanto provide additional information either experimentally (e.g., binding studies or with resistant colonies) or using another analysis. Alternatively, Monsanto could revise the CEW model submitted with the original MON 89034 IRM plan (Head 2006) to support 20% refuge in cotton-growing regions. This model simulated CEW resistance to MON 89034 and assumed complete cross resistance between Cry1A.105 and Cry1Ac; the model could be adapted to evaluate a 5% refuge in the Corn Belt with similar assumptions.

ii) Modeling

As part of the review of Monsanto's initial IRM plan for MON 89034, BPPD identified the need for additional species-specific (e.g., ECB and SWCB for the Corn Belt), spatially-explicit, landscape modeling to explore the durability of MON 89034 versus single-protein *Bt* corn products (BPPD 2007). Previously, Monsanto had cited the modeling work of Roush (1998) to demonstrate that a 5% refuge was justified with a two toxin pyramided product. Roush's model made a number of key assumptions, particularly in terms of the toxin expression level in pyramided product. For homozygote susceptible insects, the model assumed 95% mortality and 70% mortality for heterozygotes (with one resistance allele) for each toxin. The dose information provided by Monsanto for MON 89034, however, was not sufficient to demonstrate that each protein would kill 95% of the homozygous susceptible insects and 70% of the heterozygotes (see BPPD 2007). BPPD recommended that Monsanto further characterize the dose expression for the MON 89034 toxins for the major target pests of the Corn Belt (ECB and

SWCB). Given the dose uncertainties, BPPD could not at the time of registration support the use of Roush's model to justify a lower 5% refuge for MON 89034 (BPPD 2007).

Rather than re-run dose studies for Cry1A.105 or Cry2Ab2, Monsanto created a deterministic model for ECB and SWCB using dose mortality estimates consistent with the previously conducted studies. The model (Gustafson & Head 2008; contained in MRID# 474748-01) included the toxins from other registered *Bt* corn products (Cry1Ab, Cry1F) and had a number of assumptions and parameters:

- Dose mortality for ECB: 99.9% for Cry1 (Cry1Ab, Cry1F, Cry1A.105) and Cry2Ab2 toxins (one mortality scenario was modeled);
- Dose mortality for SWCB: 99 - 99.5% for Cry1 and 85 - 95% for Cry2Ab2 (six dose mortality scenarios were modeled);
- Complete resistance to Cry2Ab2 and Cry1A.105 (i.e., survival probability of heterozygote resistant individuals = 1) with no fitness costs;
- Heterozygotes (i.e., with one resistance allele) survival probability is twice that for homozygote susceptible insects;
- Three cross resistance scenarios: 1) Cry1A.105 and Cry1Ab fully cross resistant (but not Cry1F) (the "base case" scenario); 2) Cry1A.105 and Cry1F fully cross resistant (but not Cry1Ab) (alternate "base case" scenario), and 3) Cry1A.105, Cry1Ab, and Cry1F all fully cross resistant (worst case scenario);
- All resistance alleles (Cry1, Cry1A.105, and Cry2Ab2) have initial frequencies of 0.005. Cry1Ab and Cry1F are modeled as one output (i.e., estimated time to resistance for Yieldgard/Herculex);
- MON 89034 was assumed to have a refuge of 5%; other single gene products (Yieldgard and Herculex) were assumed to have 20% refuge;
- ECB and SWCB have no natural refuge (i.e., wild hosts or other cultivated crops that could serve as a source of susceptible insects) and have two generations per year on corn;
- A range of market share adoption values for MON 89034 and other products (Herculex and Yieldgard) were included in the model simulations. MKT 1 = 100% MON 89034; MKT 2 = 50% MON 89034, 25% MON 810, 25% TC1507; MKT 3 = 0% MON 89034, 50% MON 810, 50% TC1507.

Most of the assumptions above were conservative estimates, with the possible exception of the dose mortality parameters for SWCB (see discussion in the BPPD review section below). Simulations were run with both ECB and SWCB to estimate the time to resistance (in years; up to a maximum of 30 years) and resistance allele frequency for each of the three cross resistance scenarios described above. Within each cross resistance scenario, model runs were conducted for three different market adoption contingencies of MON 89034, MON 810 (Cry1Ab Yieldgard) and TC1507 (Cry1F Herculex).

ECB Results

For ECB, the results of the model runs were relatively consistent among the different cross resistance and market adoption scenarios. In almost all cases, the durability of the MON 89034 toxins (Cry1A.105 and Cry2Ab2; assuming a 5% refuge) exceeded the 30 year time frame of the model. Only in the "worst case" cross resistance scenario (i.e., all three toxins cross resistant) was the durability of Cry1A.105 less than 30 years (29 years) for ECB -- Cry2Ab2 remained effective in all model simulations (> 30 years). For the other Cry1 toxins (Cry1Ab and Cry1F) that are expressed in other *Bt* corn products, resistance developed in less than 30 years for some of the cross resistance and market adoption scenarios. In the "base case" (Cry1Ab and Cry1A.105 cross resistant), the durability of Cry1Ab/Cry1F lasted 26 years (0% MON 89034, 50% MON 810, 50% TC1507) and 29 years (50% MON 89034, 25% MON 810, 25% TC1507). However, for the alternate base case (Cry1F and Cry1A.105 cross resistance), resistance to Cry1Ab/Cry1F did not evolve within 30 years. In the worst case scenario (all three toxins cross resistant), resistance to Cry1Ab/Cry1F developed in 29 years.

SWCB Results

For SWCB, more model simulations were run to account for a range of dose mortalities. Overall, durability of the traits was affected by the dose mortality scenarios -- the simulations with lower dose mortality frequently resulted in fewer years to resistance in Cry1A.105 and Cry1F than those with higher dose mortalities. As with ECB, Cry2Ab2 remained durable (> 30 years) in all but one of the simulations regardless of the cross resistance or market adoption scenario.

For the "base case" cross resistance scenario, the time to resistance was lowest in the market adoption scheme (MKT 3) without MON 89034 (50% MON 810, 50% TC1507) ranging from 17 years (lower dose mortalities for Cry1 and Cry2Ab2 toxins) to 20.5 years (higher dose mortalities). Once MON 89034 was added to the model (MKT 1 and 2), the time to resistance with the Cry1 toxins increased by 2 -2.5 years for all simulations. Cry1A.105 and Cry2Ab2 did not evolve resistance in any of the model runs for MKT 2, although there were two instances with MKT 1 (100% MON 89034) in which resistance evolved within 30 years. In both of these cases, lower dose mortality values for SWCB (85% for Cry2Ab2; 99% for Cry1A.105) were included in the model.

Time to resistance in the "alternate base case" (Cry1F and Cry1A.105 cross resistant) was > 30 years in almost all cases. Only in the simulation that incorporated the lowest dose mortality values (85% for Cry2Ab2 and 99% for Cry1A.105) did resistance evolve to one of the toxins (28.5 years for Cry1A.105).

In the "worst case" (Cry1Ab, Cry1F and Cry1A.105 are all cross resistant), resistance developed in all scenarios for both the Cry1 toxins and Cry1A.105. Conversely, Cry2Ab2 remained durable (> 30 years) for all of the simulations. Time to resistance in the Cry1 and Cry1A.105

toxins was lowest (17 years) in the model run using the lower SWCB dose mortality values (85% for Cry2Ab2 and 99% for Cry1A.105). Resistance also evolved for case with the higher dose mortality values, ranging up to 22 years for each toxin. A truncated summary of the results for all of the model simulations is contained in Table 8 below -- the complete results of the modeling are detailed in Tables 5 and 6 in Monsanto's submission (MRID# 474748-01).

Table 8: Results of Monsanto's model simulations of MON 89034 (5% refuge), MON 810, TC1507 (20% refuge) expressed in years to resistance (30 year maximum). Derived from data reported in MRID# 474748-01.

Pest		Cross resistance scenario				
		Base case[1]			Alt. base case[2]	Worst case[3]
		MKT 1	MKT 2	MKT 3		
ECB	Cry1A.105	>30	>30	N/A	>30	29
	Cry2Ab2	>30	>30	N/A	>30	>30
	Cry1Ab/Cry1F	N/A	29	26	>30	29
SWCB	Cry1A.105	22.5 - >30	>30	N/A	28.5 - >30	17 - 22
	Cry2Ab2	25 - >30	>30	N/A	>30	>30
	Cry1Ab/Cry1F	N/A	19 - 23	17 - 20.5	>30	17 – 22

[1] Base case = Cry1Ab and Cry1A.105 cross resistant; three different marketing scenarios included (Mkt 1 = 100% MON 89034, 0% MON 810/TC1507; Mkt 2 = 50% MON 89034, 25/25% MON 810/TC1507; Mkt 3 = 0% MON 89034, 50/50% MON 810/TC1507).
[2] Alt. base case = Cry1F and Cry1A.105 cross resistant (only Mkt 2 simulated).
[3] Worst case = Cry1A.105, Cry1Ab, and Cry1F all fully cross resistant (only Mkt 2 simulated).

Based on the model work, Monsanto concluded that the durability of the MON 89034 proteins (Cry1A.105 and Cry2Ab2) will remain strong for both ECB and SWCB. With a 5% refuge, Monsanto predicted that MON 89034 will have at least 22 years durability even under the "worst case" model assumptions. The durability of Cry2Ab2 in the model was particularly robust in almost all simulations for ECB and SWCB (only one simulation predicted less than 30 years durability). Resistance to Cry1A.105 was also rare in most simulations, although the "worst case" modeling (assuming complete cross resistance with Cry1Ab and Cry1F) showed resistance developing in less than 30 years. Monsanto also noted that in the simulations with different market adoption scenarios, the addition of MON 89034 increased the time to resistance for the previously registered Cry1 toxins (Cry1Ab and Cry1F).

BPPD Review - Modeling

BPPD agrees with Monsanto's overall conclusions that the model simulations demonstrate the effectiveness in delaying resistance of MON 89034 and provide support for the use of a 5% refuge in the Corn Belt. However, BPPD notes that some of the parameters and assumptions of the model could be revised to improve and expand the overall analysis.

For ECB, the model clearly predicts that resistance is unlikely to evolve to Cry1A.105, Cry2Ab2, or the previously-registered Cry1 toxins. Even under the worst case scenario that assumed complete cross resistance, the durability of all toxins was at least 29 years. Presumably, a large reason for this is the high dose mortality of the MON 89034 toxins against ECB. Previous mortality studies submitted by Monsanto (reviewed in BPPD 2007) showed that the Cry1A.105 and Cry2Ab2 proteins in MON 89034 each provide essentially 100% control of ECB (Monsanto assumed 99.9% mortality for each toxin in the model).

For SWCB, the model predictions were more varied, largely due to the different simulations run with the range of dose mortality assumptions. Not surprisingly, the simulations that were run with the lower mortality estimates (i.e., 85% for Cry2Ab and/or 99.0% for Cry1) resulted in less time to resistance than those using the higher dose values. In the worst case simulations with the lower dose estimates, SWCB resistance evolved in 17 years to both Cry1A.105 and Cry1Ab/Cry1F while with the higher doses resistance took 21 or 22 years to develop. As with ECB, Cry2Ab2 remained durable (>30 years) for almost all of the simulations.

A number of factors appeared to influence the model results. BPPD agrees with Monsanto that the addition of MON 89034 in the simulations testing various market adoption scenarios delayed resistance in the other previously-registered Cry1 toxins. Likely, these results were due to less selection pressure on each individual toxin because of a diverse mosaic of toxins in the landscape. Cross resistance was also an important variable. Monsanto's "base case" for cross resistance assumed cross resistance between Cry1Ab and Cry1A.105. This resulted in resistance always developing in Cry1Ab/Cry1F (i.e., within 30 years), although Cry1A.105 and Cry2Ab2 durability remained strong. On the other hand, when cross resistance between Cry1A.105 and Cry1F was assumed, resistance rarely developed in either the MON 89034 toxins or the existing Cry1 toxins. In the worst case scenario (all three toxins cross resistant), the durability of Cry1A.105 to SWCB was clearly impacted relative to the other cross resistance simulations. Conversely, Cry2Ab remained durable in almost all cases regardless of the varying assumptions and scenarios included in the model. Since Cry2Ab is not cross resistant to the Cry1 toxins, this result was not unexpected.

BPPD generally agrees with Monsanto that conservative assumptions were used in the model. BPPD notes, however, that several of the parameters could have been expanded or have included an additional degree of conservatism or additional refinement to improve the model analysis. For example, Monsanto's simulations assumed a 5% refuge for MON 89034 (while maintaining the 20% refuge for the other *Bt* toxins). Although MON 89034 is currently registered with a requirement for a 20% refuge, simulations were not run with the larger refuge size. Separate simulations with 5% and 20% MON 89034 refuges would have been useful for comparative purposes. To illustrate using the SWCB "base case" (with the three different marketing adoption cases), with no MON 89034 adoption resistance to the Cry1 toxins occurred in 17 - 20.5 years. When MON 89034 with a 5% refuge was included, the time to Cry1 resistance was 19 - 23 years -- indicating that the addition of MON 89034 provides some delay in resistance development (2 -

2.5 years). It would have been interesting to observe the impact of adoption of MON 89034 with a 20% refuge on Cry1 resistance. In all likelihood, the time to resistance would be increased, although the magnitude of such an increase is unknown. Had the difference been small, it could be argued that there is little value gained in having a 20% refuge versus a 5% refuge.

The model time frame (maximum 30 years) was another limiting parameter. Many of the simulations resulted in no resistance within the 30 year time period of the model, so it was difficult to discern the effects of certain variables (i.e., cross resistance, market adoption, dose mortality) between model runs. Had the time horizon been extended (e.g. to 50 years), differences between the various model scenarios may have been apparent.

For the SWCB simulations, Monsanto used dose mortality range of 85-95% for Cry2Ab2 and 99-99.5% for Cry1 toxins. Based on the dose data submitted for the registration of MON 89034 (reviewed in BPPD 2007), BPPD believes these estimates to be somewhat high. For example, dose data for Cry2Ab2 and SWCB suggested a mortality range of 80-90%. The Cry1A.105 protein in MON 89034 provided approximately 95% control in mortality assays, though the other registered Cry1 proteins (Cry1Ab and Cry1F) may provide closer to 99% of SWCB. Had the model simulations been run with these more conservative dose estimates, it is likely the time to resistance would have been reduced in some scenarios. The extent of this effect is unknown, although BPPD notes that the differences between the lower Cry2Ab2 dose (85%) and the highest dose (95%) in the range appeared to be negligible in the model runs (i.e. no differences in years to resistance).

iii. BPPD Review - Overall Proposal to Reduce Refuge

Taken together, Monsanto's cross resistance and modeling work provide justification for reducing the MON 89034 structured refuge requirement in the Corn Belt from 20% to 5% non-*Bt* corn. Key elements of support include a lack of cross resistance between Cry2Ab2 and Cry1 proteins and model simulations which demonstrate strong durability of Cry1A.105 and Cry2Ab2 under a variety of dose, market adoption, and cross resistance scenarios. Reducing the refuge to 5% is unlikely to increase the selection pressure for resistance in either MON 89034 or the other previously-registered Cry1Ab or Cry1F corn hybrids.

Despite a good case for a refuge reduction, BPPD notes that there are still some limitations and uncertainties in the analysis that could be addressed to provide additional support for the proposal. These areas include:

- Cross resistance between Cry1Ac and Cry1A.105. Cry1Ac is registered in *Bt* cotton products and the chimeric protein Cry1A.105 has two Cry1Ac domains. CEW feed on both corn and cotton and successive generations may have exposure to both Cry1A.105 and Cry1Ac during the same growing season;
- No model simulations were conducted to compare 5% vs. 20% refuge for MON 89034; the model assumed a 5% refuge for MON 89034;

- The model time horizon was limited to 30 years. Many of the model runs did not evolve resistance during this time precluding comparisons between some of the scenarios;
- SWCB model simulations included dose mortality estimates somewhat higher than those suggested by previously-submitted data. For Cry2Ab2, mortality ranged from 80 to 90% in dose testing submitted for MON 89034 (instead of 85-95% used in the model). Cry1A.105 caused 95% mortality in submitted dose studies, though a range of 99-99.5% was used in the model.

As a condition of registration of MON 89034, Monsanto was required to address cross resistance in existing *Bt* corn and *Bt* cotton products for Cry1A.105, Cry1Fa and Cry1Ac. Monsanto has sufficiently addressed cross resistance for Cry1A.105 and Cry1Fa, but there are lingering questions regarding Cry1Ac and Cry1A.105. The amendment submission included only a circumstantial discussion of Cry1Ac cross resistance with an assumption that the protein will behave similarly to Cry1Ab. But, since Cry1A.105 contains domains I and II and the C-terminal from Cry1Ac, BPPD is still concerned about the potential for cross resistance. As such, BPPD recommends additional work (as described in the cross resistance section above) to satisfy the condition of registration. Should additional cross resistance work (as previously described) demonstrate little or no cross resistance potential between Cry1A.105 and Cry1Ac, further support could be provided for the use of a 5% refuge in the Corn Belt.

In terms of resistance risk for MON 89034, cross resistance between Cry1Ac and Cry1A.105 is an issue primarily for CEW. This insect is known to feed on both corn and cotton during the same growing season and could be exposed to Cry1A.105 (in corn) and then Cry1Ac (in Bollgard cotton) later in the growing season. Theoretically, CEW could develop resistance to Cry1Ac due to exposure in cotton -- should there be a degree of cross resistance between Cry1Ac and Cry1A.105, MON 89034 could functionally have only Cry2Ab2 remaining as an effective toxin against CEW. With a reduced refuge (5%), selection pressure could be increased for resistance to MON 89034 and Cry2Ab2 (which also is expressed in Bollgard II cotton). While these are legitimate concerns (and reason for additional analysis), BPPD notes that there are several mitigating factors that reduce the overall resistance risk for CEW and MON 89034. First, CEW is generally a lesser pest in the Corn Belt than ECB (and in some areas SWCB), primarily due to poor overwintering capability in much of the Corn Belt (i.e., north of Virginia, Tennessee, and Missouri). Therefore, selection pressure for resistance will likely be less for CEW than ECB which does overwinter in the Corn Belt. On the other hand, in cotton-growing regions south of the Corn Belt where CEW can overwinter, conditions for resistance development may be more probable. In these areas, a 20% refuge (approved with the initial registration of MON 89034) will still be required. Along these lines, in Monsanto's original MON 89034 IRM submission, modeling was conducted to support the use of a 20% refuge for CEW in southern cotton-growing regions (see discussion in BPPD 2007).

A second mitigating factor is that CEW is a highly polyphagous insect and is known to feed on a wide variety of plants including weeds, wild hosts, and other cultivated crops (unlike ECB and SWCB which feed primarily on corn). Analysis conducted for Bollgard II cotton determined

that a natural refuge is present for CEW (CBW) in cotton growing areas in the southeastern U.S. (see BPPD 2004b and 2006b). It is likely that in the Corn Belt, there is also at least some degree of natural refuge that could supplement a 5% structured refuge to help reduce the overall selection pressure on CEW and MON 89034. BPPD emphasizes that natural refuge for CEW has been quantified only in cotton-growing regions and that host utilization patterns in the Corn Belt are speculative.

The other modeling parameter uncertainties detailed above are relatively minor, though a more expanded model analysis could have provided stronger support for the proposal. Separate model runs with 5% and 20% MON 89034 refuges would have been useful to compare potential differences in times to resistance. Although since most of the simulations did not result in resistance within 30 years, any differences would have been difficult to detect. Expanding the time horizon of the model (for example, from 30 years to 60 years) possibly could have fleshed out variation between model scenarios and provided a more thorough basis for comparison. Finally, BPPD would have preferred if Monsanto had used the more conservative estimates of SWCB dose mortality (based on the MON 89034 dose data), though the impact on the model output would likely have been relatively small.

MON 89034 was originally registered as a conditional time-limited registration (with an expiration date of September 30, 2010) and BPPD recommends reevaluating 5% refuge if warranted by cross resistance data or other information during this interim period.

3. Conditional IRM Data Submitted for MON 89034 and MON 89034 x MON 88017 (2010)

Monsanto has submitted a number of reports to EPA to satisfy the Insect Resistance Management conditions of registration for MON 89034 and MON 89034 x MON 88017. These submissions are summarized in Table 9 below.

Table 9: Conditional IRM data submitted by Monsanto for MON 89034 and MON 89034 x MON 88017 corn.

Date	Submission	MRID No.
7/29/08	Copies of grower agreements	None
9/22/08	Grower education: copy of Monsanto's Technology Use Guide (2009)	None
10/6/08	Compliance Assurance Program (ABSTC plan for lepidoptera and MON 863 plan for corn rootworm)	None
6/29/09	Cross resistance data: comparative binding of Cry1A.105 and Cry1Ac proteins in tobacco budworm and cotton bollworm	477912-01
6/29/09	Resistance monitoring: baseline susceptibility data for Cry1A.105 and ECB	477912-02
6/29/09	Resistance monitoring: baseline susceptibility data for Cry1A.105 and CEW	477912-03

11/3/09	Compliance, grower education, resistance monitoring, remedial action for MON 89034: updated materials for 5% refuge	479035-01
11/9/09	Compliance, grower education, resistance monitoring, remedial action for MON 89034 x MON 88017: updated materials for 5% refuge	479083-01

In a letter to EPA dated September 28, 2009, Monsanto requested additional time to fulfill some of the conditional data requirements. One of these requirements is baseline susceptibility (resistance monitoring) data for southwestern corn borer. This report was submitted on August 27, 2010.

Each of the submissions detailed in Table 9 was reviewed in BPPD (2010a) and is addressed below by discipline.

Cross Resistance

For the original registration of MON 89034 corn, Monsanto demonstrated low likelihood of cross resistance between the expressed toxins Cry1A.105 and Cry2Ab2 (see review in BPPD 2007). BPPD was concerned, however about potential cross resistance between Cry1A.105 in MON 89034 and Cry1F and Cry1Ac toxins that are expressed in other registered *Bt* corn and cotton PIPs. A condition of registration was imposed to conduct additional cross resistance studies on these toxins (documented in the June 10, 2008 MON 89034 registration notice). As part of the their amendment request for a 5% refuge Monsanto provided sufficient data to demonstrate that cross resistance is unlikely between Cry1A.105 and Cry1F, though BPPD still had uncertainties about Cry1Ac (see review in BPPD 2008a). Given this concern, the condition to conduct cross resistance data with Cry1A.105 and Cry1Ac was maintained for the 5% refuge amendment approval (see December 15, 2008 approval letter).

Monsanto addressed this concern by submitting a report: "Comparative Binding of the *Bacillus thuringiensis* Cry1A.105 and Cry1Ac Proteins to Cotton Bollworm (*Helicoverpa zea*) and Tobacco Budworm (*Heliothis virescens*) Brush Border Membranes" (MRID# 477912-01).

Summary of Monsanto's Cross Resistance Submission (MRID# 477912-01)

Monsanto addressed the question of potential cross resistance between Cry1A.105 and Cry1Ac by conducting binding assays using brush border membranes (BBM) from two cotton pests, tobacco budworm (TBW) and cotton bollworm (CBW). To accomplish this objective, BBMs were obtained from homogenized and filtered whole body third instar larvae (centrifuged into pellets) using established procedures (English et al. 1991). The BBM proteins were then suspended onto nitrocellulose membranes and separated using gel electrophoresis (SDS-PAGE) techniques. Membranes containing the separated BBMs were exposed to incubations containing either Cry1A.105 or Cry1Ac. Bound protein was detected using a mammalian (goat) antibody capable of recognizing both Cry1A.105 and Cry1Ac.

An analysis of the BBMs of TBW and CBW revealed that both insects have similar protein components (as detected by SDS-PAGE). Because of this, the binding experiments with Cry1A.105 and Cry1Ac showed similar patterns between the insects. The specific binding characteristics for each insect are summarized in Table 10 below.

Table 10. Binding profiles for Cry1A.105 and Cry1Ac in TBW and CBW (preferential binding sites as analyzed by SDS-PAGE). (Created from data submitted to EPA in MRID# 477912-01)

	TBW	**CBW**
Cry1A.105 unique binding sites (kDas)	20, 42, 50, 95	20, 42, 50, 90
Cry1Ac unique binding sites (kDas)	57, 63	57, 63
Common binding sites for both toxins (kDas)	40, 120, 150	40, 120, 150

Although there are shared binding sites for Cry1A.105 and Cry1Ac in both TBW and CBW, Monsanto concluded that "...these proteins have different insecticidal modes of action." Each toxin also has a number of unique bands such that the binding profiles were sufficiently different between the two toxins.

BPPD Review

BPPD agrees with Monsanto that there appear to be distinct binding sites for each toxin in both TBW and CBW (see Table 10 above). The presence of three common binding sites could indicate the potential for some cross resistance between Cry1A.105 and Cry1Ac. The 120 and 150 kDA bands appeared to be relatively strong (as or more intense than any of the unique binding bands) in the photographs of the gel plates.

While some shared binding sites between two toxins is not a definitive indicator of cross resistance, without other supporting data (e.g., assays with toxin-resistant colonies) the possibility cannot be eliminated. To BPPD's knowledge, no additional studies have been conducted with Cry1A.105 and Cry1Ac. As noted in BPPD (2008a), Cry1A.105 is a chimeric protein that contains domains I and II and the C-terminal from Cry1Ac. Cross-resistance can result when proteins share key structural features, which allows one resistance mechanism to confer resistance to more than one protein (Tabashnik 1994; Gould et al. 1995). Because of the structural similarities, some shared binding sites may be expected. Since there are a number of unique binding sites for each toxin, any cross resistance due to common binding sites will likely be at low levels.

Cry1Ac could be a potential concern due to the presence of *Bt* cotton PIPs that express the toxin (Cry1Ac is not found in any presently registered *Bt* corn PIPs). TBW is not likely to be at risk for cross resistance because it is not known to be a corn pest. Similarly, corn pests such as ECB

and SWCB are unlikely to be exposed to Cry1Ac in cotton. On the other hand, successive generations of CBW can feed on both corn and cotton during the same growing season and may be exposed to *Bt* toxins in both crops. BPPD notes, however that there are several mitigating factors that could lower the impact of any cross resistance development in CBW to Cry1A.105/Cry1Ac. First, CBW is generally a lesser pest in the Corn Belt than ECB, primarily due to poor overwintering capability in much of the region (i.e., north of Virginia, Tennessee, and Missouri). Therefore, the potential for resistance to evolve will likely be less for CBW than ECB (which does overwinter in the Corn Belt). For cotton-growing regions south of the Corn Belt, where CBW can overwinter and conditions for resistance development may be more favorable, a 20% refuge is required for MON 89034.

Resistance Monitoring

As part of the terms of registration, Monsanto was required to implement a resistance monitoring program for MON 89034 and MON 89034 x MON 88017. For MON 89034, resistance monitoring was required for the main lepidopteran pests (ECB, CEW, and SWCB) and the two expressed toxins (Cry1A.105 and Cry2Ab2). In addition to the lepidopteran pests, monitoring for corn rootworm (CRW) and the Cry3Bb1 toxin was also mandated with the MON 89034 x MON 88017 registration.

Monsanto was directed to utilize existing monitoring strategies for both pest complexes that had been developed for previous PIP registrations, although a revised monitoring plan for CRW was requested as a term of registration for MON 89034 x MON 88017. The core components of the monitoring program include insect sampling in areas of high risk of resistance development, bioassays to detect resistant individuals, and investigations of report of unexpected pest damage. To support these objectives, baseline susceptibility data were required to be submitted for Cry1A.105 and Cry2Ab2 activity against ECB (Cry1A.105 only), CEW, and SWCB.

Resistance monitoring was also required for fall armyworm (FAW) with sweet corn uses of the MON 89034 and MON 89034 x MON 88017. FAW monitoring is triggered if acreage exceeds 5,000 in any county known to support overwintering of the insect. A proposed monitoring plan and FAW baseline susceptibility data to Cry1A.105 and Cry2Ab2 were required as terms of registration.

Monsanto's Responses to Resistance Management Requirements

To address the resistance monitoring terms and conditions of registration, Monsanto submitted two baseline susceptibility studies for ECB (MRID# 477912-02) and CEW (MRID# 477912-03). Both studies were conducted with Cry1A.105 only -- for Cry2Ab2, Monsanto cited previously submitted studies for ECB (part of the original MON 89034 IRM submission, MRID# 469514-30) and CEW (submitted with *Bt* cotton monitoring data, see review in BPPD 2005a). The company requested additional time (until August 31, 2010) to conduct baseline studies for SWCB (letter to EPA dated September 24, 2009). Separately, Monsanto asked for an extension

(to April 1, 2012) to submit FAW baseline data (letter to EPA dated June 3, 2010). Baseline studies for SWCB were submitted to the Agency and will be evaluated in the future.

Monsanto also provided a revised lepidopteran monitoring plan (contained in MRID# 479035-01) that mirrors a previously-submitted plan submitted by the Agricultural Biotechnology Stewardship Technical Committee (ABSTC) (MRID# 474070-01). For CRW monitoring, Monsanto cited the plan (MRID# 478836-03) submitted for SmartStax corn (EPA Reg. No. 524-581), a separate *Bt* corn product that targets rootworm.

ECB Baseline Susceptibility Data - Cry1A.105 (MRID# 477912-02)

Baseline susceptibility studies for ECB and Cry1A.105 were conducted by Dr. Blair Siegfried and Dr. Terrence Spencer of the University of Nebraska. Dr. Siegfried's laboratory has conducted much of the ECB resistance monitoring work since *Bt* corn PIPs were registered in the mid 1990's. The methodology used for the Cry1A.105 testing was similar to the procedures employed for the annual ABSTC corn monitoring program.

ECB were collected from sites consistent with the ABSTC sampling strategy for *Bt* corn. A total of 16 populations were collected as either adults or diapausing larvae from five states (Illinois, Iowa, Minnesota, Nebraska, and South Dakota). Collected ECB were reared in the lab to produce progeny for testing (F_0, F_1, or F_2 neonate larvae were tested). A susceptible laboratory ECB strain was tested as well. Bioassays were conducted with dilutions (range 0.06 to 4.0 ng/cm^2) of Cry1A.105 toxin overlaid onto artificial diet. The toxin was microbially-produced (by *E. coli*) and supplied by Monsanto. Results were tabulated after seven days by assessing mortality (non-molting 1st instar larvae were considered dead) and larval weight.

An initial test with a susceptible laboratory colony showed that 96.9% mortality was achieved at a concentration of 2.0 ng Cry1A.105/cm^2 and 100% mortality with all concentrations exceeding 6.25 ng/cm^2. Of the field collected populations, results were reported for 10 populations; the remaining six populations collected as diapausing larvae were delayed. The susceptibility assays (Table 3) for the reported populations resulted in a LC_{50} range of 0.52 - 1.02 ng Cry1A.105/cm^2 and a LC_{90} range of 1.66 - 4.04 ng/cm^2. EC_{50} values (determined from larval weights) ranged from ~ 0.17 to 0.46 ng/cm^2. By comparison, the laboratory colony LC_{50} was 0.40 ng/cm^2, the LC_{90} was 1.06 ng/cm^2, and the EC_{50} was 0.19 ng/cm^2. The study authors attributed the differences in susceptibility (only two fold) between field-collected populations to natural variability similar to that seen for other *Bt* toxins. A diagnostic concentration for Cry1A.105 was not reported in the results.

CEW Baseline Susceptibility Data - Cry1A.105 (MRID# 477912-03)

Bioassays for CEW were conducted by Bruce Lang of Custom Bio-Products, who have conducted the CEW monitoring for *Bt* corn toxins since 2001. Custom Bio-Products used similar sampling and testing procedures for assessing CEW susceptibility to Cry1A.105.

CEW were collected from 24 locations in nine states (Alabama, Arkansas, Georgia, Illinois, Iowa, Louisiana, Mississippi, North Carolina, and Texas) creating 26 total populations (two sites had multiple collections). Populations were collected as larvae which were returned to Monsanto's facility for rearing. Monsanto supplied eggs from subsequent generations (F_2 to F_5) to Custom Bio-Products for the bioassays. A susceptible laboratory colony was also included in the experiments. Susceptibility was assessed with diet bioassays incorporating dilutions of toxin overlays (concentration range of 0.1 to 6 µg Cry1A.105/cm^2). Cry1A.105 toxin used in the testing was provided by Monsanto. Neonates were exposed to the diet for seven days and then evaluated for mortality (larvae < 10 mg were considered dead) and larval weight.

All of the collected populations were included in the testing. The baseline susceptibility assays (Table 11) for the field-collected populations resulted in the following ranges: LC_{50}: 0.010 - 0.540 µg/cm^2; LC_{90}: 0.042 - 2.118 µg/cm^2; LC_{99}: 0.114 - 6.457 µg/cm^2; EC_{50}: 0.0016 - 0.0190 µg/cm^2; EC_{95}: 0.0184 - 1.1092 µg/cm^2; EC_{99}: 0.0482 - 10.8520 µg/cm^2. For the laboratory colony, the susceptibility results were: LC_{50}: 0.256 µg/cm^2; LC_{90}: 1.296 µg/cm^2; LC_{99}: 4.857 µg/cm^2; EC_{50}: 0.0034 µg/cm^2; EC_{95}: 0.2255 µg/cm^2; EC_{99}: 2.3628 µg/cm^2. The study author did not suggest a potential value for a CEW diagnostic concentration based on these results.

Table 11. ECB and CEW baseline susceptibility to Cry1A.105 (Created from data submitted to EPA in MRID# 477912-02 and -03)

	Susceptibility to Cry1A.105			
	LC_{50}[1]	LC_{90}[1]	EC_{50}[1]	EC_{95}[1]
ECB - Field Collected	0.52 - 1.02	1.66 - 4.04	0.17 to 0.46	--
ECB - Lab Strain	0.40	1.06	0.19	--
CEW - Field Collected	0.010 - 0.540	0.042 - 2.118	0.0016 - 0.0190	0.0184 - 1.1092
CEW - Lab Strain	0.256	1.296	0.0034	0.2255

[1] Units are ng Cry1A.105/cm^2 for ECB and µg Cry1A.105/cm^2 for CEW

BPPD Review

For MON 89034 and MON 89034 x MON 88017, Monsanto has proposed to use existing programs to monitor for resistance among lepidopteran and corn rootworm pests. Regarding the lepidopteran pests, the Agricultural Biotechnology Stewardship Technical Committee (ABSTC), a consortium representing *Bt* corn registrants, has been responsible for conducting resistance monitoring activities. ABSTC submitted a unified plan for lepidopteran resistance monitoring that covered all registered *Bt* corn PIPs in 2003 (see ABSTC 2003; reviewed in BPPD 2004a). This plan has formed the basis of all monitoring activities since its submission including insect sampling, bioassays, procedures for unexpected pest damage, definitions of pest resistance, and steps to confirm cases of suspected resistance. ABSTC amended the monitoring plan in 2008 (MRID# 474070-01; see review in BPPD 2008b) to adjust the sampling strategy for ECB and

SWCB and modify the procedures for determining resistance (mainly in CEW). This revised monitoring program was integrated into the amended registration terms for both products after the approval of 5% lepidopteran refuge (letters to Monsanto dated December 15, 2008).

Monsanto submitted lepidopteran resistance monitoring program (MRID# 479035-01) largely follows the revised (2008) ABSTC plan and now required by the terms of registration. In Monsanto's submission there are several differences from the ABSTC plan and some components have been adapted to Cry1A.105 and Cry2Ab2. For ECB, Monsanto indicated that diagnostic concentrations have been developed ("upper 95% confidence limit of the LC_{99} or EC_{99}") for the toxins and will be used in the assays, although the specific values were not reported. Survival of >1% on the diagnostic concentration will trigger follow-up investigations of the population. For SWCB, Monsanto reported that a diagnostic concentration has not been developed. Rather, dose-response assays (i.e., LC_{50} and EC_{50}) will be used to assess field collected populations relative to historical data for susceptible populations. With CEW, Monsanto stated that "high diagnostic concentrations are not practical or relevant," but that diagnostic concentrations will be developed for Cry1A.105 and Cry2Ab2 since they have higher activity. Dose-response parameters (LC_{50} and EC_{50}) will also be used for comparisons with historical data.

BPPD believes that diagnostic concentrations are an integral part of pest monitoring as a means to distinguish susceptible and potentially resistant individuals. Monsanto submitted acceptable Cry1A.105 baseline susceptibility studies for ECB and CEW but diagnostic concentrations were not proposed in the reports and additional work may be needed to develop functional standards. For ECB, 96.9% mortality was achieved at a concentration of 2.0 ng/cm^2 and 100% mortality with all concentrations exceeding 6.25 ng/cm^2, so a diagnostic concentration based on an LC_{99} should be easy to extrapolate. As is typical for the species, the CEW baseline results revealed high variability (as much as several orders of magnitude) in LC_{99} and EC_{99} ranges. Data are still pending for SWCB and FAW (Monsanto was granted an extension to fulfill the data needs for these insects). BPPD recommends that Monsanto continue to work towards developing susceptibility data and diagnostic concentrations for these pests and report the results with the annual ABSTC monitoring reports.

Monsanto's submitted monitoring plan for MON 89034 (MRID# 479035-01) also did not reference definitions of resistance (suspected and confirmed) and the steps to verify resistance that are detailed in the revised ABSTC program. To confirm resistance, a pest population must demonstrate: 1) 30% survival and commensurate insect feeding in a bioassay representative of field exposure to *Bt* corn (ECB and SWCB only); 2) survival on a laboratory diagnostic concentration that demonstrates a genetic basis for the tolerance and a resistance allele frequency ≥ 0.1; 3) a LC_{50} in a standardized laboratory bioassay that exceeds the upper 95% LC_{50} confidence interval for a susceptible population. BPPD notes that these criteria are now required by the amended terms of registration for both MON 89034 and MON 89034 x MON 88017 (approval letters to Monsanto dated December 15, 2008).

For CRW resistance monitoring with the Cry3Bb1 toxin (expressed in MON 89034 x MON 88017), Monsanto cited to the plan developed for the MON 88017 x MON 810 registration (MRID# 473547-01) and revised for SmartStax corn (MRID# 478875-03). Both of these documents have been reviewed separately (see BPPD 2009 and 2010b) for their respective registrations. Specific aspects of CRW resistance monitoring were required as conditions of registration including development of a diagnostic dose assay and rootworm damage guidelines (for unexpected pest damage). These conditions were also required for the existing Monsanto rootworm *Bt* corn registrations and were addressed with previous submissions.

CRW resistance monitoring remains a work in progress as methodologies are developed to assess the pest complex. Developing functional detection bioassays have been complicated by rootworm biology and difficulties rearing and maintaining colonies in laboratory environments. BPPD recommends that Monsanto continue to work on improvements to the CRW monitoring program with the goal of implementing a harmonized program for all *Bt* corn PIPs (similar to the ABSTC program for lepidoptera).

Remedial Action

Similar to resistance monitoring, Monsanto was required to utilize existing paradigms to address remedial action plans for lepidoptera and corn rootworm. For lepidoptera, a plan was developed by ABSTC in 2001 and modified in 2008 (MRID# 474070-01; reviewed in BPPD 2008b). The modified remedial action strategy was incorporated into the terms of registration for both MON 89034 and MON 89034 x MON 88017 with the approval of 5% lepidopteran refuge (approval letters to Monsanto dated December 15, 2008). Monsanto submitted a version of this plan with the lepidopteran monitoring program (MRID# 479035-01).

The lepidopteran remedial action plan submitted by Monsanto contains a description of procedures to confirm the heritability and field relevancy of resistance, estimate the frequency of resistance alleles, determine the geographic boundaries of the resistance, and, in cases where resistance allele frequencies are increasing or proliferating, creation of "an appropriate remedial action plan based on the knowledge of the genetics and level of resistance it confers in the field."

Monsanto's described remedial action plan for MON 89034 differs significantly from that required by the amended terms of registration. The terms of registration require that Monsanto (paraphrased from EPA's December 15, 2008 letter):

- Notify EPA, affected customers, and extension agents within 30 days of resistance confirmation;
- Increase resistance monitoring in the affected area;
- Utilize alternate control measures in the area including insecticides or other control measures if appropriate;
- Stop sales of relevant *Bt* corn PIPs in the area until an EPA-approved mitigation measure is in place;

- Develop a case-specific mitigation plan within 90 days and notify affected parties of the plan;
- Maintain the sales suspension and alternate control strategy into future growing seasons until an EPA-approved mitigation plan is implemented.

Although Monsanto's submitted plan does not address many of these elements, they are still required by the terms of registration. Therefore, the registration requirements for lepidopteran remedial action essentially supersede the version submitted by Monsanto.

For CRW, Monsanto (as required by the terms of registration for MON 89034 x MON 88017) referenced the remedial action plan previously developed for Cry3Bb1 registrations. This plan was originally developed for MON 863 and was subsequently carried over to MON 88017 and SmartStax, both of which also express Cry3Bb1 for CRW control.

Conceptually, the CRW remedial action plan (submitted in MRID# 473547-01; reviewed in BPPD 2009) is similar to the strategy for lepidoptera. Activities are centered on assessing the genetics (heritability, r-allele frequency) and geographic scope of the resistance event prior to "design an appropriate remedial action plan." The actual remedial action plan to be deployed is based on the one originally created for MON 863 corn (see review in BPPD 2004c).

Compliance

For the initial registrations of MON 89034 and MON 89034 x MON 88017 Monsanto was required to design and submit a compliance assurance program (CAP) to ensure adherence to refuge requirements by growers. The CAP was to be based on a "phased compliance approach" to address non-compliant growers, include annual surveys (anonymous and on-farm) to assess compliance, and provide a means to investigate "tips and complaints" of out-of-compliance growers. In addition, Monsanto was required to utilize (and provide copies to EPA) a grower agreement to contractually bind growers to plant refuges.

Compliance Assurance Program (MRID# 479035-01, 479083-01, other submissions with no MRID#)

Monsanto responded to these terms of registration by submitting copies of the CAP developed by ABSTC for lepidopteran *Bt* corn (dated September 23, 2002) and the CAP designed for MON 863 and CRW (dated July 7, 2005) as part of a non-MRID submission dated October 6, 2008. These documents have been previously reviewed by BPPD -- see BPPD 2005b (2002 lepidopteran CAP), BPPD 2006a (2005 CRW CAP), and BPPD 2004c (review of the original 2003 CRW CAP). CAP activities have been conducted by ABSTC since the 2002 growing season. The core elements of the CAP are the same for lepidopteran and corn rootworm PIPs (as well as stacked *Bt* corn PIPs targeting both pest complexes).

The major components of the ABSTC CAP for *Bt* corn are as follows (paraphrased from ABSTC 2002):

- Annual IRM survey: The survey (conducted anonymously by an independent research firm) is intended to provide a statistically representative sample of growers from various corn-growing regions in the U.S. Results from the survey can assess not only levels of grower compliances with refuges but also grower motivations, attitudes, and insights into IRM for *Bt* corn.
- Tips and complaints: Registrants establish a means for the reporting and investigation of incidences of refuge non-compliance.
- On-farm assessments: Trained personnel from each company make on-site visits to farms growing *Bt* corn. During these visits, compliance with refuge requirements is assessed and growers out of compliance are identified for corrective action under the Phased Compliance Approach.
- Phase Compliance Approach (PCA): The PCA provides a stepwise set of procedures to address non-compliance with the goal of bringing growers back into compliance. Separate protocols are established for "significant deviations" (or <2/3 refuge fields within ½ mile of *Bt* fields) and "other deviations" (i.e., less than the significant deviations). Significant deviations include one or more of the following:
    - <15% refuge (for 20% requirement) or <40% refuge (for 50% requirement);
    - < 2/3 refuge fields planted within ½ of *Bt* fields (lepidoptera);
    - < 2/3 refuge fields planted within adjacent to *Bt* fields (rootworm);
    - < 2/3 in-field strips planted at least 6 rows wide (rootworm)

    Responses for both significant and other deviations include warning letters, compliance assistance visits, educational efforts, and other measures. Growers who have significant deviations for two years in a row will be denied access to the *Bt* corn product for at least one growing season.
- Other measures: Alternate approaches including addressing large scale non-compliance on a geographic scale and taking action against seed dealers not in adherence with IRM requirements are detailed in the CAP.

Subsequent to the original registration, MON 89034 was amended to allow for a 5% refuge in the U.S. Corn Belt (20% refuge is still required in cotton regions). Because of the new refuge requirements, Monsanto submitted revised CAPs for MON 89034 (MRID# 479035-01) and MON 89034 x MON 88017 (MRID# 479083-01). The revised CAP follows the framework of the ABSTC (2002) program with several modifications to address the reduced refuge. Monsanto removed the distinctions between "significant" and "other" deviations in the phased compliance approach; instead, all instances of non-compliance are vetted equally. All growers found to be non-compliant will be issued a warning letter, receive a "compliance assistance" visit, and provided additional IRM educational materials. As with the ABSTC plan, any grower out of compliance for two consecutive years will be prevented from purchasing MON 89034 varieties for at least one growing season.

BPPD agrees that the modification to eliminate tiered levels of non-compliance for MON 89034 is reasonable given the small (5% refuge). Any deviation from such a refuge will likely be significant -- a 1% refuge deviation for a 5% requirement functionally results in 20% less refuge. Further, Monsanto's plan to address non-compliant growers in the MON 89034 CAP is the practical equivalent of the approach for growers with "significant deviations" in the original ABSTC plan.

Grower Agreements

As required by the terms of registration, Monsanto submitted copies of the "grower agreements" used with MON 89034 and MON 89034 x MON 88017 customers. These contractual documents obligate growers to adhere to IRM requirements as well as other conditions imposed by the registrant (and not under the purview of EPA). Two sets of grower agreements were submitted: one (for the 2009 growing season) in response to approval of the original registration (attached with letter to EPA dated July 29, 2008) and the second (2010 growing season) for approval of the amendment allowing 5% refuge (contained in MRID# 479035-01 and 479083-01).

The form appears to be updated annually and is generically written to cover all Monsanto agricultural biotechnology products. Portions of the 2010 contract pertinent to IRM are quoted below:

In the "Grower Agrees" section:

- "To implement an Insect Resistance Management ('IRM') program as specified in the applicable.....YieldGard® corn sections of the most recent Technology Use Guide ('TUG') and the Grower and Insect Resistance Management Guide ('IRM/Grower Guide') and to incorporate and comply with these IRM programs."
- "To read and follow the applicable sections of the TUG and IRM/Grower Guide, which are incorporated into and is a part of this Agreement, for specific requirements relating to the terms of this Agreement, and to abide by and be bound by the terms of the TUG and the IRM/Grower Guide as it may be amended from time to time."

In the "Grower Understands" section:

- "Insect Resistance Management: When planting any YieldGard®.....products, grower must implement an IRM program according to the size and distance guidelines specified in the TUG and the IRM/Grower Guide, including any supplemental amendments. Grower may lose grower's limited use license for these products if grower fails to follow the IRM program required by this Agreement."

In the "General Terms" section:

- "Grower acknowledges that grower has received a copy of Monsanto's Technology Use Guide ('TUG') and the Grower and Insect Resistance Management Guide ('IRM/Grower Guide')."

The 2009 and 2010 versions of the contract are functionally the same although the 2010 grower agreement expands some of the IRM terms.

Grower Education

Monsanto was required by the terms of registration to "design and implement a comprehensive, ongoing IRM education program" for MON 89034 and MON 89034 x MON 88017. Specific requirements include at least one communication to growers per year to inform them of current IRM requirements and the use of multiple media to convey educational messages (e.g., mailings, bag tags, internet communications, radio/TV ads).

Monsanto Grower Education Submissions (MRID# 479035-01, 479083-01, other submissions with no MRID#)

To address grower education, Monsanto submitted a copy of its Technology Use Guide (TUG) after registration (submission dated September 22, 2008; No MRID#). The TUG covers Monsanto's complete agricultural biotechnology line including MON 89034 and MON 89034 x MON 88017 (trade name YieldGard VT). A second submission was made in 2009 that includes a more complete description of the grower education program for MON 89034 (MRID# 479035-01) and MON 89034 x MON 88017 (MRID# 479083-01).

The initial TUG provided covered the 2009 season when the original refuge requirement of 20% was in place for all corn-growing areas nationwide. A supplemental section detailing this refuge requirement (then unique to MON 89034) was included with the TUG. The 2009 TUG was a comprehensive document (50 pg.) that addressed topics such as biotechnology, stewardship, IRM, weed resistance, and crop-specific information for corn, cotton, and herbicide-resistant soybean, alfalfa, canola, and sugarbeet. In terms of IRM, the TUG provided general information on its importance and emphasized the need to follow refuge requirements. Growers were warned that non-compliance could lead to loss of access to Monsanto's products and implementation a monitoring program for refuge planting. Specific information on IRM requirements was included in the TUG regarding refuge percentage and deployment. Diagrams were provided to illustrate acceptable refuge configurations such as separate fields, blocks, and strips. Additional information was supplied on requirements for insecticide treatments of refuges and other aspects of refuge management. Each Monsanto *Bt* corn platform (corn borer control only, rootworm only, and stacked corn borer/rootworm control) was addressed separately in the TUG. For the stacked YieldGard Triple VT Triple product (i.e., MON 89034 x MON 88017), the TUG includes descriptions of how to deploy "common" and "separate" refuges for both lepidopteran (corn borer) and rootworm pest complexes.

Monsanto's latter submissions (MRID# 479035-01 and 479083-01) contained a more detailed description of their educational activities for growers. The following components were described:

- Grower Agreements: Contractual arrangements between Monsanto and growers (described in the previous section on compliance).
- Annual Affirmation: Monsanto employs a "bag tag" system in which growers affirm their obligation to comply with refuge requirements when they open the seed bag.
- Grower Education Program: Monsanto uses a multi-faceted system comprised of these elements:
    - Use of an IRM logo and development of the "Respect the Refuge" advertising campaign.
    - A comprehensive grower guide (TUG).
    - Advertising including in or on billboards, seed catalogs, and websites.
    - IRM training for sales representatives and communications with seed dealers.
    - Published articles and news releases in farm media to inform growers of IRM responsibilities.

Subsequent to the registration of MON 89034 and MON 89034 x MON 88017, EPA approved an amendment to lower the refuge to 5% for lepidopteran pests for the Corn Belt (required refuge remains 20% in southern cotton-growing regions). Given the new refuge requirement, Monsanto provided additional information regarding their grower educational efforts to facilitate this change. To accomplish this, Monsanto will partner with ABSTC and the National Corn Grower Association to harmonize educational messages. The TUG for the products (2010 version) was revised to include details on which products and regions are eligible to employ the 5% refuge, although only an excerpt (one page) was provided in the submission. Seed catalogs and bags will be clearly marked to display the required refuge size and training will be provided for the seed distribution network (including an "IRM Quick Guide" for sales representatives). Finally, Monsanto developed an on-line calculator to help growers make accurate refuge determinations.

BPPD could find no errors or omissions in the education materials submitted for MON 89034 and MON 89034 x MON 88017. But, a complete version of the 2010 grower guide with all of the IRM information was not provided, so it is not possible to fully verify the revisions made for the 5% refuge approval. In addition, the grower guide provides little information on IRM principles (i.e., how *Bt* corn is at risk for resistance and why refuges can mitigate the threat of resistance). Such information could be beneficial to growers' understanding of the importance and need for IRM.

F. BENEFITS AND PUBLIC INTEREST FINDING

To grant a conditional registration under Section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), EPA must determine that such conditional registration will, *inter*

alia, be in the public interest. EPA determines whether conditional registration of a pesticide is in the public interest in accordance with the criteria set forth at 51 Fed. Reg. 7628 (*Conditional Registration of New Pesticides*, March 5, 1986). There is a presumption that registration of a pesticide is in the public interest if one of the following criteria are met: (i) the use is for a minor crop; (ii) the use is a replacement for another pesticide that is of continuing concern to EPA; (iii) the use is one for which an emergency exemption under FIFRA Section 18 has been granted (the basis for the exemption was lack of a registered alternative product); or (iv) the use is against a pest of public health significance. Notwithstanding whether a registration of a pesticide may be presumed to be in the public interest, EPA may determine that such a registration is in the public interest on the basis of one of the following criteria: (i) there is a need for the new chemical that is not being met by currently registered pesticides; (ii) the new pesticide is comparatively less risky to health or the environment than currently registered pesticides; or (iii) the benefits (including economic benefits) from the use of the new active ingredient exceed those of alternative registered pesticides and other available non-chemical techniques.

MON 89034 and MON 89034 x MON 88017 do not meet any of the criteria for a presumption of public interest; however, BPPD has determined that MON 89034 and MON 89034 x MON 88017 are in the public interest based on criteria (ii) and (iii) mentioned above. Specifically, under criteria (ii), both MON 89034 and MON 89034 x MON 88017 should allow growers the opportunity to reduce the use of higher risk, and often less effective and more expensive, conventional pesticides. A reduction in use of conventional pesticides equates to less potential for adverse effects to human health and the environment. Additionally, MON 89034 and MON 89034 x MON 88017 provide a wider spectrum of protection against primary and secondary corn pests, which should facilitate greater grain quality, a reduction of mycotoxin contamination, increased yield and ultimately have positive implications for human health.

1. Agricultural Benefits

MON 89034

BPPD recognizes that MON 89034's unique combination of Cry1A.105 and Cry2Ab2 proteins expands the spectrum of protection for corn against lepidopteran pests - past that offered by already-registered MON 810 (BPPD, 2007a). In addition to providing protection against primary pests such as European corn borer (*Ostrinia nubilalis*, ECB), MON 89034 also protects against secondary corn pests such as corn earworm (*Helicoverpa zea*, CEW), fall armyworm (*Spodoptera frugiperda*, FAW), and black cutworm (*Agrotis ipsilon*, BCW) (BPPD, 2007a; BPPD, 2007c; BPPD, 2007d). Use of MON 89034 could reduce or eliminate the need for conventional pesticide applications on acreage infested with secondary pests, although most growers do not use conventional pesticides to treat pests that are not part of the soil pest complex (BPPD, 2001). Finally, yield appears to be comparable to other *Bt* insect-protected corn. In situations of increased lepidopteran pressure, yield could be higher than other *Bt* insect-protected corn because of the presence of two insecticidal toxins and the effective protection against particular primary and secondary corn pests.

MON 89034 x MON 88017

In addition to the agricultural benefits mentioned above for MON 89034, MON 89034 x MON 88017 provides control of corn rootworm complex (*Diabrotica spp.*, CRW) that is functionally equivalent to already-registered MON 863 and MON 88017. Use of MON 89034 x MON 88017 should encourage replacement and reduction of higher-risk conventional pesticides currently utilized for CRW control (BPPD, 2003). Additionally, MON 89034 x MON 88017, which has tolerance for glyphosate, should allow corn growers to utilize a conventional chemical, Roundup, that is recognized by the Agency as a Category E chemical (i.e., there is evidence of non-carcinogenicity for humans). Finally, yield appears to be comparable to other *Bt* insect-protected corn. In situations of increased lepidopteran and/or coleopteran pressure, yield could be higher than other *Bt* insect-protected corn because of the presence of three insecticidal toxins and the effective protection against particular primary and secondary corn pests.

2. Economic (Grower) Benefits

MON 89034

MON 89034 will offer protection against a wider spectrum of primary and secondary corn pests (including FAW and CEW); should create conditions that allow for a reduction in the amount of mycotoxin contamination; and should facilitate replacement and reduction of the amount of a small amount of conventional pesticides that may be used against particular non-soil complex corn pests. It is reasonable to believe that all of these characteristics should result in increased yield, increased grain quantity, and increased grain quality.

MON 89034 x MON 88017

Because of the presence of MON 88017, which offers protection against CRW, MON 89034 x MON 88017 should offer the same benefits as MON 89034 with perhaps more reduction in conventional pesticide use and a slight advantage over the single event for growers that require protection against lepidopteran pests and CRW.

3. Human Health and Environmental Benefits

MON 89034

Human Health

The Cry1A.105 and Cry2Ab2 proteins produced by MON 89034 should not present toxicity or allergenicity problems in humans based on the reviews of the studies submitted in support of MON 89034's conditional registration. As with other *Bt* corn products, it is reasonable to assume that the utilization of MON 89034 should reduce the use of some conventional pesticides

(BPPD, 2001). Finally, because the Cry1A.105 and Cry2Ab2 proteins target secondary corn pests - such as FAW and CEW - and protect the ear from damage caused by these pests, decreased amounts of mycotoxin contamination should be recognized as a substantial benefit.

Environmental

Generally, there should be no risk from the proposed uses for MON 89034 to non-target organisms, including, mammalian wildlife species, aquatic species, avian species, non-target insects, and endangered species (BPPD, 2007i; BPPD, 2007j). Finally, use of MON 89034 should encourage a small reduction in the use of conventional pesticides. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers.

MON 89034 x MON 88017

Human Health

In addition to Cry1A.105 and Cry2Ab2 proteins produced in MON 89034, the introduction of MON 88017 in the pyramided product results in production of Cry3Bb1 protein. Human risk assessment data has previously been reviewed for MON 88017, and BPPD concluded that there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children (BPPD, 2007g). Additionally, an exemption from tolerance was established for Cry3Bb1 protein under 40 CFR 174.518. As with other *Bt* corn products, it is reasonable to assume that the utilization of MON 89034 x MON 88017 should reduce the use of conventional pesticides. In particular, the use of MON 89034 x MON 88017 should result in the reduction of many conventional pesticides that are currently used, which have significant adverse effects on human health (BPPD, 2003).

Environment

Cry3Bb1 protein, produced in MON 88017, posed no significant risk to test organisms (BPPD, 2003). The only potential concern, brought to BPPD's attention by a recently published study, relates to MON 89034 and will be dealt with by submission of 7-14-day *Daphnia* study. Additionally, use of MON 89034 x MON 88017 should reduce the amount of conventional pesticides used in the environment. All of the conventional pesticides used for CRW control or suppression currently cause significant adverse environmental effects under conditions of normal use (BPPD, 2003). Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers.

4. Insect Resistance Management (IRM) Benefits

MON 89034 (mention of MON 89034 assumes the same conclusions for MON 89034 x MON 88017 also)

BPPD concludes that MON 89034's two modes of action are better than a single mode of action for mitigating the development of insect resistance. But, because of uncertainties in the data submitted to support the MON 89034 IRM plan, a 5% refuge cannot be established until additional data is submitted to support such a reduction. Instead, BPPD recommends that the separate refuge option include a 20% lepidopteran refuge (as has been required for other *Bt* products). A 20% refuge is likely to be supported for MON 89034 in cotton-growing regions of the southeastern U.S. where a 50% refuge has been previously required. This will likely provide an economic benefit to certain growers, since they will be required to plant less structured refuge. In addition, the Cry1A.105 and Cry2Ab2 toxins are new proteins targeting lepidopteran pests in corn. These additional modes of action will likely provide a benefit to IRM programs (i.e., a toxin "mosaic" in corn-growing regions may reduce the likelihood of resistance developing in individual toxins). Also, the use of pyramided *Bt* corn products (containing 2 or more toxins targeting the same pest) should further reduce the potential for resistance (BPPD, 2007b; BPPD, 2007h).

BACKGROUND

1. General Information

Corn (*Zea mays* L.) is the largest cultivated crop grown in the United States in terms of acreage planted and net value. Monsanto states that 93.6 million of acres of corn were planted in the U.S. during 2007 and that the net value of the 2006 corn crop was 33.7 billion dollars. The corn industry suffers substantial economic losses from damage caused by specific lepidopteran and coleopteran pests.

Two primary corn pests of particular concern to growers are corn rootworm complex (*Diabrotica spp.*, CRW) and European corn borer (*Ostrinia nubilalis*, ECB). According to Monsanto, CRW causes damage to all portions of the plant (i.e., those above and below ground) depending on the insect's life stage. In 2003, EPA estimated that approximately 28 million acres of corn were infested with CRW and that untreated corn could result in severe yield loss, which was typically in the range of 8 -16% reduction, but could be as high as 28% (BPPD, 2003). ECB has been identified as the second most important insect pest of corn after CRW. ECB causes damage to the plant based upon the generation: (i) the first generation causes leaf and stalk damage; (ii) the second generation causes stalk, leaf sheath, collar, and ear damage; and (iii) the third generation causes leaf sheath, collar, and ear damage. Monsanto estimates that the average annual U.S. yield loss from ECB infestation is within the range of 3-7%. Deviations from the range are attributed to level of infestation and region.

Two secondary corn pests of particular concern to growers are corn earworm (*Helicoverpa zea*, CEW) and fall armyworm (*Spodoptera frugiperda*, FAW). FAW typically has a limited range as it is primarily found in the Gulf States and overwinters only in extreme southern Texas and Florida. Monsanto provides an estimate that FAW damage to untreated acreage in Georgia between 1991 and 1997 resulted in average yield loss of approximately 10%. No average yield loss for all of the U.S. due to FAW damage was provided. On the other hand, CEW is found throughout the U.S. corn-growing region, but Monsanto cites its economic damage as being low and dependent on timing of infestation, region, and number of moth flights per year.

2. MON 89034

Monsanto has developed MON 89034, a corn product that produces *Bacillus thuringiensis* (*Bt*)-derived insecticidal proteins Cry1A.105 and Cry2Ab2. The Cry1A.105 toxin is a "chimeric" protein containing domains I and II and the C-terminal from Cry1Ac and domain III from Cry1F. The Cry2Ab2 protein is functionally equivalent to that currently expressed in Monsanto's Bollgard II cotton. MON 89034 is protected from damage caused by larval feeding of ECB, southwestern corn borer (*Diatraea grandiosella*, SCWB), Sugarcane borer (*Diatraea saccharalis*, SCB), FAW, and CEW (BPPD, 2007a).

3. MON 89034 x MON 88017

Monsanto has also developed a second generation corn product, MON 89034 x MON 88017. MON 88017 (EPA Reg. No. 524-551) (plasmid vector ZMIR39) expresses the Cry3Bb1 *Bt* toxin and is targeted against CRW larvae. The toxin is the same as expressed by MON 863 corn (EPA Reg. No. 525-528), which was registered by Monsanto for the 2003 growing season. The Cry3Bb1 protein produced in MON 88017 and MON 863 is a variant of the wild-type Cry3Bb1 protein from *Bt* subsp. *kumamotoensis*. When compared by amino acid sequencing, the Cry3Bb1 protein expressed in MON 88017 has been reported to be 99.8% similar to the Cry3Bb1 protein expressed in MON 863. The primary difference between the two hybrids is that MON 88017 also expresses a gene for resistance to glyphosate (Roundup)-based herbicides (BPPD, 2005). By crossing MON 89034 and MON 88017 through conventional breeding, Monsanto has obtained an insect-protected corn product that expresses the Cry1A.105, Cry2Ab2, and Cry3Bb1 *Bt* toxins, is targeted against lepidopteran corn pests including ECB, SWCB, CEW, and FAW as well as coleopteran CRW, and is tolerant of glyphosate (BPPD, 2007b).

4. Monsanto's Public Interest Assertions for MON 89034 and MON 89034 x MON 88017

In the introduction of their public interest document (PID), Monsanto outlines the following reasons why MON 89034 and MON 89034 x MON 88017 are in the public interest according to some of the criteria set forth in 51 Fed. Reg. 7628:

- *Enhanced spectrum of control*. MON 89034 provides protection against

an expanded spectrum of lepidopteran pests when compared to current *Bt* corn products. MON 89034 x MON 88017 protects against both particular lepidopteran and coleopteran pests. The increased protection found in both products improves overall grain quality and limits yield losses due to root, leaf, stalk, and ear damage.

- *Reduced mycotoxin levels*. Because MON 89034 and MON 89034 x MON 88017 control the secondary pests, FAW and CEW, the opportunity for fungal infections to thrive due to plant damage is reduced. This leads to less mycotoxin contamination.

- *Improved breeding efficiency*. Vector-stacking, which increases the efficiency of breeding multiple traits into new corn hybrids, was utilized in the creation of MON 89034 and MON 89034 x MON 88017.

- *Compatibility with integrated pest management (IPM) systems.* Both MON 89034 and MON 89034 x MON 88017 provide two different modes of action in a single plant and reduce the probability of lepidopteran pests developing resistance to the *Bt* proteins. This allows for a smaller refuge, helps the product maintain efficacy, and guards against potential insect resistance to *Bt* crops.

- *Reduced use of chemical pesticides.* MON 89034 and MON 89034 x MON 88017 reduce the use of conventional chemicals, which saves costs and protects human health and the environment.

- *Easy implementation*. No additional labor or machinery is needed to plant, grow, or harvest MON 89034 and MON 89034 x MON 88017 relative to conventional corn.

- *Presence of glyphosate tolerance*. MON 89034 x MON 88017 produces 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. Strain CP4, which confers tolerance to glyphosate. Therefore, the agricultural herbicide, Roundup, can be utilized on MON 89034 x MON 88017 corn to control weeds and enhance the ability of the corn plants to access soil nutrients. EPA has classified glyphosate as a Category E Chemical, meaning there is evidence of non-carcinogenicity for humans.

This review will evaluate most of the assertions presented above in order to determine if MON 89034 and MON 89034 x MON 88017 are in the public interest.

EPA'S REVIEW OF MONSANTO'S PUBLIC INTEREST DOCUMENT

Monsanto submitted a public interest document in support of the Section 3(c)(7)(C) registrations of MON 89034 and MON 89034 x MON 88017 (Crawford and Bogdanova 2007, MRID 472797-01). The main portion of this document is divided into the following five sections: (i) agricultural benefits; (ii) economic (grower) benefits; (iii) human health benefits; (iv) environmental benefits; and (v) insect resistance management benefits. This document also includes three appendices: Appendix I provides a summary of reports submitted by Monsanto to the EPA that support registration of MON 89034 and MON 89034 x MON 88017, Appendix II contains a study that analyzes the mycotoxin levels in grain of MON 89034 corn exposed to lepidopteran insect infestation and inoculation with *Aspergillus flavus* or F*usarium verticillioides*, and Appendix III contains an assessment of efficacy of MON 89034 x MON 88017 corn against corn rootworm complex (*Diabrotica spp.*, CRW) in the US during 2005 and 2006. Information provided by Monsanto will be discussed below, as applicable.

1. Agricultural Benefits

a) Pest Spectrum and Efficacy - Monsanto's Summary (MRID 472797-01)

MON 89034

MON 89034 exhibits the Cry proteins, Cry1A.105 and Cry2Ab2, which specifically target lepidopteran pests (See Tables 7 and 8). The primary benefit of MON 89034 is that it provides equal (as compared to MON 810) or improved protection (as compared to MON 810, other *Bt* corn products, and non-*Bt* corn products) from feeding damage caused by particular lepidopteran pest larvae. The spectrum of protection against lepidopteran insects includes the following: European corn borer (*Ostrinia nubilalis*, ECB), southwestern corn borer (*Diatraea grandiosella*, SCWB), Sugarcane borer (*Diatraea saccharalis*, SCB), fall armyworm (*Spodoptera frugiperda*, FAW), black cutworm (*Agrotis ipsilon*, BCW), and corn earworm (*Helicoverpa zea*, CEW).

During the 2003 and 2004 growing seasons, Monsanto conducted efficacy field trials in the U.S., Puerto Rico, and Argentina. MON 89034's control of ECB, SWCB, and SCB was found to be comparable to MON 810 (See Table 11). Because of the production of Cry1A.105 protein by MON 89034 and the subsequent control of FAW throughout the season and not just the plant's vegetative growth phase, Monsanto claims a higher level of protection and increased activity against FAW are shown by MON 89034 as opposed to MON 810 (See Table 11). Of particular note, under heavy FAW pressure, MON 810 did not provide the significant amount of protection from leaf damage that MON 89034 exhibited. Finally, the Cry2Ab2 protein produced by MON 89034 provided improved control from CEW, when compared to the activity of MON 810's Cry1Ab protein (See Table 11).

Table 9. Summary of arthropod LC_{50} values for the Cry1A.105 protein exposure in diet bioassays

In the event that no adverse effect was observed, the LC_{50} value is considered to be greater than the maximum concentration tested.

Test Insect (Order/Family/Species)	Insect Stage Tested	Assay Duration (days)	Diet Assay Type	Maximum Concentration tested (µg/mL or g diet)	LC_{50} (µg/mL or g diet)[1]
Lepidoptera					
Noctuidae					
Helicoverpa zea	larvae	7	Incorporation	N/A	6
Agrotis ipsilon	larvae	7	Incorporation	N/A	33
Spodoptera frugiperda	larvae	7	Incorporation	N/A	6.9
Crambidae					
Diatraea grandiosella	larvae	12	Incorporation	N/A	37
Ostrinia nubilalis	larvae	12	Incorporation	N/A	0.43
Collembola					
Folsomia candida	nymphs	28	Overlay	80[2]	>80
Coleoptera					
Curculinoidae					
Anthonomus grandis grandis	larvae	7	Overlay	100	>100
Chrysomelidae					
Diabrotica undecimpunctata howardi **Coccinellidae**	larvae	5	Overlay	100	>100
Coleomegilla maculata	larvae	20	Incorporation	240	>240

*Table from page 15 of MRID 472797-01

[1] LC_{50} values with a greater than sign represent the highest dose tested.

[2] Assay was performed with lyophilized leaf tissue from MON 89034.

Table 9. (cont). Summary of arthropod LC_{50} values for the Cry1A.105 protein exposure in diet bioassays

In the event that no adverse effect was observed, the LC_{50} value is considered to be greater than the maximum concentration tested.

Test Insect (Order/Family/Species)	Insect Stage Tested	Assay Duration (days)	Diet Assay Type	Maximum Concentration tested (µg/mL or g diet)	LC_{50} (µg/mL or g diet)[1]
Hymenoptera					
Ichneumonidae					
Ichneumon promissorius	adults	21	Incorporation	240	>240
Apidae					
Apis mellifera	adults	18	Incorporation	550	>550
Apis mellifera	larvae	18	Overlay	1100 µg/mL as a single dose	>1100 µg/mL as a single dose
Hemiptera					
Aphididae					
Myzus persiscae	adults/ nymphs	5	Incorporation	80	>80
Miridae					
Lygus hesperus	nymphs	5	Incorporation	80	>80
Anthocoridae					
Orius insidiosus	nymphs	14	Incorporation	N/A	240[2]

*Table from page 16 of MRID 472797-01

[1] LC_{50} values with a greater than sign represent the highest dose tested.

[2] The no observed effect concentration was determined to be 120 µg/g diet.

Table 10. Summary of arthropod LC50 values for the Cry2Ab2 protein exposure in diet bioassays

In the event that no adverse effect was observed, the LC_{50} value is considered to be greater than the maximum concentration tested.

Test Insect (Order/Family/Species)	Insect Stage Tested	Assay Duration (days)	Diet Assay Type	Maximum Concentration tested (µg/mL or g diet)	LC_{50} (µg/mL or g diet)[1]
Lepidoptera					
Noctuidae					
Helicoverpa zea	larvae	7	Incorporation	N/A	9.9
Agrotis ipsilon	larvae	5	Overlay	N/A	>100[2]
Spodoptera frugiperda	larvae	7	Overlay	N/A	<50[5]
Crambidae *Ostrinia nubilalis*	larvae	12	Incorporation	N/A	1.5
Diatraea grandiosella	larvae	7	Incorporation	N/A	>100[4]
Collembola					
Folsomia candida	nymphs	28	Incorporation	70[5]	>70
Coleoptera					
Curculinoidae					
Anthonomus grandis grandis	larvae	7	Overlay	100	>100
Chrysomelidae					
Dibrotica undecimpunctata howardi	larvae	5	Overlay	100	>100

*Table from page 17 of MRID 472797-01

[1] LC_{50} values with a greater than sign represent the highest dose tested.

[2] 42% mortality was observed at the lowest tested dose of 100 µg/mL diet.

[3] 61% mortality was observed at the lowest tested dose of 50 µg/mL diet.

[4] Significant mortality was not observed at the highest tested dose of 100 ug/mL diet, however, at the highest tested dose of 100 ug/mL diet >95% growth inhibition was observed relative to the control treatment in three independent assays

[5] Assay was performed with lyophilized leaf tissue derived from MON 89034.

Table 10. (cont). Summary of Arthropod LC_{50} Values for the Cry2Ab2 Protein Exposure in Diet Bioassays

In the event that no adverse effect was observed, the LC_{50} value is considered to be greater than the maximum concentration tested.

Test Insect (Order/Family/Species)	Insect Stage Tested	Assay Duration (days)	Diet Assay Type	Maximum Concentration tested (µg/mL or g diet)	LC_{50} (µg/mL or g diet)[1]
Coccinellidae					
Coleomegilla maculata	larvae	20	Incorporation	120	>120
Hymenoptera					
Ichneumonidae					
Ichneumon promissorius	adults	21	Incorporation	100	>100
Nasonia vetripennis	adults	10	Incorporation	4500	>4500
Apidae					
Apis mellifera	adults	19	Incorporation	68	>68
Apis mellifera	larvae	12	Overlay	100 µg/mL (as a single dose)	>100 µg/mL (as a single dose)
Hemiptera					
Aphididae					
Myzus persiscae	adults/ nymphs	5	Overlay	80	>80
Miridae					
Lygus hesperus	nymphs	5	Overlay	80	>80
Anthocoridae					
Orius insidiosus	nymphs	14	Incorporation	100	>100

*Table from page 18 of MRID 472797-01

[1] LC_{50} values with a greater than sign represent the highest dose tested.

Table 11. Summary of field efficacy of MON 89034, MON 810 and control corn against major lepidopteran pests during the 2003-2004 growing season

Field Location	Infestation method	Damage measured	Infestation level	Trait performance[1]
Fall Armyworm				
Puerto Rico (I)	Natural	Leaf	High	MON 89034>MON 810>Control
Puerto Rico (II)	Natural	Leaf	Severe	MON 89034>MON 810=Control
U.S.	Artificial	Leaf	50 larvae /plant	MON 89034>MON 810>Control
Argentina	Natural	Leaf	Low	MON 89034>MON 810>Control
Corn Earworm				
Puerto Rico (I)	Natural	Ear	Moderate	MON 89034>MON 810>Control
U.S.	Artificial	Ear	15 larvae /plant	MON 89034>MON 810>Control
Argentina	Natural	Ear	Low-moderate	MON 89034>MON 810>Control
Southwest Corn Borer				
U.S.	Artificial	Stalk tunneling	7 larvae /plant	MON 89034=MON 810>Control
European Corn Borer				
U.S.	Artificial	Stalk tunneling	50 larvae /plant	MON 89034=MON 810>Control
Sugarcane Borer				
Argentina	Natural	Stalk tunneling	Moderate-high	MON 89034=MON 810>Control

*Table from page 20 of MRID 472797-01

[1] Level of protection against lepidopteran pest damage.
> represents statistically significantly improved performance compared to other treatment
= represents no statistically significant difference in performance

MON 89034 x MON 88017

In addition to producing the Cry1A.105 and Cry2Ab2 proteins, MON 89034 x MON 88017 also produces the insecticidal protein, Cry3Bb1, that controls damage caused by CRW.

The efficacy of MON 89034 x MON 88017 against CRW was compared in field trials in the U.S. in 2005 and 2006, and against lepidopteran pests in 2006. The pyramided product showed protection from feeding damage by lepidopteran pests that was comparable to MON 89034, as well as protection from damage by CRW that was comparable to MON 88017. The average root damage rating (RDR) for MON 88017 and MON 89034 x MON 88017 was significantly less than the RDR for non-CRW protected controls (See Table 12).

Table 12. Field efficacy of MON 88017 and MON 89034 x MON 88017 and non-CRW-protected control corn against corn rootworm tested in 2005 and 2006

Entry	RDR[1,2]
2005	
Control	1.399 A
MON 88017	0.165 B
MON 89034 x MON 88017	0.164 B
2006	
Control	0.774 A
MON 89034 x MON 88017	0.092 B

*Table from page 21 of MRID 472797-01

[1] RDR - Root damage rating calculated as a least-square mean of n=5 plants per plot in 2005 and n = 6 plants per plot in 2006.
[2] Values indicated by the same letter in the same column are not statistically different (Fisher's protected LSD p=0.05 level).

The efficacy of MON 89034 x MON 88017 against ECB was also assessed in 2005 U.S. trials. Significantly less feeding was observed on MON 89034 and MON 89034 x MON 88017 and these two insect-protected corn crops also provided a high level of control against leaf damage and stalk tunneling by ECB.

a) Pest Spectrum and Efficacy – BPPD's Response

MON 89034

BPPD agrees with Monsanto that MON 89034 targets lepidopteran pests specifically. In two studies conducted by Monsanto, both the Cry1A.105 protein and Cry2Ab2 protein exhibited insecticidal activity in the order Lepidoptera but not in the orders Coleoptera and Hemiptera (BPPD, 2007c; BPPD, 2007d). The Cry1A.105 protein, administered at 50 µg/mL and 100 µg/mL concentrations to insects in the orders Lepidoptera, Coleoptera, and Hemiptera, caused a range of mortality of 32% to 96% in CEW, ECB, and FAW (BPPD, 2007c). Additionally, all four lepidopteran insects (CEW, ECB, FAW, and BCW) had a range of 32% to 100% growth stunting (BPPD, 2007c). On the other hand, the Cry2Ab2 protein, administered at 50 µg/mL and 100 µg/mL concentrations to insects in the orders Lepidoptera, Coleoptera, and Hemiptera, caused at least 61% mortality (corrected) against CEW, ECB, and FAW (BPPD, 2007d). All four lepidopteran insects (CEW, ECB, FAW, and BCW) had a range of 97 to 100% growth stunting (BPPD, 2007d).

BPPD agrees with Monsanto's conclusions from the field trials conducted in the U.S., Puerto Rico, and Argentina. Across all geographies, the efficacy of MON 89034 against ECB, SWCB, CEW, FAW, and SCB was equal to or greater than that of YieldGard Corn Borer (MON 810), a lepidopteran control corn product that expresses the Cry1Ab protein. However, MON 89034 did offer a broader spectrum of insect protection activity than MON 810 and demonstrated better control of CEW, FAW, and SCB than MON 810 in these trials (BPPD, 2007a).

MON 89034 x MON 88017

In the past, BPPD has concluded that MON 88017 is functionally equivalent to MON 863 for CRW control (BPPD, 2005). Therefore, BPPD finds the efficacy benefits of MON 89034 x MON 88017 are similar to the efficacy benefits of MON 863 (BPPD, 2003) and MON 88017 (BPPD, 2005). A summary of these benefits can also be found in BPPD's Biopesticides Registration Action Document – *Bacillus thuringiensis* Plant-incorporated Protectants (BPPD, 2001). BPPD agrees that the efficacy of MON 89034 x MON 88017 should be comparable to efficacy of the MON 89034 and MON 88017 isolines for FAW and western corn rootworm (WCRW, *Diabrotica virgifera)* and to the MON 89034 isoline for ECB, SWCB, CEW, and FAW. Furthermore, similar to MON 89034, MON 89034 x MON 88017 provides a broader spectrum of efficacy than MON 810 against lepidopteran pests. Although no SCB, WBCW, or BCW field trials were conducted with MON 89034 x MON 88017, based on the efficacy against the other pests, it is reasonable to assume comparable efficacy to MON 89034 for these pests as well (BPPD, 2007b).

b) Yield – Monsanto's Summary (MRID 472797-01)

MON 89034 and MON 89034 x MON 88017

In 2006, Monsanto conducted field trials with the objective of comparing yield between MON 89034, MON 89034 x MON 88017, MON 810, MON 810 x MON 88017, and other hybrids not producing *Bt* proteins. Results showed comparable yield across several hybrids tested for insect-protected hybrids and higher yields compared to hybrids that did not produce *Bt* proteins (See Table 13). Additionally, the assumption is made that under intense lepidopteran pressure, the yield benefit from MON 89034 would be significantly higher. Although no economic benefits can be assessed for MON 89034 until it is actually used by growers, Monsanto predicts that the economic benefits would be equal or even more advantageous depending on the level and type of pest infestation that occurs.

Table 13. Yield comparison between MON 89034, MON 89034 x MON 88017, MON 810, MON 810 x MON 88017, and non-*Bt* hybrids grown in the U.S. during 2006

Product	Yield (Bu/Acre)	Number of hybrids tested
MON 89034	180.4	60
MON 810	180.6	29
MON 89034 x MON 88017	189.3	80
MON 810 x MON 88017	185.7	36
Non-*Bt*	171.5	2

*Table from page 13 of MRID 472797-01

c) Yield – BPPD's Response

BPPD believes that it is reasonable for Monsanto to assume that significant pressure from lepidopteran pests would cause the yield benefit for MON 89034 to be higher because of the presence of 2 insecticidal toxins, Cry1A.105 and Cry2Ab2. Furthermore, if the pressure is from secondary corn pests such as FAW and CEW, then increase of yield is even more logical. Although Monsanto assumes that economic benefits will be equal or more advantageous for MON 89034 and is probably correct, BPPD will not assume complete validity of this assumption until MON 89034 is used over the course of several years and reliable yield data is available. Although use of MON 89034 x MON 88017 could also result in an overall increase in yield, BPPD does not expect an increase in yield that exceeds that of the previously-registered single gene MON 88017 as any increase in yield will result mostly from the characteristics of MON 89034: expanded pest spectrum and the presence of two toxins instead of one.

2. Economic (Grower) Benefits

a) Monsanto's Summary (MRID 472797-01)

MON 89034

For economic benefits, Monsanto cites to National Center for Food and Agricultural Policy statistics that are based on planted acreage of MON 810 corn in 2005. Monsanto estimated that MON 810 increased corn production by 103.9 million bushels because of the corn borer resistant trait. Net returns, decreased costs (fuel, labor, and conventional pesticides purchased), and premium price of protected seeds were estimated to be 197 million dollars. The decreased use of conventional pesticides was estimated at 4.85 million pounds. In percentage terms, MON 810 planted in 2005 resulted in a 24% increase in yield, a 27% decrease in pesticide use, and increased monetary gain of 26% when compared to 2004.

The major economic benefits of MON 89034 are the following: (i) a wider spectrum of pest protection (to include FAW and CEW), which results in increased grain quality and increased yield; (ii) reduction in mycotoxin contamination levels which contributes to economic recovery; (iii) protection that is more effective in controlling corn borers and therefore results in increased yield, grain quality, and grain quantity; and (iv) reduction in conventional pesticide use that results in less costs (See Table 14).

Table 14. Summary of economic benefits to growers using corn-borer protected corn

Benefit	Per acre benefit ($)	Total benefit ($ Millions)
Yield increase	13.59 (-3.67 – 48.76)	217 (-59 – 780)
Pesticide reduction	1.99 (1.00 – 2.98)	32 (16 – 48)
Mycotoxin reduction	1.98 (0.52 – 7.12)	32 (8.3 – 114)

*Table from page 29 of MRID 472797-01

MON 89034 x MON 88017

Monsanto states that the pyramided product will offer the combined benefits of the individual parents, MON 89034 and MON 88017. The addition of MON 88017 creates enhanced protection against CRW, a primary corn pest that can cause total yield losses that exceed $1 billion dollars annually, and adds the glyphosate tolerance trait that limits yield loss from weed pressure. Overall, MON 89034 x MON 88017 will limit yield losses from corn borer insects, CRW, and weed pressure, reduce conventional pesticide use, and reduce mycotoxin contamination while increasing yield, grain quantity, and grain quality.

b) BPPD's Response

MON 89034

Overall, BPPD agrees that MON 89034 should produce economic benefits for many growers. Monsanto's numbers are estimates and actual economic benefits may be affected by factors including pest pressure, climatic fluctuations, and commodity pricing. MON 89034 is effective against a wider spectrum of corn pests and it is reasonable to assume that this should result in increased yield. Additionally, BPPD has concluded that a slight decrease in pesticide use should be realized with the use of *Bt* corn products similar to MON 89034 (BPPD, 2001); therefore, BPPD agrees with Monsanto's assertion of possible conventional pesticide use reduction and associated reduced costs.

BPPD agrees with Monsanto's conclusion that MON 89034 should reduce mycotoxin contamination. Overall, if primary and secondary corn pest pressure is reduced, then less mycotoxin contamination will be present, which will in turn lead to increased yield, grain quantity, and grain quality (BPPD, 2001). Furthermore, field evidence has demonstrated the ability of *Bt* corn to reduce the infestation rates of certain mycotoxins (Wu, 2008). This article specifically associates CEW with aflatoxin accumulation in corn and claims that *Bt* corn varieties, perhaps those such as MON 89034, are being developed to combat this insect pest in order to reduce particular mycotoxin contamination.

MON 89034 x MON 88017

BPPD believes that use of MON 89034 x MON 88017, much like MON 89034, should produce the same economic benefits mentioned above. Additionally, the combination with MON 88017, which protects corn against CRW and exhibits glyphosate tolerance, can be expected to create slightly greater economic benefits than MON 89034 for growers needing to treat both lepidopteran pests and CRW. The economic benefits of MON 88017 (minus an evaluation of glyphosate tolerance) have previously been assessed in MON 863's public interest finding document (BPPD, 2003).

3. Human Health and Environmental Benefits

a) Monsanto's Summary (MRID 472797-01)

MON 89034

Human Health

Monsanto states that the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034 are structurally and functionally related to Cry proteins that have a history of use both as active ingredients in *Bt* microbial pesticides and bio-tech derived food and feed. Furthermore, they

state that *Bt* has been commercially used in the U.S. since 1958 to produce microbial-derived pesticides and no adverse effects on humans or animals has been reported during their use on food or feed crops. Additionally, Monsanto asserts that Cry1A.105 and Cry2Ab2 proteins are highly unlikely to create any concerns of toxicity or allergenicity to humans. These assertions are based on acute oral toxicity data, which produced results that agreed with literature showing that *Bt* proteins only impact insect species and that no mammalian toxicity or issues have been reported in nearly 60 years of *Bt* protein insecticide use, and a comparison to known allergens, which indicated no allergenicity in the Cry1A.105 and Cry2Ab2 proteins.

Two of the specific human health benefits that Monsanto attributes to use of MON 89034 are pesticide reduction and mycotoxin reduction. Using numbers from an article from the National Center for Food and Agricultural Policy, Monsanto demonstrates current usage levels of MON 810, a functional equivalent of MON 89034, results in an estimated decrease in use of approximately 4.85 million pounds of conventional pesticides per year (equivalent to a 27% decrease in conventional pesticide use to control corn-boring pests). According to Monsanto, mycotoxin reduction is also evident with the use of MON 89034 because of its ability to suppress or control secondary corn pests, such as CEW and FAW, that play a role in damaging corn ears and facilitating the inoculation and growth of mycotoxin-producing fungi. Monsanto cites to two types of fungi, *Fusarium* and *Aspergillus* that produce fumonisin and aflatoxin, respectively. A study conducted by Monsanto indicates that MON 89034 is subject to less damage from corn pests and subsequently, it suffers less mycotoxin contamination, particularly from *Aspergillus*.

Environmental

Monsanto states that Cry1A.105 and Cry2Ab2 have no toxic effects on non-target organisms (to include the following: mammalian wildlife species, aquatic species, avian species, non-target insects, and endangered species) based on studies they submitted to the Agency in conjunction with the registration application for MON 89034. Additionally, the proteins rapidly degrade in soil which also minimizes exposure to non-target species.

MON 89034 x MON 88017

Human Health

Since MON 89034 is present in the pyramided product, Monsanto's contentions for the safety of MON 89034 with regard to human health also apply to MON 89034 x MON 88017. In addition to the presence of the Cry1A.105 and Cry2Ab2 proteins found in MON 89034, the pyramided product also produces Cry3Bb1 protein. Given that MON 88017 is already a product registered by the EPA, Monsanto states that the Cry3Bb1 protein produced by MON 89034 x MON 88017 already has an exemption from tolerance. Furthermore, human risk assessment data reviewed by the EPA for registration of MON 88017 has resulted in a conclusion that there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children. As with MON 89034, MON 89034 x MON 88017 is expected to result in

both reduced pesticide use (and perhaps more because of MON 88017's protection from primary corn pest, CRW) and mycotoxin contamination.

Environmental

Since MON 89034 is present in the pyramided product, Monsanto's contentions for the safety of MON 89034 with regard to environmental effects also apply to MON 89034 x MON 88017. MON 89034 x MON 88017 also produces the Cry3Bb1 protein, which is in the previously registered MON 88017. Upon review of the environmental effects data for MON 88017's registration, EPA concluded that no unreasonable adverse effects are expected to the environment from the cultivation of MON 88017 and MON 88017 x MON 810 corn.

b) BPPD's Response

MON 89034

Human Health

BPPD agrees with Monsanto's conclusions that the Cry1A.105 and Cry2Ab2 proteins produced by MON 89034 should not cause toxicity or allergenicity problems in humans. The data submitted and cited regarding potential health effects for the Cry1A.105 and Cry2Ab2 proteins include the characterization of the expressed proteins in corn, as well as acute oral toxicity studies, amino acid sequence comparisons to known allergens and toxins, and in vitro digestibility of the proteins. The results of these studies were used to evaluate human risk (BPPD, 2007e).

The acute oral toxicity data submitted support the prediction that the Cry1A.105 and Cry2Ab2 proteins would be non-toxic to humans. When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels. Since no treatment-related adverse effects were shown to be caused by the Cry1A.105 and Cry2Ab2 proteins, even at relatively high dose levels, the Cry1A.105 and Cry2Ab2 proteins are not considered toxic. Basing this conclusion on acute oral toxicity data without requiring further toxicity testing or residue data is similar to the Agency position regarding toxicity and the requirement of residue data for the microbial *Bt* products from which this plant-incorporated protectant was derived (See 40 CFR 158.740(b)(2)(i)) (BPPD, 2007e).

Since Cry1A.105 and Cry2Ab2 are proteins, potential allergenicity was also considered as part of the toxicity assessment. Considering all of the available information (1) Cry1A.105 and Cry2Ab2 originate from a non-allergenic sources; (2) Cry1A.105 and Cry2Ab2 have no sequence similarities with known allergens; (3) Cry1A.105 and Cry2Ab2 are not glycosylated; and (4) Cry1A.105 and Cry2Ab2 are rapidly digested in simulated gastric fluid; EPA has concluded that the potential for Cry1A.105 and Cry2Ab2 to be a food allergens is minimal (BPPD, 2007e).

The lack of mammalian toxicity at high levels of exposure to the Cry1A.105 and Cry2Ab2 proteins, as well as the minimal potential to be a food allergens, demonstrate the safety of the product at levels well above possible maximum exposure levels anticipated (BPPD, 2007e).

BPPD agrees with Monsanto's conclusion that MON 89034 should reduce mycotoxin contamination. Overall, if primary and secondary corn pest pressure is reduced, then less mycotoxin contamination will be present, which will in turn lead to increased yield, grain quantity, and grain quality (BPPD, 2001). Further, field evidence has demonstrated the ability of *Bt* corn to reduce the infestation rates of certain mycotoxins (Wu, 2008). This article specifically associates CEW with aflatoxin accumulation in corn and claims that *Bt* corn varieties, perhaps such as MON 89034, are being developed to combat this insect pest in order to reduce particular mycotoxin contamination.

Environmental

BPPD agrees with Monsanto's assessment that there should be no risk from the proposed uses for MON 89034 to non-target organisms, including mammalian wildlife species, aquatic species, avian species, non-target insects, and endangered species (BPPD, 2007i; BPPD, 2007j). In addition to Monsanto's submitted rationale for environmental benefits, Monsanto could have included a reference to MON 89034 use potentially reducing the amount of conventional pesticides applied in the environment and the subsequent environmental benefits. BPPD believes that cultivation of MON 89034 corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, MON 89034 corn should require substantially fewer applications of chemical pesticides, compared to production of non-*Bt* corn. Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers.

MON 89034 x MON 88017

Human Health

BPPD agrees with Monsanto that the use of MON 89034 x MON 88017 should not result in any unreasonable adverse effects to human health. In addition to Cry1A.105 and Cry2Ab2 proteins produced in MON 89034, the introduction of MON 88017 results in production of Cry3Bb1 protein. BPPD has already reviewed human risk assessment data for MON 88017 and reached a conclusion that there is reasonable certainty that no harm will result from aggregate exposure to the U.S. population, including infants and children (BPPD, 2007g). Additionally, Cry3Bb1 currently has an exemption from tolerance established under 40 CFR 174.518.

BPPD also agrees with the claim that there should be a reduction of some pesticide use (BPPD, 2001). Additionally, Cry3Bb1 protein primarily protects corn plants against CRW. Virtually all of the registered conventional insecticides used to control CRW are of special concern to the EPA because of risks to humans. Each year, there are confirmed reports of human illness associated with these registered conventional chemicals (See BPPD, 2003).

Finally, BPPD agrees with the claim that use of MON 89034 x MON 88017 should combat mycotoxin contamination because of the production of the Cry1A.105 and Cry2Ab2 proteins (as mentioned previously).

Environmental

BPPD agrees with Monsanto's assessment that there should be no risk from the proposed uses for MON 89034 x MON 88017 to non-target organisms, including mammalian wildlife species, aquatic species, avian species, non-target insects, and endangered species. The only potential concern will be addressed through a 21-day *Daphnia* study. For the registration of MON 88017, a series of studies were completed by Monsanto that exposed non-target organisms to high doses of leaf tissue, grain, or pollen containing a plant-produced Cry3Bb1 variant or to an artificial diet containing a *Bt*-produced Cry3Bb1 variant. Results indicated that the Cry3Bb1 protein posed no significant risk to test organisms (BPPD, 2003). Additionally, a study was conducted on MON 89034 x MON 88017 to ensure that the interaction between Cry1A.105, Cry2Ab2, and Cry3Bb1 proteins in the pyramided product would not change the overall properties of each individual component. BPPD concluded that the activity of Cry1A.105 and Cry2Ab2 proteins was not significantly altered by the presence of Cry3Bb1, and the activity of Cry3Bb1 was not significantly altered by the presence of Cry1A.105 and/or Cry2Ab2. The study, along with the previously reviewed interaction study between Cry1A.105 and Cry2Ab2, indicated that MON 89034 x MON 88017 corn should not result in any unexpected interaction with regards to target and non-target insects (BPPD, 2007f).

In addition to the submitted rationale for environmental benefits, Monsanto could have included reference to MON 89034 x MON 88017 use reducing the amount of conventional pesticides applied in the environment and the subsequent environmental benefits. BPPD believes that cultivation of MON 89034 x MON 88017 corn may have fewer adverse impacts on non-target organisms than use of chemical pesticides for corn production, because under normal circumstances, MON 89034 x MON 88017 corn should require substantially fewer applications of chemical pesticides, compared to production of non-*Bt* corn. The reduction in conventional pesticide use should essentially be the same seen from MON 88017 and MON 863 use (BPPD, 2003). Fewer chemical insecticide applications generally result in increased populations of beneficial organisms that control secondary pests, such as aphids and leafhoppers. Furthermore, all of the conventional pesticides used for CRW control or suppression cause significant adverse environmental effects under conditions of normal use (BPPD, 2003).

4. Insect Resistance Management (IRM)

a) Monsanto's Summary (MRID 472797-01)

MON 89034 (mention of MON 89034 assumes the same conclusions for MON 89034 x MON 88017 also)

Monsanto establishes that MON 89034, which produces Cry1A.105 and Cry2Ab2 proteins, has two different modes of action against lepidopterans, particularly in the way the proteins bind to the midgut. Therefore, based on the distinct modes of action of the two proteins and reduced likelihood of insect resistance, Monsanto proposes that a reduced structured refuge is possible: 5% for the corn belt, down from 20% and 20% for cotton-growing regions, down from 50%.

b) BPPD's Response

MON 89034 (mention of MON 89034 assumes the same conclusions for MON 89034 x MON 88017 also)

BPPD agrees with Monsanto in that two modes of action are better than one for reducing the risk of insect resistance to MON 89034. But, due to uncertainties in the data submitted to support the MON 89034 IRM plan, a 5% refuge cannot be established until additional data is submitted to support such a reduction. Instead, BPPD recommends that the separate refuge option include a 20% lepidopteran refuge (as has been required for other *Bt* products). A 20% refuge is likely to be supported for MON 89034 in cotton-growing regions of the southeastern U.S. where a 50% refuge has been previously required (BPPD, 2007b; BPPD, 2007h).

Overall, MON 89034 should present two immediate IRM benefits: (i) dual (distinct) modes of action for *Bt* corn and (ii) reduced refuge in cotton regions (and the resulting economic benefits to growers). These benefits can likely be achieved without an unreasonable risk of resistance to Cry1A.105 and Cry2Ab2. Additional grower benefits may be realized in the long term, if a 5% refuge can be supported.

5. Efficacy Studies

In addition to the efficacy studies referenced in the preceding sections, the following studies were submitted and are considered for registration of MON 89034. These studies demonstrate the efficacy of MON 89034 corn and the individual *Bt* proteins (Cry1A.105 and Cry2Ab2) against a range of lepidopteran corn pests including European corn borer (ECB), corn earworm (CEW), southwestern corn borer (SWCB), fall armyworm (FAW), and sugarcane borer (SCB).

MRID 46951413

In laboratory bioassays, the insecticidal activity of Cry1A.105 protein was tested against agronomically important insects from the orders Lepidoptera (four species), Coleoptera (two

species), and Hemiptera (two species). Neonate larvae, nymphs or adults (eggs for western bean cutworm prior to hatching) were fed artificial diets containing the appropriate doses of 50 or 100 µg Cry1A.105/mL of insect diet in the diet-overlay bioassays for fall armyworm, black cutworm, European corn borer, corn earworm, Southern corn rootworm, and boll weevil or 40 or 80 µg Cry1A.105/mL of insect diet in diet-incorporation bioassays for western tarnished plant bug and green peach aphid. Mortality and a reduction in weight or honeydew production over a five or seven day period depending on the insect were the endpoints used to indicate insecticidal activity. The 50 µg/mL and 100 µg/mL Cry1A.105 concentrations caused a range of 32 to 96% mortality in three (corn earworm, European corn borer, and fall armyworm) of the four lepidopterans. All four lepidopteran insects had a range of 32-100% stunting. The Cry1A.105 protein had activity against all four lepidopteran insects, but no activity against the two coleopteran or two hemipteran insects tested. **Classification: Acceptable**

MRID 46951414

In laboratory bioassays, the insecticidal activity of Cry2Ab2 protein was tested against agronomically important insects from the orders Lepidoptera (four species), Coleoptera (two species), and Hemiptera (two species). Neonate larvae, nymphs or adults (eggs for western bean cutworm prior to hatching) were fed artificial diets containing the appropriate doses of 50 or 100 µg Cry2Ab2/mL of insect diet in the diet-overlay bioassays for fall armyworm, black cutworm, European corn borer, corn earworm, Southern corn rootworm, and boll weevil or 40 or 80 µg Cry2Ab2/mL of insect diet in diet-incorporation bioassays for western tarnished plant bug and green peach aphid. Mortality and a reduction in weight or honeydew production over a five or seven day period depending on the insect were the endpoints used to indicate insecticidal activity. In the diet-overlay bioassays, both the 50 µg/mL and 100 µg/mL Cry2Ab2 concentrations caused at least 61% mortality (corrected) against corn earworm, European corn borer, and fall armyworm; while stunting was at least 97% for all four lepidopteran insects tested. Only the 28% black cutworm mortality resulting from testing against the 50 µg/mL Cry2Ab2 concentration failed to meet the study criterion of >30% mortality. The Cry2Ab2 protein had activity against all four lepidopteran insects, but no activity against the two coleopteran or two hemipteran insects tested. **Classification: Acceptable**

MRID 46951415

Field trials were conducted in 2003-2004 seasons in Puerto Rico, the United States and Argentina to determine the efficacy of MON 89034 corn (Cry1A.105 and Cry2Ab2) and MON 89597 (Cry1A.105 and Cry2Ab2) corn against European corn borer (ECB), corn earworm (CEW), southwestern corn borer (SWCB), fall armyworm (FAW), and sugarcane borer (SCB). Across all geographies the efficacy of the MON 89034 and MON 89597 against ECB, SWCB, CEW, FAW, and SCB was equal to or greater than that of YieldGard® Corn Borer (MON 810), a lepidopteran control corn product that expresses the Cry1Ab protein. For all geographies tested, there was no significant difference in efficacy between MON 89034 and MON 89597 with the exception of CEW damage in PR I, (2003 testing), where MON 89034 demonstrated significantly better control than MON 89597. MON 89034 and MON 89597 offer a broader spectrum of insect activity than MON 810. MON 89034 and MON 89597 demonstrated

significantly better control of CEW, FAW, and SCB than MON 810. Details of the field trials are found below. Data are **acceptable** for the Puerto Rico and U.S. trials. Data are **supplemental** for the Argentina trials due to lack of sufficient rationale as to why certain locations were excluded from the analysis. No additional data are required.

III. REGULATORY POSITION FOR Cry1A.105, AND Cry2Ab2

A) Original 3(c)(7)(C) Assessment

Pursuant to FIFRA section 3(c)(7)(C), EPA may conditionally register a new pesticide active ingredient for a period of time reasonably sufficient for the generation and submission of required data that are lacking because insufficient time has elapsed since the imposition of the data requirement for those data to be developed. EPA may grant such conditional registration only if EPA determines that (1) the use of the pesticide product during the period of the conditional registration will not cause any unreasonable adverse effect on the environment, and (2) the registration and use of the pesticide during the conditional registration is in the public interest. EPA determines that all of these criteria have been fulfilled.

The first criterion under FIFRA Section 3(c)(7)(C) mentioned above has been met because insufficient time has elapsed since the imposition of the data requirements for:

1) An independent lab validation of the analytical method for the detection of Cry2Ab2 and/or Cry1A.105 to satisfy residue analytical method in plants requirements for event MON 89034 corn and event MON 89034 x MON 88017 corn.

2) A 7 to 14 day *Daphnia* study as per the 885 Series OPPTS Guidelines or alternatively, a dietary study of the effects on an aquatic invertebrate, representing the functional group of a leaf shredder in headwater streams.

3) Additional information on cross-resistance of Cry1A.105 and Cry1Fa and Cry1Ac (preferably including binding site models and use of resistant colonies) for the target pests and determine how such cross-resistance may impact the durability of MON 89034.

4) Baseline susceptibility studies and/or a discriminating concentration assay that are required for the Cry1A.105 protein against ECB, SWCB, and CEW and for the Cry2Ab2 protein against SWCB, CEW.

5) Baseline susceptibility studies to support sweet corn uses that must be conducted on FAW populations collected from sweet corn growing areas; Monitoring studies that will be conducted on FAW populations collected from sweet corn distribution areas in states in which Monsanto MON 89034 and/or MON 89034 x MON 88017 sweet corn plantings exceed 1000 acres; and monitoring of the collected populations of FAW for changes in susceptibility to the Cry1A.105 and Cry2Ab2 proteins.

The applicants submitted or cited data sufficient for EPA to determine that conditional registration of *Bacillus thuringiensis* Cry2Ab2 and Cry 1A.105 proteins and the genetic material necessary for their production in event MON 89034 field corn and sweet corn under FIFRA 3(c)(7)(C) will not result in unreasonable adverse effects to the environment, as discussed above.

The applicants submitted and/or cited satisfactory data pertaining to the proposed use. The human health effects data and nontarget organism effects data are considered sufficient for the period of the conditional registration. These data demonstrate that no foreseeable human health hazards or ecological effects are likely to arise from the use of the product and that the risk of resistance developing to Cry2Ab2 and Cry 1A.105 proteins, during the conditional registrations are not expected to be significant.

Registration of *Bacillus thuringiensis* Cry2Ab2 and Cry 1A.105 proteins and the genetic material necessary for their production in event MON 89034 field corn and sweet corn is in the public interest because:

(1) Registration of MON 89034 is expected to result in the reduction of the use of higher risk, and often less effective and more expensive, conventional pesticides. A reduction in use of conventional pesticides equates to less potential for adverse effects to human health and the environment.

(2) Additionally, MON 89034 provide a wider spectrum of protection against primary and secondary corn pests, which should facilitate greater grain quality, a reduction of mycotoxin contamination, increased yield and ultimately have positive implications for human health.

In view of these minimal risks and the clear benefits related to *Bacillus thuringiensis* Cry2Ab2 and Cry 1A.105 proteins and the genetic material necessary for their production in event MON 89034 field corn and sweet corn , EPA believes that the use of the product during the limited period of the conditional registration will not cause any unreasonable adverse effects.

Although the data with respect to this particular new active ingredient are satisfactory, they are not sufficient to support an unconditional registration under FIFRA 3(c)(5). Additional data are necessary to evaluate the risk posed by the continued use of this product. Consequently, EPA is imposing the data requirements specified earlier in Section III.

EPA has determined, as explained in section II.F., that the third criterion for a FIFRA 3(c)(7)(C) conditional registration has been fulfilled because the use of *Bacillus thuringiensis* Cry2Ab2 and Cry 1A.105 proteins and the genetic material necessary for their production in event MON 89034 field corn and sweet corn under this registration is in the public interest.

The submitted data in support of this registration under section 3(c)(7)(C) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) have been reviewed and determined to be adequate. Studies mentioned above are included in the terms, conditions, and limitations of these registrations. This registration will not cause unreasonable adverse effects to man or the environment and is in the public interest.

The expiration date of the registrations has been set to September 30, 2010.

B) 2010 3(c)(7)(A) Assessment

Section 3(c)(7)(A) of FIFRA provides for the registration or amendment of a pesticide when the pesticide and proposed use "…are identical or substantially similar to any currently registered pesticide and use thereof, or differ only in ways that would not significantly increase the risk of unreasonable adverse effects on the environment, and (ii) approving the registration or amendment in the manner proposed by the applicant would not significantly increase the risk of any unreasonable adverse effect on the environment." Unreasonable adverse effects on the environment are defined under section 2(bb) of FIFRA as "… any unreasonable risk to man or the environment, taking into account the economic, social, and environmental costs and benefits of the use of any pesticide…" Thus, pursuant to section 3(c)(7)(A), EPA may conditionally register a pesticide if (1) the pesticide and its proposed use are identical or substantially similar to a currently registered pesticide; or (2) the pesticide and its proposed use differ only in ways that would not significantly increase the risk of unreasonable adverse effects; and (3) approving the registration would not significantly increase the risk of any unreasonable adverse effect.

The Agency concludes that the following Cry1A.105 and Cry2AB2 corn product registrations, that were set to expire on September 30, 2010 and described in-depth throughout this BRAD, meet both criteria (1) and (2):

(1) Event MON 89034 with Cry1A.105 and CryAb2 (EPA Reg. No. 524-575)
(2) Events MON 89034 with MON 88017, Cry1A.105, Cry2Ab2, and Cry3Bb1 (EPA Reg. No. 524-576)

These Cry1A.105 and Cry2AB2 corn products are identical in both composition and use (corn) to plant-incorporated protectants that are currently registered. Thus, criterion (1) has been fulfilled.

With regard to criterion (2), the Agency maintains, as was previously determined for the original registration of these particular products, that cultivation of Cry1A.105 and Cry2AB2-containing corn will not cause unreasonable adverse effects on the environment. The conditional environmental effects data, submitted in response to terms and conditions of registration strengthen the Agency's initial position and also confirm that long-term effects on non-target organisms are not anticipated. Lastly, the continued use of these products will likely still provide many of the benefits as were evaluated in section II(F) of this BRAD to support the 2005 registration of these products.

In conclusion, as the expiring Cry1A.105 and Cry2AB2 products have met the required criteria under section 3(c)(7)(A) of FIFRA, the Agency is amending these registrations to extend their respective expiration dates as follows:

Product Name (EPA Reg. No.)	Expiration Date
Event MON 89034 with Cry1A.105 and Cry2Ab2 (524-575)	September 30, 2022
Events MON 89034 with MON 88017, Cry1A.105, Cry2Ab2, and Cry3Bb1 (524-576)	September 30, 2015

Although data provided were satisfactory to make the determinations required by section 3(c)(7)(A) of FIFRA, they were not sufficient to support an unconditional registration under FIFRA section 3(c)(5). Additional data, specifically in relation to insect resistance management,are necessary for a finding of registrability under FIFRA section 3(c)(5) and remain as terms or conditions for the purposes of the amendments.

C) Period of Registration

In the 2001 *Bt* Corn reassessment, EPA determined that it was appropriate to amend the then-existing registrations to extend the period of registration of those products to an expiration date of October 15, 2008. All of the products being assessed at that time were efficacious against lepidopteran pests. EPA based this action on the finding that use of Cry1A.105 and Cry2AB2 expressed in corn will not significantly increase the risk of unreasonable adverse effects on the environment "for the limited time period of 7 additional years (to October 15, 2008)." These registrations were later amended to extend the period of registration to an expiration date of September 30, 2010. EPA subsequently granted time-limited registrations to products efficacious against coleopteran corn rootworm pests. For example, EPA registered Cry3Bb1 on February 24, 2003, to May 1, 2004, and extended that registration twice, to February 24, 2008, and September 30, 2010.

As set forth elsewhere in this document, EPA's primary concern for the *Bt* protected transgenic corn products is the possibility that target pests will develop resistance to one or more of the PIP toxins. Development of resistance to a *Bt* toxin would be a grave adverse effect, and, for over 15 years, EPA has imposed stringent requirements intended to countermand the potential development of resistance. Registrants similarly have been busily developing various products, product mixes (i.e., so-called "pyramids" and "stacks"), and resistance strategies, to maximize agronomic benefits and address resistance management issues. The result has been a vast array of product combinations and, occurring over the past couple of years, a re-emergence of varying refuge requirements for different products.

As discussed in the 2001 *Bt* PIP BRAD (at IID13), the earliest *Bt* corn registrations did not include mandatory refuge requirements. There was a lack of scientific consensus as to what the appropriate refuge requirement should be, and, it was assumed that the limited market penetration of these early crops would be so low as to guarantee that adequate natural refuges would be available from neighboring non-*Bt* corn fields. From 1995 to 1997, *Bt* corn

registrations included voluntary refuge requirements of 0% to 20% in the corn belt. In 1999, the ABSTC, in conjunction with the National Corn Growers Association, proposed uniform IRM requirements for *Bt* corn registrations. With some modifications, this proposal, put in place for the 2000 growing season, formed the baseline IRM requirements for almost all *Bt* corn registrations for the better part of a decade: farmers were required to plant a 20% refuge that could be treated for insects, or a 50% treated refuge in cotton-growing areas; all refuges to be planted within one-half mile of the *Bt* corn field.

These uniform requirements brought certainty and consistency to the market after the initial period where many *Bt* corn products had different refuge requirements. Recently, however, as product developers have begun to conceive of products with different combinations of "pyramided" products (i.e., products containing two or more toxins efficacious against the same pest) and "stacked" products (i.e., products combining toxins efficacious against different pests), the refuge requirements have begun to vary. For example, certain products require a 20% external refuge; some products permit a 5% external refuge; one product incorporates a 10% seed blend refuge; we have applications in process for products that propose to incorporate a 5% seed blend refuge; and other permutations are possible.

Given the profusion of various toxin combinations and refuge options, we can no longer proceed on the basis that, as concerns insect resistance management, all products are equal. It was a relatively simple proposition when the default requirement of a 20% sprayed refuge applied to almost all of the *Bt* corn crops in the market. Under those circumstances, the relative durability of products against the development of resistance was functionally equivalent, and, as a consequence, imposing functionally equivalent registration periods was appropriate. That is now no longer the case.

As part of our continually evolving regulatory approach to the continually evolving product mix wrought by developers, we think it appropriate to revise our regulatory requirements in scientifically defensible ways to reflect the comparative level of risks posed by the products that we regulate. Here, for example, where we've determined that a particular product, or category of products, likely will pose less risk of insect resistance developing to a particular PIP protein, we think it appropriate to grant that particular product, or category of products, a registration for a period greater than that granted a corresponding product that poses a greater risk of insect resistance developing. This approach is reflective of complementary principles: first, to ensure that we apply our limited resources to the products that pose greater risk of adverse effects to the environment; and, second, to conserve the resources that registrants and applicants must expend in amending the registrations of products that pose less risk of adverse effects to the environment.

The scheme that we are following includes registration periods of five, eight, and twelve years; a fifteen year registration period will also be available, if adequately supported by our science assessment. In this scheme, (i) a product with a single PIP toxin, and a 20% external refuge, qualifies for a five year registration; (ii) a product with pyramided PIP toxins (i.e., two or more

toxins with distinct, non-cross reacting modes of action), that are non-high dose (the definition for a high dose product remains unchanged), with either a seed blend or external refuge, qualifies for an eight year registration; (iii) a product with pyramided PIP toxins (i.e., two or more toxins with distinct, non-cross reacting modes of action), that are **high-dose**, with either a seed blend or external refuge, qualifies for a twelve year registration; (iv) a product with pyramided PIP toxins (i.e., two or more toxins with distinct non-cross reacting modes of actions), with either a seed blend or external refuge, that has been determined by EPA's science assessment to be 150% as durable as the baseline single toxin product with a 20% external refuge, would qualify for a fifteen year registration. Products determined by EPA's science assessment to be less than 100% as durable as the baseline single toxin product with a 20% external refuge would not qualify for a five year registration and the registration period for such products will be determined on a case-by-case basis consistent with the level of risk they pose. Similarly, instances where other risk issues may arise, or where novel resistance concerns may be present, would also be determined on a case-by-case basis, as will novel refuge configurations that may present unique durability profiles.

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